Screening for Antimicrobial Substance Producing Actinomycetes from Soil

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ชื่อวิทยานิพนธ์ การคัดเลือกเชื้อแอคติโนมัยสีทที่สร้างสารต้านจุลินทรีย์ที่แยกได้จากดิน

ผู้เขียน นางสาวสุนันทา สวัสดี

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บทคัดย่อ

เชื้อแอคติโนมัยสีท 100 ไอโซเลทที่แยกได้จากดินใน 4 จังหวัดทางภาคใต้ของประเทศไทย ได้ถูกนำไปทำการศึกษาความสามารถในการสร้างสารต้านจุลินทรีย์โดยวิธี cross streak และ hyphal growth inhibition กับเชื้อก่อโรคในคน 10 สายพันธุ์ ได้แก่ *Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* SK1*, Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 27853*, Cryptococcus neoformans* ATCC 90112 และ ATCC 90113, *Candida albicans* ATCC 90028 และ NCPF 3153, *Microsporum gypseum* และ *Penicillium marneffei* ที่แยกได้จากผู้ป่วย พบว่า 80% ของแอคติโนมัยสีทสามารถยับยั้งเชื้อก่อโรคได้อย่างน้อย 1 สายพันธุ์ โดยมี 8 และ 32% แสดงฤทธิ์ต้านแบคทีเรีย และฤทธิ์ต้านรา เท่านั้นตามลำดับ และ 40% สามารถยับยั้งได้ทั้งแบคทีเรีย และเชื้อรา สำหรับฤทธิ์ต้านแบคทีเรียพบว่า 40% ของแอคติโนมัยสีทสามารถยับยั้งการเจริญของ *S. aureus* ทั้งสองสายพันธุ์ มีเพียง 9 และ 15% ที่สามารถยับยั้งการเจริญของ *E. coli* และ *P. aeruginosa* ตามลำดับ ส่วนฤทธิ์ต้านเชื้อราพบว่า 49, 41, 30 และ 21% สามารถยับยั้งการเจริญของ *C. neoformans, P. marneffei*, *C. albicans*, และ *M. gypseum* ตามลำดับ คัดเลือกแอคติโนมัยสีทที่ให้ค่า inhibition zone มากกว่า 25 mm และที่ยับยั้งการเจริญของเส้นใยของเชื้อราก่อโรคได้มากกว่า 80 % รวม 46 ไอโซเลท นำมาเพาะเลี้ยงในอาหารเหลว Yeast Malt

Extract (YME) และสกัดสารออกฤทธิ์ทางชีวภาพด้วยวิธีทางเคมี นำสารสกัดมาทดสอบหาค่า minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) และ minimal fungicidal concentration (MFC) โดยวิธี colorimetric broth microdilution พบว่าสารสกัด 90 สาร จากทั้งหมด 138 สาร (65%) จากแอคติโนมัยสีททั้ง 46 ไอโซเลท (100%) แสดงฤทธิ์ต้านจุลินทรีย์ให้ค่า MIC/MBC-MFC อยู่ในช่วง 0.5-200/2->200 µg/ml โดยสารสกัดส่วนใหญ่มีฤทธิ์ต้านแบคทีเรียมากกว่าต้านเชื้อรา สารสกัด ACK21CH ที่สกัดจากส่วนเซลล์แอคติโนมัยสีท ACK21 ด้วยเฮกเซน ต้านแบคทีเรียได้ดีที่สุด โดยยับยั้ง *S. aureus* และ MRSA มีค่า MIC/MBC 0.5/4 และ 0.5/8 µg/ml ตามลำดับ ตามด้วยสารสกัด ACK20CE จากแอคติโนมัยสีท ACK20 ที่สกัดด้วยเอธิลอะซิเตทต้านแบคทีเรียได้ดีรองลงมา (MIC/MBC ต่อ *S. aureus* และ MRSA 2/8 และ 0.5/2 µg/ml ตามลำดับ) นอกจากนี้ ACK21CH ยังยับยั้ง *C. albicans* NCPF3153 ได้ดีที่สุด (MIC/MFC 4/128 µg/ml) เมื่อศึกษาผลของสารสกัดต่อเซลล์แบคทีเรียด้วยกล้องจุลทรรศน์อิเลคตรอนชนิดส่องกราด (scanning electron microscopy) พบว่าสารสกัด ACK21CH และ ACK20CE สามารถทำลายเซลล์ของ *S. aureus* โดยทำให้เกิดการรั่วไหลของ cytoplasm และทำให้เกิดการตายของเซลล์ เมื่อศึกษาสภาวะที่เหมาะสมในการสร้างสารต้านจุลินทรีย์ของแอคติโนมัยสีท ACK21 และ ACK20 ต่อเชื้อ *S. aureus* ทั้งสองสายพันธุ์ โดยศึกษาปัจจัยที่เกี่ยวข้อง 3 ปัจจัย คือ การเขย่า อุณหภูมิ และ พีเอชเริ่มต้นของอาหารเลี้ยงเชื้อ พบว่าสภาวะที่ดีที่สุดในการสร้างสารต้านจุลินทรีย์ของ ACK21 คือ การเลี้ยงเชื้อแบบไม่เขย่า อาหารเลี้ยงเชื้อมีค่าพีเอชเริ่มต้น 7 และอุณหภูมิ 30 องศาเซลเซียส ส่วน ACK20 คือ การเลี้ยงเชื้อแบบไม่เขย่า อาหารเลี้ยงเชื้อมีค่าพีเอชเริ่มต้น 6 และ 7 และอุณหภูมิ 25 องศาเซลเซียส ผลการจำแนกชนิดโดยอาศัยลักษณะทางสัณฐานวิทยาและการวิเคราะห์ 16S rDNA พบว่าเชื้อ แอคติโนมัยสีท ACK21 จัดเป็น *Streptomyces* sp. และ ACK20 เป็นเชื้อ *Amycolatopsis echigonensis.*

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ABSTRACT

A total of 100 actinomycetes isolated from soils from four provinces in southern Thailand were screened for their ability to produce antimicrobial substances by cross streak and hyphal growth inhibition tests against ten human pathogens: *Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* SK1, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Cryptococcus neoformans* ATCC 90112 and ATCC 90113, *Candida albicans* ATCC 90028 and NCPF 3153, *Microsporum gypseum* and *Penicillium marneffei* clinical isolates. Eighty percents of the isolates showed antimicrobial activity against at least one test microorganism. Among them, 8% exhibited selective antibacterial activity, 32% had only antifungal activity and 40% displayed both antibacterial and antifungal activities. For antibacterial activity, 40% of soil actinomycetes inhibited both strains of *S. aureus* and only 9 and 15% inhibited *E. coli* and *P. aeruginosa*, respectively. For antifungal activity, 49, 41, 30 and 21% inhibited *C. neoformans, P. marneffei*, *C. albicans* and *M. gypseum*, respectively. Forty-six active isolates that showed inhibition zone over 25 mm and hyphal growth inhibition over 80% were selected and cultured in Yeast Malt Extract (YME) broth for extraction of bioactive compound. Crude extracts were then tested for their minimal inhibitory concentrations (MICs), minimal bactericidal concentrations (MBCs) and minimal fungicidal concentrations (MFCs) by colorimetric broth microdilution methods. Ninety extracts out of 138 extracts (65%) from 46 actinomycete isolates (100%) were inhibitory with MIC/MBC-MFC in the range of 0.5-200/2->200 µg/ml. The extracts were more effective against bacteria than fungi. Crude hexane extract from the cells of ACK21

(ACK21CH) exhibited the strongest antibacterial activity against *S. aureus* and MRSA with MIC/MBC 0.5/4 and 0.5/8 µg/ml, respectively followed by crude ethyl acetate extract from the cells of ACK20 (ACK20CE) against *S. aureus* and MRSA with MIC/MBC 2/8 and 0.5/2 µg/ml, respectively. In addition, ACK21CH also showed the strongest activity against *C. albicans* NCPF 3153 (MIC/MFC 4/128 µg/ml). Furthermore scanning electron microscopic study showed that ACK21CH and ACK20CE strongly destroyed *S. aureus* cells causing cytoplasm leakage and cell death. The effects of agitation, temperature and initial pH of culture medium on the production of antimicrobial metabolites by the isolates ACK21 and ACK20 were investigated. The optimum condition for ACK21 was observed at the static condition, pH7 and temperature 30^{o}C and ACK20 at the static condition, pH6 and 7 and temperature 25^{o}C Assayed against both strains of *S. aureus*. Based on morphological characteristics and 16S rDNA analysis, ACK21 was identified as *Streptomyces* sp. and ACK20 as *Amycolatopsis echigonensis*.

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THE RELEVANCE OF THE RESEARCH WORK TO THAILAND

Clinically-important bacteria, such as *Staphylococcus aureus*, are becoming resistant to commonly used antibiotics. Nowadays, new resistant strains emerge more quickly while the rate of discovery of new antibiotics is slowing down. Because of this, many scientists have focused on screening programs of microorganisms, primarily of actinomycetes, for their potential to produce new antibiotics against resistant strains. Many of actinomycetes are known to have the capacity to synthesize bioactive secondary metabolites, especially antibiotics. Almost 80% of the world´s antibiotics are known to come from actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora*. Over 50 different antibiotics have been isolated from *Streptomyces* sp. The purpose of this research aimed to isolate actinomycetes from soils and test for their ability to produce antimicrobial substances against human pathogens. In this study, crude hexane extract from the cells of ACK21 (ACK21CH) exhibited the strongest antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) with MIC/MBC 0.5/4 and 0.5/8 µg/ml, respectively followed by the ethyl acetate extract from the cells of ACK20 (ACK20CE) against *S. aureus* and MRSA with MIC/MBC 2/8 µg/ml, 0.5/2 µg/ml, respectively which were comparable to vancomycin (MIC 0.5-1 µg/ml). In addition, scanning electron microscopic study showed that ACK21CH and ACK20CE strongly destroyed *S. aureus* cells causing cytoplasm leakage and cell death. The culture conditions of both strains on the production of antimicrobial metabolites were also investigated. These two strains are a good source of antibacterial agents. They were identified based on morphological characteristics and the analysis of 16S rDNA to be *Amycolatopsis echigonensis* ACK20 and *Streptomyces* sp. ACK21. The isolation of active compounds and their structural elucidation are under investigation by the chemists. The mechanisms of action of bioactive compounds from ACK21 and ACK20 will be investigated.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	=	Degree Celsius
g	=	Gram
mg	=	Milligram
µ	=	Micro
µg	=	Microgram
µl	=	Microliter
ml	=	Milliliter
DMSO	=	Dimethyl sulfoxide
EtOAc	=	Ethyl acetate
Na_2SO_4	=	Sodium sulfate
RT	=	Room temperature

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Infectious diseases caused by drug-resistant bacteria and fungi are a major worldwide problem (Alanis, 2005). There is a need to find new antimicrobial agents to combat them. Actinomycetes are an important source of bioactive substances that are important for both medical and economic values, particularly in biotechnology (Mitchell *et al*., 2004). About two-thirds of antibiotics are from actinomycetes (Takizawa *et al*., 1993), most of which were produced by various *Streptomyces* species. This group of bacteria is interesting because it has complex life cycle and its members are antibiotic producers with a number of species (Kelemen and Buttner, 1999; Chater, 2001; Bentley *et al*., 2002; Willey *et al*., 2006; Nguyen *et al*., 2007). Many commercially available antibiotics are produced by *Streptomyces* spp. such as the antibacterial agents chloramphenicol, clindamycin, erythromycin, imipenem, streptomycin, tetracycline as well as antifungal agents amphotericin B and nystatin (Todar, 2012). Other bioactive compounds include anti-cancer compounds such as echinosporin (Morimoto and Imai, 1985) and limocrocin, an immunosuppressive agent, rapamycin, an insecticide macrolide compound (avermectin), and herbicides (phosphinothricin) are also produced by *Streptomyces* spp. (Goodfellow *et al*., 1988). Ruan (1994) reported that about 1,000 of the rare species of actinomycetes including *Micromonospora* 400 species, *Nocardia* 270 species, *Actinomadura* 170 species, *Actinoplanes* 150 species, *Saccharopolyspora* 50 species and *Streptosporangium* 40 species produced antibiotics. The commercial production of antibiotics from rare actinomycetes such as the rifamycins produced by *Amycolatopsis mediterranei*, erythromycin produced by *Saccharopolyspora erythraea*, teicoplanin produced by *Actinoplanes teichomymyceticus*, and gentamicin and vancomycin from *Amycolatopsis*

orientalis and *Micromonospora purpurea*, respectively have also been reported (Lazzarini *et al.,* 2000). However, in the last 20 years, the rates of discovery of new antibiotics from various sources have declined while the demand for antibiotics for the treatment of drug resistant pathogens and opportunistic diseases in AIDS patients and patients with organ transplantation is increasing all the time. Thus, seeking sources of new types of antibiotics is important and necessary (Schumacher *et al.,* 2003) and actinomycetes are still probably the most interesting source. Screening programmes for antimicrobial agents from actinomycetes are still fairly common and one new biologically active agent (resistoflavine) has recently been isolated from *Streptomyces chibaensis* AUBN1/7. This compound showed a potent cytotoxic activity against cell lines viz. HMO2 (gastric adenocarcinoma) and HePG2 (hepatic carcinoma) (Gorajana *et al.,* 2007) and showed both antibacterial and antifungal activity (Arasu *et al.,* 2008, Yadav *et al.,* 2009, Duraipandiyan *et al.,* 2010, Aouiche *et al.,* 2012, Dasari *et al.,* 2012)

1.2 Review of the literature

1.2.1 The importance of antibiotics

Antibiotics are substances normally of low molecular weight capable of inhibiting or slowing the growth of pathogenic microorganisms. They are often secondary metabolite produced by microorganisms and seem to have no definite role in the growth of the cell source. Microorganisms produce antibiotics normally during their late log phase of growth until their stationary phase. One of their key benefits to the source organism is said to be their ability to inhibit the growth of other microorganisms growing in the same environment in nature hence providing the source with a competitive advantage. Antibiotic producing microorganisms can then compete with others and survive in nature for a long time (Onlamoon, 2008)

1.2.2 The source of bioactive compound.

1.2.2.1 Bioactive compound from chemical synthesis

Antimicrobial compounds obtained from chemical synthesis are fewer in number than antibiotics from biological sources. Perhaps the best examples of antimicrobial agent from chemical synthesis are the sulfonamides (Figure 1a), the first antimicrobial agent synthesized in 1930. In 1962 nalidixic acid, a quinolone antimicrobial drug derived from chloroquine was discovered. After that many analogues have been synthesized and fluoroquinolone derivatives such as ciprofloxacin (Figure 1b) have been successfully developed. In 1979, a synthetic substance oxazolidinone was synthesized and linezolid (Figure 1c) was the first commercially available 1,3-oxazolidinone antibiotic (Blunt *et al.*, 2005). It is very effective against Gram-positive bacteria including drug resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA).

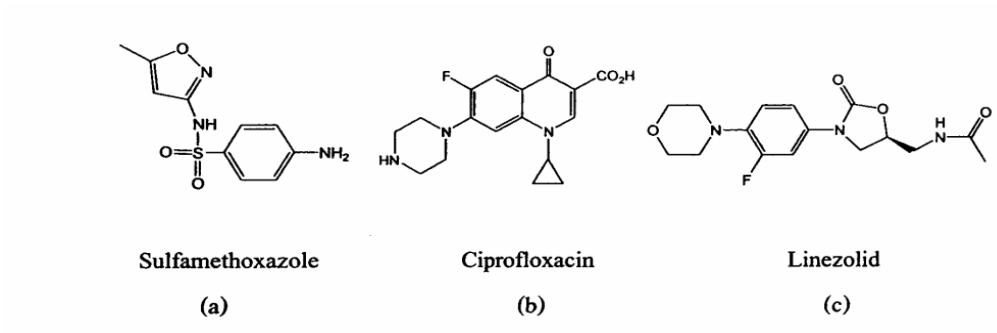


Figure 1 Chemical structures of sulfamethoxazole (a), ciprofloxacin (b), and linezolid (c).

Source: Blunt *et al.* (2005)

1.2.2.2 Bioactive compound from natural sources

Natural sources of biologically important compounds are plants, animals and microorganisms. The use of plant bioactive compounds as therapeutic drugs has developed over a long time from such chemicals as aspirin, morphine and quinine (Figure 2), etc. Natural products derived from animals such as clarhamnoside

produced by *Agelas clathorodes* (sponge), carijenone produced by *Carijoa multiflora* (coral), and violatinctamine produced by *Cystodytes violatinctus* (sea squirt). Recently, many studies have focused on natural products from marine organisms including microorganisms for the active ingredients of new developments in anticancer drugs. In 2009, approximately 1000 new compounds with biological activities were isolated (Blunt *et al.*, 2005).

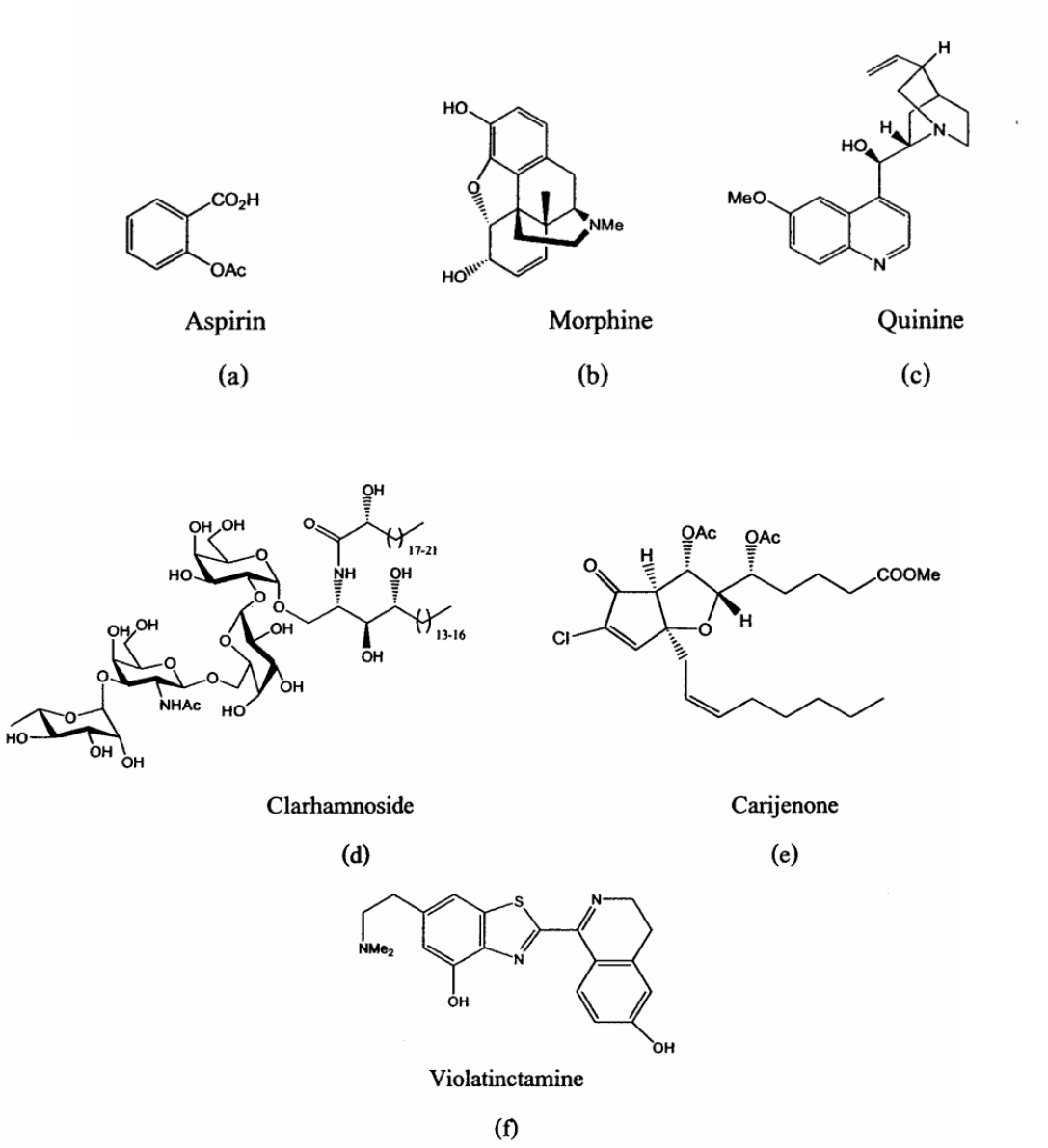


Figure 2 Chemical structures of aspirin (a), morphine (b), quinine (c), clarhamnoside (d), carijenone (e) and violatinctamine (f)

Source: Blunt *et al.* (2005)

1.2.2.2.1 Antimicrobial substances produced by fungi

Fungi are a good source of antibitoics. Antibiotics produced by fungi are such as penicillin, the first antibiotic produced by *Penicillium notatum* that can inhibit the growth of a wide range of bacteria, cephalosporin produced by *Cephalosporium* spp., fusidic acid produced by *Fusidium coccineum* and cyclosporine A produced by *Trichoderma polysporum*, etc (Onlamoon, 2008).

1.2.2.2.2 Antimicrobial substances produced by bacteria.

1.2.2.2.2.1 Gram-negative bacteria.

Gram-negative bacteria including a number of *Pseudomonas* spp. produce antibiotics such as mupiracin, pirrolnitrin and sulfazecin while *Myxobacteria* can produce althiomycin, pyrrolnitrin, ambruticin, sorangicin A and B, etc (Onlamoon, 2008).

1.2.2.2.2.2 Gram-positive bacteria

The Gram-positive bacteria that can produce antibiotics include the majority of *Bacillus* such as *B. licheniformis* (bacitracin), *B. polymyxa* (polymyxin) etc. However, the Gram-positive bacteria group of actinomycetes is the most important one for the production of antibiotics. It has been reported that approximately 75 % of antibiotics are derived from actinomycetes isolated from soil, such as *Nocardia lactamdurans* produce nocardicin, rifamycins, and ristocetin etc. Examples of antibiotics from Streptomycetes such as actinomycin D, echinomycin, sporaviridin Al, filipin, enterocin, maltophilin and pyridindolol etc., and antibiotics from *Micromonospora* found both on land and sea, such as the BU-4664L and lkarugamycin etc (Onlamoon, 2008).

A number of bioactive natural products produced by microorganisms are shown in Table 1.

Table 1 Approximate number of bioactive microbial natural products (2002) according to their producers

Source	Antibiotic	Other bioactive metabolites	Total bioactive metabolites	Practically used (in human therapy)	Inactive metabolites
Bacteria	2900	900	3800	10-12 (8-10)	3000 - 5000
Actinomycetales	8700	1400	10100	100-120 (70~75)	5000 - 10000
Fungi	4900	3700	8600	30-35 (13-15)	2000 - 15000
Total	16500	6000	22500	140-160 (~100)	20000 - 25000

Source: Berdy (2005)

Actinomycetes are the most widely distributed groups of microorganism in nature. They are a large part of the microbial population of the soil (Oskay *et al.*, 2004). Among actinomycetes, *Streptomyces* is the dominant genus. About 90% of the actinomycetes isolated from soil can be assigned to the genus *Streptomyces* (Paul and Clark, 1989).

Obviously various actinomycetales, in particular the *Streptomyces* species and filamentous fungi, and to a lesser extent several bacterial species are the most noteworthy producers both in respect of numbers, versatility and diversity of structures of the produced metabolites. The significance and frequency of these main types of microoganisms as producers of bioactive metabolites had varied significantly during the last decades. In the beginning of the antibiotic era, antibiotics from fungal sources (penicillin, griseofulvin) and bacteria (gramicidin) were in the foreground of interest, but after the discovery of streptomycin and later chloramphenicol, tetracyclines and macrolides the attention turned to the *Streptomyces* species. In the fifties and sixties the majority (70%) of antibiotics were discovered from these species. In the next two decades the significance of the non-*Streptomyces*

actinomycetales species (rare actinos) increased, producing up to a 25-30% share of all known antibiotics (Figure 3).

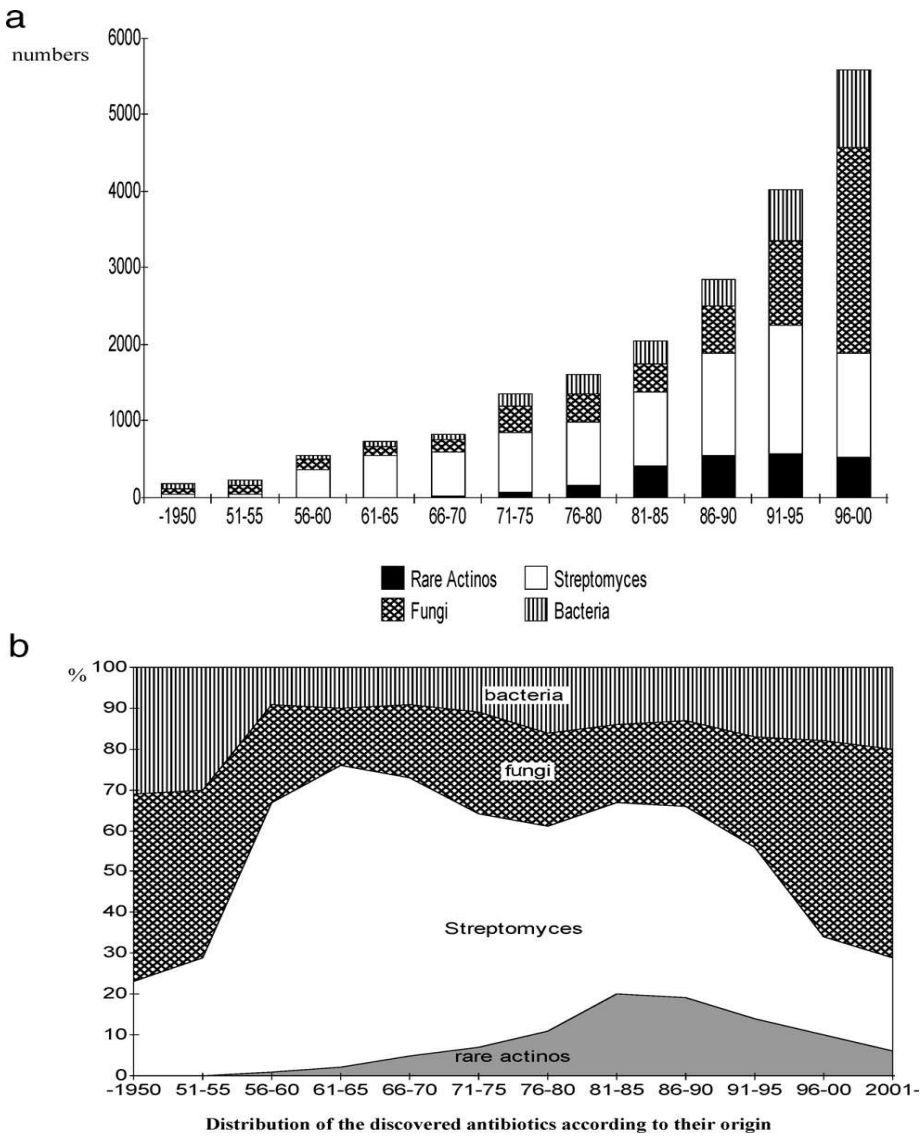


Figure 3 Distribution of the discovered antibiotics according to their origin.
Source: Berdy (2005)

From the early nineties the number of bioactive compounds isolated from various filamentous and other microorganisms and higher fungal species had continuously increased up to more than 50% by the turn of the millennium (2000). The interest in bacteria in recent years had only slightly increased. However, 45% of the presently known bioactive metabolites, from over 10,000 compounds were still isolated from various actinomycetales species, 34% from *Streptomyces* and 11% from the rare actinos. The most frequent producers, the *Streptomyces* species produce 7,600 compounds (74% of all actinomycetales), while the rare actinos represent 26%, altogether 2,500 compounds. The representation of rare actinos products in 1970 was only 5%. In this group *Micromonospora*, *Actinomadura*, *Streptoverticillium*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora* and *Streptosporangium* species were the most frequent producers, each produces several hundreds of antibiotics. The numbers of actinomycetales species, including the all rare actinos, known to produce bioactive metabolites, are summarized in Table 2.

Table 2 Number of actinomycetales species producing bioactive microbial metabolites

Actinomycetales	**No. of species**	**Actinomycetales**	**No. of species**
Streptomycetaceae:		**Thermomonosporaceae**:	
Streptomyces	~8000	*Actinomadura*	345
Streptoverticillium	258	*Saccharothrix*	68
Kitasatosporia	37	*Microbispora*	54
Chainia	30	*Actinosynnema*	51
Microellobosporia	11	*Nocardiopsis*	41
Nocardioides	9	*Microtetraspora*/*Nonomuria*	26/21
		Thermomonospora	19
Micromonosporaceae:		*Micropolyspora*/*Faenia*	13/3
(Actinoplanetes)		*Thermoactinomyces*	14
Micromonospora	740	*Thermopolyspora*	1
Actinoplanes	248	*Thermoactinopolyspora*	1
Dactylosporangium	58		

Table 2 (Cont.) Number of actinomycetales species producing bioactive microbial metabolites

Actinomycetales	No. of species	Actinomycetales	No. of species
Micromonosporaceae: (Actinoplanetes)		**Mycobacteriaceae: (Actinobacteria)**	
Ampullariella	9	*Nocardia*	357
Glycomyces	2	*Mycobacterium*	57
Catenuloplanes	3	*Arthrobacter*	25
Catellatospora	1	*Brevibacterium*	17
		Proactinomyces	14
Pseudonocardiaceae:		*Rhodococcus*	13
Saccharopolyspora	131	**Other (unclassified) species**:	
Amycalotopsis/*Nocardia*	120/357	*Actinosporangium*	30
Kibdellosporangium	34	*Microellobosporia*	11
Pseudonocardia	27	*Frankia*	7
Amycolata	12	*Westerdykella*	6
Saccharomonospora	2	*Kitasatoa*	5
Actinopolyspora	1	*Synnenomyces*	4
		Sebekia	3
Streptosporangiaceae: (Maduromycete)		*Elaktomyces*	3
		Excelsospora	3
Streptosporangium	79	*Waksmania*	3
Streptoalloteichus	48	*Alkalomyces*	1
Spirillospora	11	*Catellatospora*	1
Planobispora	10	*Erythrosporangium*	1
Kutzneria	4	*Streptoplanospora*	1
Planomonospora	2	*Microechinospora*	1
		Salinospora	1

Source: Berdy (2005)

In the light of our accumulated knowledge and from statistical data, the potency of the *Streptomyces* species should not be underestimated. Their capacity to produce promising new compounds will certainly be unsurpassed for a long time and they still have been producing the majority of the antibiotics used in chemotherapy (Berdy**,** 2005). Many investigators are still looking for new bioactive compounds from *Streptomyces* spp. and have recently found that particular *Streptomyces* spp. can produce antibacterial activities against *S. aureus*, MRSA, vancomycin-resistant *S. aureus* (VRSA), *B. subtilis, S. epidermidis, Enterococcus faecalis*, *Micrococcus luteus*, *E. coli, P. aeruginosa*, *Klebsiella* sp. (Arasu *et al.*, 2008; Selvameenal *et al.*, 2009; Yadav *et al.*, 2009; Duraipandiyan *et al.*, 2010; Aouiche *et al.*, 2012; Dasari *et al.*, 2012).

1.2.2.2.3 Bioactive substances from actinomycetes

1.2.2.2.3.1 Antimicrobial substances from actinomycetes

Actinomycetes especially the genus *Streptomyces* are a good source of antibiotics (Table 3). The most common antibiotics used to treat infections are from actinomycetes (45%), followed by fungi (38%) and other bacteria (17%). Although there are many commercially available antibiotics, there is a need to find new antimicrobial substances because of the problem of drug resistance. In Table 4 there are examples of studies for the screening of antibiotic producing actinomycetes and their antimicrobial compounds since the year 2000.

Table 3 Some antibiotics produced by *Streptomyces* spp. and their properties

Antibiotic	Source	Spectrum	Mode of action
Amphotericin B	*S. nodosus*	Fungi	Inactivate membranes containing sterol
Chloramphenicol	*S. venezuelae*	Gram-positive and Gram-negative bacteria	Inhibits protein synthesis (translation step)
Chlortetracycline	*S. aureofaciens*	Gram-positive and Gram-negative bacteria and rickettsias	Inhibits protein synthesis (translation step)
Clindamycin	*S. lincolnensis*	Gram-positive and Gram-negative bacteria esp. anaerobic *Bacteroides*	Inhibits protein synthesis (translation step)
Erythromycin	*S. erythraeus*	Gram-positive bacteria, Gram-negative bacteria not enterics, *Neisseria, Legionella, Mycoplasma*	Inhibits protein synthesis (translation step)
Imipenem	*S. cattleya*	Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Nystatin	*S. noursei*	Fungi (*Candida*)	Inactivate membranes containing sterols
Rifampicin	*S. mediterranei*	Gram-positive and Gram-negative bacteria, *Mycobacterium tuberculosis*	Inhibits transcription (bacterial RNA polymerase)
Streptomycin	*S. griseus*	Gram-positive and Gram-negative bacteria	Inhibits protein synthesis (translation step)
Tetracycline	*S. viridifaciens*	Gram-positive and Gram-negative bacteria, Rickettsias	Inhibits protein synthesis (translation step)

Source: Todar (2012)

Table 4 Bioactive substances from actinomycetes with antibacterial and antifungal activities

Investigator	Actinomycetes	Substances produced/activity
Capon *et al*., 2000	Actinomycetes species MST-MA 190	- Lorneamides A and B, a new type of aromatic aminde compound belongs to tri-alkyl-substituted benzenes. - Lorneamides A can inhibit the growth of *B. subtilis* (LD_{99} 50.0 μg/ml).
Cho *et al*., 2001	*Streptomyces* sp.	- Lactone metabolites comprising 3 butanomides and 3-hydroxy-Y-butyrolactones. - 3-hydroxy-Y-butyrolactones (50.0 μg/disk) can inhibit the growth of *C. albicans* with an inhibition zone of 19.0 mm.
Schumacher *et al*., 2001	*Nocardiopsis dassonvillei*	- Kahakamides A and B - Kahakamide A can inhibit *B. subtilis*.
Woo *et al*., 2002	*Streptomyces* sp. strain AP77	- Heterologous protein consisting of 3 units of the SAP1, SAP2, and SAP3 - This protein could inhibit the growth of *Pythium porphyrae* the disease red rot in seaweed *Porphyra* spp. (MIC of 1.65 μg/disk) and *Pythium ultimum* (MIC of 6.3 μg/disk).
Schumacher *et al*., 2003	*Streptomyces* sp. strain BD21-2	- Bonactin from culture filtrate - Bonactin can inhibit the growth of *Bacillus megaterium, M. luteus, Klebsiella pneumoniae, S. aureus, Alcaligenes faecalis, E. coli* and *Saccharomyces cerevisiae* with inhibition zones in the range 7-10 mm.

Table 4 (cont.) Bioactive substances from actinomycetes with antibacterial and antifungal activities

Investigator	Actinomycetes	Substances produced/activity
Stritzke *et al*., 2004	*Streptomyces* sp. strain B6007	- Two new caprolactones: (R)-10-methyl-6-undecanolide and (6R, 10S)-10-methyl-6-dodecanolide. - Caprolactones have the ability to inhibit the growth of *Streptomyces viridochromogenes, S. aureus, Mucor miehei,* and *C. albicans*. No activity against *E. coli* and *B. subtilis*.
Sae-lim, 2005	Actinomycetes CNA053C isolated from sea in Thailand.	- Extract F5 can inhibit *S. aureus, B. subtilis* and *E. faecalis* with MIC values of 0.58, 0.29 and 0.29 μg/ml, respectively. F5.2 extract inhibited *S. aureus, B. subtilis, E. faecalis, P. aeruginosa, S. typhi* and *S. sonnei*, with MIC values of 0.58, 0.146, 0.29, 9.38, 150 and 300 μg/ml, respectively.
Singh *et al.*, 2006	*Streptomyces* spp. isolated from soil in India	- Broad spetrum *Streptomyces* spp. (12 isolates) inhibited *B. subtilis, S. aureus, E. coli* and *Fusarium moniliforme*.
Parungao *et al.*, 2007	*Micromonospora* isolated form sea	- Actinomycetes 54 isolates inhibited *E.coli, S. aureus, C. utilis* and *Aspergillus niger*. 52% of actinomycetes had anti- *E. coli, S. aureus, C. utilis* and 13% had antifungal activity against *A. niger*.
Arasu *et al.*, 2008	*Streptomyces* spp. ERI-26	- ERI-26 showed activity against bacteria such as *B. subtilis, S. aureus, S. epidermidis, E. faecalis* and fungi such as *C. albicans, A. niger* and *A. flavus.* - MIC of ERI-26 against *S. epidermidis* was 375 μg/ml and against *C. albicans* was 500 μg/ml

Table 4 (cont.) Bioactive substances from actinomycetes with antibacterial and antifungal activities

Investigator	Actinomycetes	Substances produced/inhibition.
Gandhimathi *et al.,* 2008	*Streptomyces* sp.	- Isolate CPI 13 produced anti- *K. pneumonia* with an inhibition zone of 65.94 mm and extract from CPI 13 inhibited *C. tropicalis* with MIC and MFC values of 10 and 12.5 μg/ml, respectively.
Sawasdee, 2008	*Streptomyces* spp. from soils from Thailand.	- Crude ethyl acetate extracts had antifungal activity against *Cryptococcus neoformans* with MIC values in the range of 2-128 and 1-32 μg/ml, respectively and against *S. aureus* and MRSA with MIC values of 128 and 200 μg/ml. No extracts inhibited *E. coli, P. aeruginosa* and *C. albicans*.
Manjula *et al.,* 2009	Actinomycetes isolated from soil	- Strain A_2, A_3, A_5 inhibited Gram-positive and Gram-negative bacteria. The immobilized microorganisms were more effective than the free cell.
Selvameenal *et al.,* 2009	*Streptomyces hygroscopicus*	- Production of anti-MRSA, VRSA, *E. coli, P. aeruginosa, Klebsiella* sp.
Yadav *et al.,* 2009	*Streptomyces* isolated from soil from Bay of Bengal, India	- Strains A160, A161 and A164 inhibited growth of the Gram-positive bacteria, *B. subtilis, S. aureus* and *M. luteus* as well as few fungal pathogens, *A. niger, A. flavus, C. albicans, F. semitectum, Rhizoctonia solani* and *Botrytis cinera.* - A161 inhibited the growth of the Gram-negative bacteria

Table 4 (cont.) Bioactive substances from actinomycetes with antibacterial and antifungal activities

Investigator	Actinomycetes	Substances produced/inhibition.
Duraipandiyan *et al.,* 2010	Actinomycetes from soil of Himalaya	- ERIH-44 showed both antibacterial and antifungal activities. - The antimicrobial activity was tested against bacteria and fungi, and showed following MIC values: *B. subtilis* (<15.62 μg/ml), *S. aureus* (<15.62 μg/ml), *E. coli* (125 μg/ml) and *P. aeruginosa* (500 μg/ml), *B. cinerea* (500 μg/ml) and *Trichophyton mentagrophytes* (1000 μg/ml).
Aouiche *et al.,* 2012	*Streptomyces* sp. PAL111	- Isolate PAL111 showed a strong activity against *C. albicans*, filamentous fungi, Gram-positive and Gram-negative bacteria. The MIC were observed between 2 and 20 μg/ml for yeast, 10 and 50 μg/ml for filamentous fungi, 2and 10 μg/ml for Gram-positive and 20 and 75 μg/ml for Gram-negative bacteria.

1.2.2.2.3.2 Substances that inhibit the growth of cancer cells

In addition to the ability to produce antimicrobial substances, actinomycetes can also produce substances capable of inhibiting the growth of cancer cells and also chemicals with antioxidant property as shown in Table 5.

Table 5 Bioactive substances from actinomycetes that inhibit the growth of cancer cells

Investigator	Actinomycetes	Substances produced/inhibition.
Hardt *et al.*, 2000	Actinomycetes strain CNH-099	- A new substance neomarinone and 3 derivatives: isomarinone, hydroxydebromomarinone and methoxydebromomarinone inhibited the growth of colon cancer cells (HCT-116 colon carcinoma) with IC_{50} of 8.0 μg/ml.
Woo *et al*., 2002	*Streptomyces* sp. strain AP77	- The heterologous protein consisting of SAP1, SAP2, and SAP3 were toxic to *Porphyra yezoensis* at concentrations greater than 700.0. μg/ml in 24 hours and toxic to dermal fibroblasts at the concentration of 250.0 μg/ml in 12 hours.
Shin *et al*., 2003	Nocardioforms	- MKN-349A a new type of cyclic tetra peptide inhibited the growth of cancer cells (leukemia cell line K-562) with an LC_{50} of less than 0.05 μg/ml.
Mitchell *et al*., 2004	*Streptomyces aureoverticillatus*	- Aureoverticillactam inhibited the growth of cancer cells: HT-29, B16-F10, Jurkat cells with an EC_{50} of 3.6±2.6, 2.2±0.9 and 2.3±1.1 M, respectively.

Table 5 (Cont.) Bioactive substances from actinomycetes that inhibit the growth of cancer cells

Investigator	Actinomycetes	Substances produced/inhibition.
Dasari *et al.*, 2012	*Amycolatopsis alba* var. nov. DVR D4	- Cytotoxic compound of DVR D4, which was identified as 1(10-aminidecyl) pyridinium salt antibiotic. - The compound showed potent cytotoxic activity against cancer cell line of cervix (HeLa), breast (MCF7) and brain (U87 MG) and exhibited antibacterial activities against Gram-positive and Gram-negative bacteria.
Ravikumar *et al.*, 2012	Actinomycetes isolates	- ACT01 and ACT02 showed the IC_{50} value with 10.13±0.92 and 22.34±5.82 µg/ml, respectively for MCF-7 cell line at 48 h, and ACT01 showed the minimum level of IC_{50} value (18.54±2.49 µg/ml) with MDA-MB-231 cell line.

1.2.3 Characteristic of actinomycetes

Actinomycetes are Gram-positive bacteria in the phylum Actinobacteria, class Actinobacteria, and order Actinomycetales (Miyadoh, 1997). They have been placed in a group of bacteria since they have no nuclear membrane and mitochondria (Goodfellow and Brand, 1980). They are classified as true bacteria because their major cell wall components contain layers of peptidoglycan, muramic acid, diaminopimeric acid but no chitin and cellulose. Actinomycetes have a filamentous and branching growth pattern resulting in an extensive colony or mycelium (aerial mycelium and substrate mycelium). The mycelium in some species may break apart to form rod- or coccoid-shaped forms. Many genera also form spores; the sporangia, or spore chain may be found on aerial hyphae, on the colony surface, or free within the environment. Most members are aerobes but a few, such as *Actinomyces israelii*, can grow under anaerobic conditions. Morphological characteristics are important for the classification of actinomycetes in the Family to the Genus levels. In addition, chemotaxonomy such as the analysis of amino acids and type of sugar, key components of the cell wall is also important in their classification. The analyses of 16S and 23S ribosomal RNA genes are helpful in identification in the species level. Another important characteristic of actinomycetes is the high percentage ratio of guanine and cytosine in DNA (>55%), while other Gram-positive bacteria such as *Bacillus*, *Clostridium*, *Staphylococcus* and *Streptococcus* are less than 50, (Glazer and Nikaido, 1994).

Streptomycetes are the most widely studied and well known genus of the actinomycete family. Their life cycle shown in Figure 4 starts from spore germination and growth of the substrate mycelium. Substrate mycelium utilizes nutrients in the medium for growth and develops of aerial mycelium. After maturation, aerial mycelium develops spores. In most actinomycetes, secondary metabolites are produced during the sporulation phase.

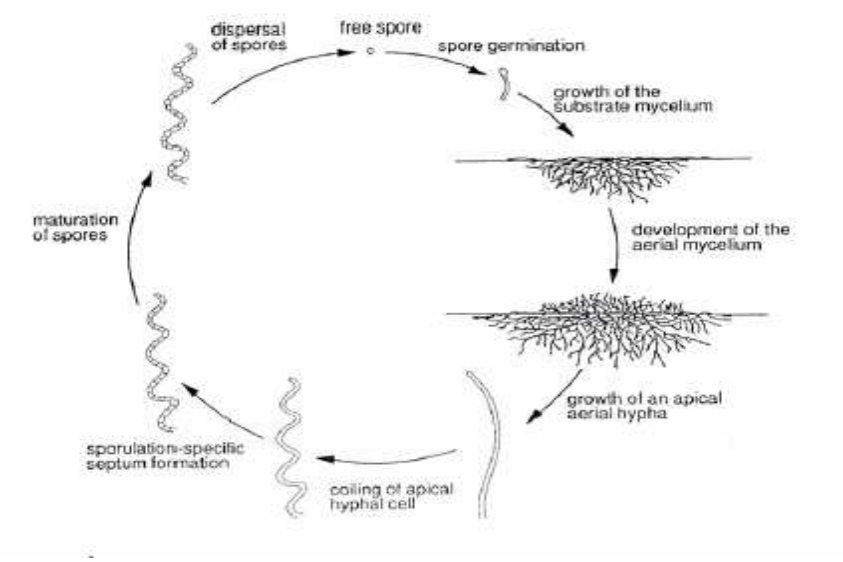


Figure 4 The life cycle of actinomycetes.
Source: Thong-Oon (2008).

The growth of actinomycetes on the solid (surface culture) and in liquid (submerged culture) culture media has different characteristics. In liquid medium cells grow as a group of mycelium, called pellets, but some actinomycetes, such as *Nocardia coralline* when grown in liquid culture medium with shaking become rod shaped and can multiply by binary fission and fragmentation, while the growth on the agar surface with the same components as the liquid medium is produced in a filamentous form with branching mycelium and fragmentation when aging (Thong-Oon, 2008).

1.2.3.1 The morphology of the actinomycetes

1.2.3.1.1 The mycelial structure (Vobis, 1997)

The mycelium of actinomycetes is similar to fungi but smaller, 0.4 to 1.2 m in size and septate with branching at the end of each mycelium. The ultrastructure of actinomycetes is not fundamentally different from bacteria (Figure 5). The main structures in the mycelium cytoplasm contains DNA, ribosomes, and others organelles such as fat or polysaccharides, polyphosphates. Mesosomes are derived from the cytoplasmic membrane and are adjacent to the cell wall. The cell wall of the mycelium is a single layer about 10-20 nm thick.

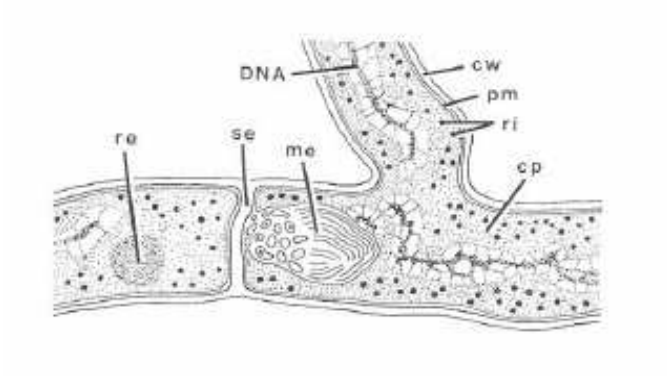


Figure 5 Cytological organization of the aerial mycelium of actinomycetes.

cp: cytoplasm	pm: plasmamembrane
cw: cell wall	me: mesosome
se: septum	ri: ribosome
DNA: nucleoid region	re: reserve material

Source: Vobis (1997)

1.2.3.1.2 Colony development (Vobis, 1997)

Spores or mycelial fragment of certain parts of the colony of origin (Figure 6A) are developed as a dietary mycelium (substrate mycelium) (Figure 6B), then the mycelia are grown through the air (aerial mycelium) (Figure 6C), which is a part directly exposed to the air and produces spore.

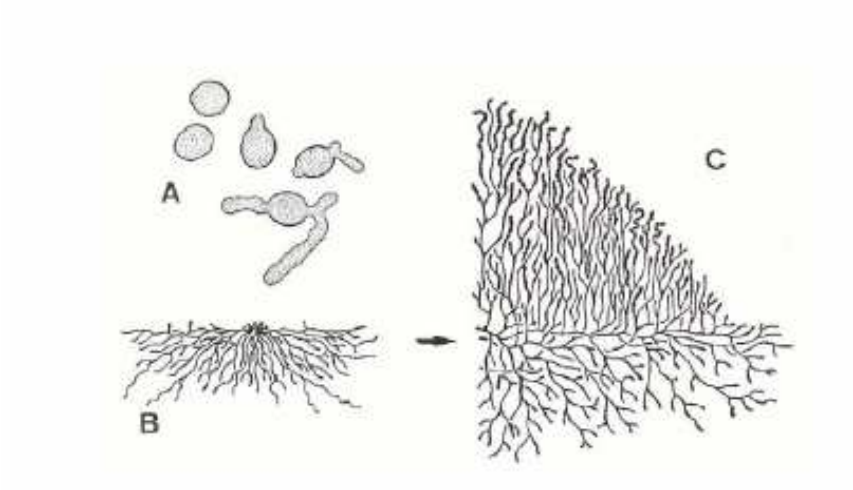


Figure 6 Development of colony of actinomycetes

A: spores or mycelial fragment of certain parts of the colony of origin

B: substrate mycelium

C: aerial mycelium

Source: Vobis (1997)

The colonies of actinomyetes are very different in different species. *Streptomyces* has tough, leathery, frequently pigmented colonies and has filamentous growth with aerial mycelium and substrate mycelium and produces an earthy odor. *Micromonospora* growing on solid media forms only substrate mycelium, which is raised and folded with areas of different colors. *Nocardia* colonies have a variable appearance, but most species appear to have aerial hyphae when viewed with a dissecting microscope, particularly when they have been grown on nutritionally limiting media. The color of actinomycete colony may be white, yellow, pink, red, brown and black.

1.2.3.1.3 Spore types (Vobis, 1997)

Actinomycete spores are formed either by subdivision of existing hyphae, by fragmentation or swelling or by endogenous spore formation. The hyphae that subdivide into spores can be sheathless or have a sheath, which partly remains on the spores after fragmentation. Spores are formed as single spore, spore chains, and spore in a sporangium.

(1.) Single spore

Single spore or the monosporous type is found in the genera *Thermomonospora*, *Micromonospora*, and *Saccharomonospora*. Single spores are produced on branched and unbranched aerial mycelium as shown in Figure 7.

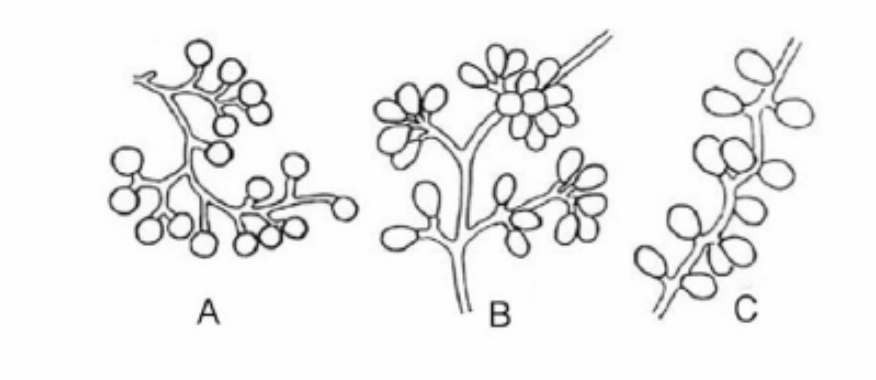


Figure 7 Single spore production

A. *Micromonospora*

B. *Thermommonospora*

C. *Saccharomonospora*

Source: Vobis (1997)

(2.) Spore chains

Most actinomycetes produce spore chains which can be divided into sections based on the length or the number of spores as disposrous or bisporous, oligosporous and polysporous (Figure 8).

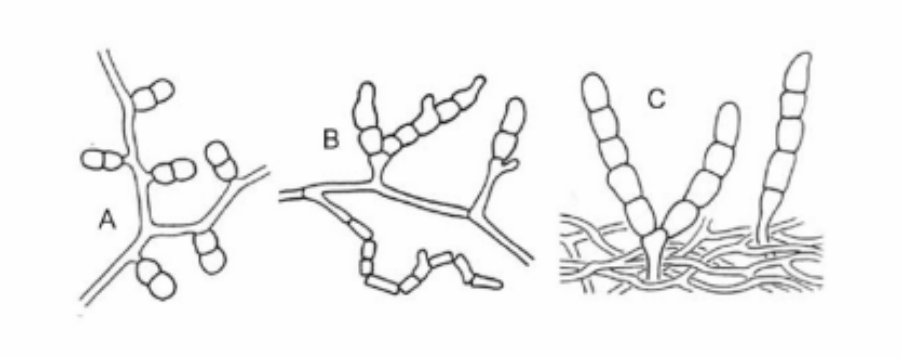


Figure 8 Dispore and oligosporous production of actinomycetes

A: Disporous of *Microbiospora*

B: Oligosporous of *Nocardia brevicatena*

C: Oligosporous of *Catellatospore*

Source: Vobis (1997)

Streptomyces and other actinomycetes can produce many spores called arthrospores. These arthrospores are similar to those produced by mitosporic fungi by fragmentation. Morphology of the spore chains can be used to identify actinomycetes. Figure 9 shows four types of long chain spores of actinomyetes.

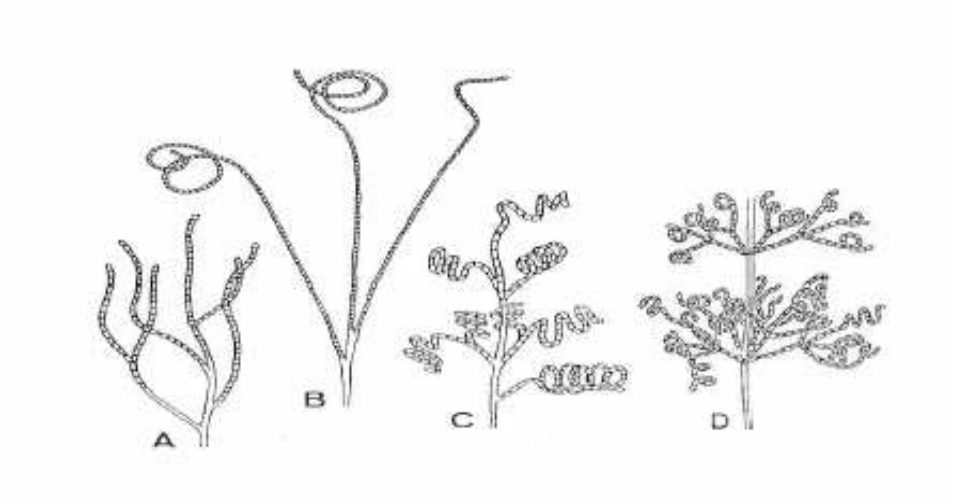


Figure 9 Spore production in long chains of actinomycetes.

A: Rectiflexibiles

B: Retinaculiaperti

C: Spira

D: Verticillati

Source: Vobis (1997)

(3) Spores in sporangia

Some actinomycetes produce spores in sporangia. The spore production in sporangia can be divided into two groups: sporangia on the mycelium surface and sporangia developed on aerial mycelium.

(3.1) Sporangia on the mycelium surface

In the genus *Actinoplanes*, sporangia are spherical with diameters of 5-15 µm, spores are in chains and branching within sporangia. It was found that *Ampullariella* can produce sporangia on substrate mycelium in a variety of forms such as spheres, cylinders, etc., as shown in Figure 10, with an average spore width of 10 µm and 15 µm long.

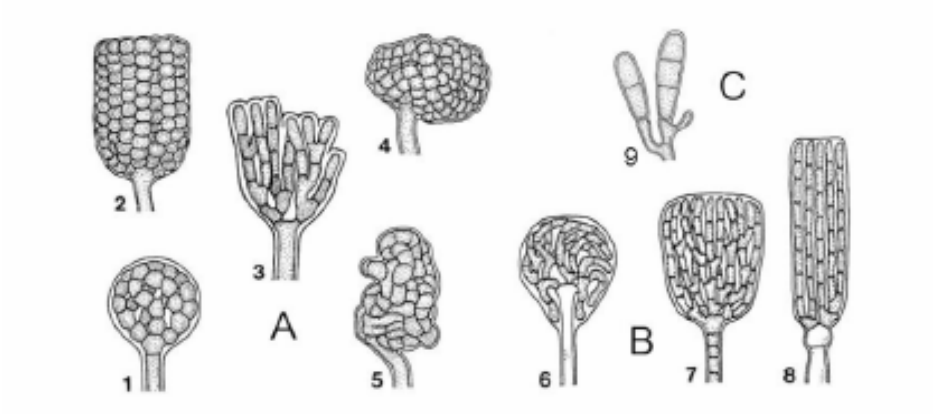


Figure 10 Spore production within sporangia

A. *Actinoplanes* (*Ampullariella*): polysporous

1. globose 2. cylindrical 3. lobate 4. subglobose 5. irregular

B. *Pilimelia*

6. ovoid 7. campanulate 8. cylindrical

C. *Dactylosporangium*: oligosporous

9. claviform

Source: Vobis (1997)

(3.2) Sporangia developed on aerial mycelia

Some genera produce sporangia on aerial mycelium such as *Planomonospora* and *Planobispora* produce cylindrical sporangia containing a single spore and disporous, respectively. *Streptosporangium* produce large spherical sporangia (~10 m in diameter) and the nonmotile spores or sporangiospores are formed by septation of coiled unbranched hyphae within the sporangium as shown in Figure 11 (Thong-Oon, 2008).

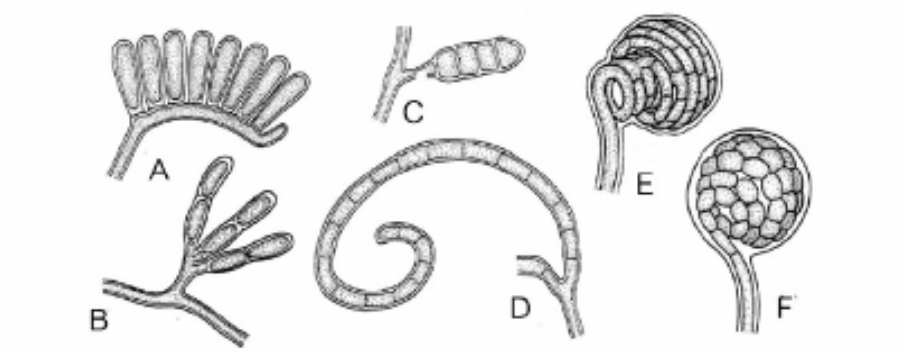


Figure 11 Sporangia developed on aerial mycelia

A. *Planomonospora* : monosporous

B. *Planobispora* : disporous

C. *Planotatraspora* : tetrasporous

D. *Planopolyspora* : polysporous

E. *Spirillospora* : polysporous

F. *Streptosporangium* : polysporous

Source: Vobis (1997)

1.2.3.1.4 The principle chemical components of the actinomycetes

The chemical components of the cell wall including major wall amino acids and the types of sugar are the key features for the chemotaxonomy of actinomycetes. Cell wall types according to major amino acids can be divided into four types as shown in Table 6 and the sugar patterns of actinomycetes are shown in Table 7.

Table 6 Chemotaxonomic characteristics of actinomycete cell wall

Type	Major amino acid	Distinguishing major constituent
I	L-Diaminopimelic acid (L-DAP)	Glycine
II	*meso*- Diaminopimelic acid (*meso*-DAP)	Glycine
III	*meso**- Diaminopimelic acid (*meso*-DAP or OH-DAP)	None
IV	*meso*- Diaminopimelic acid (*meso*-DAP)	None

*Madurose is 3-hydroxy- aminopimelic acid

Source: William (1989)

Table 7 Whole cell sugar patterns of actinomycetes

Pattern	Sugar			
	Arabinose	**Galactose**	**Madurose***	**Xylose**
A	+	+		
B			+	
C		No diagnostic sugar		
D	+			+

*Madurose is 3-O-methyl-D-galactose

Source: William (1989)

Table 8 Characteristics of the seven groups of actinomycetes

Group	Cell wall type	Cell sugar patterns	Mol % G+C	Sporangia*
Nocardioforms	IV	A	59-79	+/-
Multilocular sporangia	III	B, C, D	57-75	+/-
Actinoplanetes	II	D	71-73	+
Streptomycetes	I	-	69-78	-
Maduromycetes	III	B, C	64-74	+/-
Thermomonospora	III	major C	64-73	-
Thermoactinomycetes	III	C	52-55	-

* + with sporangia

- no sporangia

Source: Glazer and Nikaido (1994).

1.2.4 Classification of actinomycetes (William, 1989)

In general, the classification of actinomycetes is primarily based on morphology such as aerial mycelium, substrate mycelium, spores and sporangium. In addition, actinomycetes are divided into groups based on chemical analysis such as analysis of the cell wall components, analysis of the sugar components of the cell walls, types of phospholipid and types of menaquinone etc. Based on the morphology and chemical composition of cells actinomycetes can be grouped into 8 groups as follows: Nocardioform actinomycetes, Actinomycetes with multilocular sporangia, Actinoplanetes, Streptomycetes, Maduromycetes, Thermomonospora, Thermoactinomycetes, and other genera. Characteristics of seven groups of actinomycetes are shown in Table 8 and the classification actinomycetes are shown in Table 9.

1.2.4.1 Nocardioform actinomycetes

Actinomycetes in this group are diverse. Most of them have branching mycelium that breaks up into rod-shaped or coccoid elements, some strains have aerial mycelium and conidia. Actinomycetes in this group are aerobic bacteria except genus *Oerskoviae* which are facultative anaerobe. Most of the nocardioforms have a type IV (*meso*-DAP) cell wall composition. *Nocardia* and *Rhodococcus* cell walls also contain mycolic acid and sugar pattern A (arabinose and galactose).

1.2.4.2 Actinomycetes with multilocular sporangia

Actinomycetes having multilocular sporangia include 3 genera; *Dermatophilus*, *Geodermatophilus* and *Frankia*. *Geodermatophilus* has a simple rudimentary thallus. The entire thallus becomes the sporangium when spores are present. The mycelia of *Dermatophilus* are moderate to extensively developed and are almost entirely converted to long multilocular sporangium. *Frankia* produces extensive filaments and sporangia are borne intercalary, terminally or on lateral branches. All three genera have no aerial myelia. *Dermatophilus*, *Geodermatophilus* have motile spores. *Frankia* is a nitrogen-fixing genus and in microaerophilic environments they produce terminal sporangium and intercalary swellings containing nonmotile spores. All three genera have cell wall type III. *Dermatophilus* has sugar pattern B (madurose), *Geodermatophilus* sugar pattern C (no diagnostic sugars) and *Frankia* may have sugar pattern B or C.

1.2.4.3 Actinoplanetes

Actinomycetes in this group comprise five genera; *Actinoplanes*, *Ampullariella*, *Pilimelia*, *Dactylosporangium* and *Micromonospora*. Most of them have aquatic habitats and produce motile spores (zoospores) during their life cycle. Aerial mycelia are rarely developed or only sparse.

Actinoplanes, *Ampullariella*, *Pilimelia*, and *Dactylosporangium* produce motile spores within sporangia or vesicles at the tip of sporangiophore on the surface of a substrate. Spores are formed within the sporangium by fragmentation of

branched or unbranched, straight or coiled sporogenous hyphae. Multisporous sporangia have various shapes such as cylindrical, bottle shaped, flask shaped, etc. *Micromonospora* produces nonmotile spores that are borne singly, sessile or in clusters. The spores are spherical, ovoid, or ellipsoidal with much-thickened wall layers sometimes with spiny ornamentations. This group has cell wall type II (*meso*-DAP and/or 3-hydroxy-DAP) and glycine and sugar pattern D (arabinose and xylose).

1.2.4.4 Streptomycetes and related genera

Actinomycetes in this group comprise five genera including *Streptomyces*, *Streptoverticillium*, *Kineosporia*, *Intrasporangium* and *Sporichthya*. The characteristics among members of this group are very distinctive. *Sporichthya* colonies are very small. It is suggested to use microscopic examination for their recognition. They have no aerial mycelium or they are very sparse. *Kineosporia* colonies have a glistening appearance. *Kineosporia*, *Intrasporangium* and *Sporichthya* produce less aerial mycelium or no aerial mycelium. *Streptomyces* and *Streptoverticillium* produce a well developed mycelium and a long chain of arthrospores on the aerial mycelium. In *Streptoverticillium*, spore chains are arranged in a verticilliate form. *Streptomyces* produce tough, leathery, frequently pigmented colonies with a characteristic earthy odor. The surface of the colonies is wrinkled when old and spores generated on the surface of the mycelium are chalky. Bacteria in this group have cell wall type I (L-DAP and glycine).

1.2.4.5 Maduromycetes

There are seven genera of actinomycetes in this group including sporangiate actinomycetes (*Planobispora, Planomonospora, Spirillospora* and *Streptosporangium*) and actinomycetes forming paired or short chains of spores on aerial mycelia (*Actinomadura*, *Microbispora,* and *Microtetraspora*). Some species have motile spores such as *Planobispora, Planomonospora* and *Spirillospora*. They have type III cell wall (*meso*-DAP). *Planobispora*, *Planomonospora, Spirillospora* and *Streptosporangium* have sugar pattern B {3-O-methyl-D-galactose (madurose)}

while *Actinomadura, Microbispora,* and *Microtetraspora* have sugar patterns B or C (no diagnostic sugars).

1.2.4.6 Thermomonospora and related species

Actinomycetes in this group comprise 4 genera: *Thermomonospora, Actinosynnema, Nocardiopsis,* and *Streptoalloteichus.* They have common cell wall type III (*meso*-DAP), no diagnostic sugars (type C), and no mycolic acid. Their morphologies are diverse. *Thermomonospora* is thermophilic (can grow in the temperature range 40-48^{o}C) and produces single spores on aerial, and occasionally on substrate mycelium. *Actinosynnema* and *Nocardiopsis* produce spores in chain but *Actinosynnema* forms synnemata on the agar surface whereas *Nocardiopsis* has a well developed substrate mycelium that tends to be fragmented into coccoid and bacillary elements. *Streptoalloteichus* produces spores in a sporangium.

1.2.4.7 Thermoactinomycetes

This group comprises only one genus *Thermoactinomyces*. They grow well at high temperatures and produce single endospores. Their G+C content is lower than those of other actinomycetes. Their 16S rDNA sequences are closely related to *Bacillus* but they produce a well-developed mycelium, therefore they are still linked together with other actinomycetes. All species produce aerial mycelia. *T. dichotomous* produces a yellow colony and others are white. They have cell wall type III (*meso*-DAP), but without diagnostic sugars and amino acids.

1.2.4.8 Other genera

Actinomycetes in this group are not correlated with other groups. There are four genera comprising. *Glycomyces*, *Kibdelosporangium*, *Kitasatosporia* and *Saccharothrix*. All genera produce aerial mycelia.

Table 9 Classification of actinomycetes in the Order Actinomycetales

Order	Families	Genera
Actinomycetales	Actinomycetaceae	
	Micrococcaceae	
	Bogoriellaceae	*Bogoriella*
	Rarobateraceae	*Rarobacter*
	Sanguibacteriaceae	*Sanguibacter*
	Brevibacteriaceae	*Brevibacterium*
	Cellulomonadeceae	*Cellulomonas*
		Oerskovia
	Dermabacteriaceae	*Dermabacteria*
		Brachybacterium
	Dermatophilaceae	*Dermatophilus*
	Dermacoccaceae	
	Intrasporangiaceae	
	Jonesiaceaea	*Jonesia*
	Microbacteraceae	
	Beutenberggiaceae	*Beutenberggia*
	Promicromonosporaceae	*Promicromonospora*
	Corynebacteriaceae	*Corynebacterium*
	Dietziaceae	*Dietzia*
	Gordoniaceae	*Gordonia*
		Skermania
	Nocardiaceae	*Nocardia*
		Rhodococcus
	Williumsiaceae	*Williumsia*
	Micromonosporaceae	

Source: Stackbrandt *et al*. (1997)

Table 9 (Cont.) Classification of actinomycetes in Order Actinomycetales

Order	Families	Genera
Actinomycetales	Propionibacteriaceae	
	Nocardioidaceae	
	Pseudonocardioidaceae	
	Actinosynnemataceae	
	Streptomycetaceae	
	Streptosporangiaceae	
	Nocardiopsaceae	*Nocardiopsis*
		Thermobofoda
	Thermomonosporaceae	
	Frankiaceae	*Frankia*
	Geodermatophilaceae	
	Microspheraceae	
	Sporichthyaceae	*Sporichthya*
	Acidothermaceae	*Acidothermus*
	Kinesporiaceae	
	Glycomycetaceae	*Glycomyces*

Source : Stackbrandt *et al*. (1997)

1.3 Objectives of this research

1) To isolate actinomycetes from soils and screen for their ability to produce antimicrobial substances against human pathogens by a cross streak technique and hyphal growth inhibition.
2) To cultivate the selected active actinomycetes in broth medium for chemical extraction of active substances.
3) To determine the minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) or minimal fungicidal concentration (MFC) of the crude extracts.
4) To determine the effect of the best active crude extracts on the targeted microorganisms by scanning electron microscopy.
5) To optimize the culture conditions for the production of active metabolites of the top two active actinomycetes.
6) To identify the best active actinomycetes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Sampling of soil samples

Soil samples were collected from ten locations in the south of Thailand (Table 10).

Table 10 Sources of soil samples for actinomycetes isolation

Collecting site	Province
Pak Panang Basin Region	Nakhon Si Thammarat
Karom Waterfall	Nakhon Si Thammarat
Khao Maha Chai	Nakhon Si Thammarat
Lam Talumphuk	Nakhon Si Thammarat
Ton Nga Chang Waterfall	Songkhla
Songkhla Lake	Songkhla
Khao Chaison	Phattalung
Kanghurae	Phattalung
Klong Palean	Trang
Khao Phap Pha	Trang

2.1.2 Microorganisms

Tested bacteria

- *Staphylococcus aureus* ATCC 25923
- Methicillin-resistant *Staphylococcus aureus* SK1 (MRSA-SK1) isolated from patient by Pathology Department, Faculty of Medicine, Prince of Songkla University
- *Escherichia coli* ATCC 25922
- *Pseudomonas aeruginosa* ATCC 27853

Tested fungi

Yeasts

- *Candida albicans* ATCC 90028
- *Candida albicans* NCPF 3153
- *Cryptococcus neoformans* ATCC 90112
- *Cryptococcus neoformans* ATCC 90113

Filamentous fungi

- *Microsporum gypseum* SH-MU4 isolated from patient by Microbiology Department, Faculty of Medicine Siriraj Hospital, Mahidol University
- *Penicillium marneffei* isolated from patient by Pathology Department, Faculty of Medicine, Prince of Songkla University

Actinomycetes

- 40 new isolates from soils from ten locations in southern Thailand (Table 12).
- 60 isolates provided by Assoc. Prof. Dr. Vasun Petcharat, Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University.

2.1.3 Chemicals

- 0.85% NaCl, normal saline solution (NSS)
- Lacto phenol cotton blue
- McFarland Standard
- Dimethyl sulfoxide (DMSO) (Merck)
- Resazurin (Sigma Chemical Co., USA)

2.1.4 Media

- Mueller-Hinton agar (MHA) (Difco)
- Sabouraud dextrose broth (SDB) (Difco)
- Sabouraud dextrose agar (SDA) (Difco)
- Nutrient broth (NB) (Difco)
- Nutrient agar (NA) (Difco)
- RPMI-1640 without phenol-red (pH7) (Sigma Chemical Co., USA)
- Yeast extract-malt extract agar (ISP-2) (Appendix)
- Yeast extract-malt extract broth (Appendix)
- Actinomycete Isolation Agar (AIA) (Difco)

2.1.5 Antibiotics

- Vancomycin (Fujisawa, USA)
- Gentamicin (Oxoid)
- Amphotericin B (Bristol-Myer Squibb Co., USA)
- Miconazole (Sigma Chemical Co., USA)

2.1.6 Equipment

- Autoclave (Tomy, SS-320)
- Hot air oven (Sanyo, MOV212)
- Centrifuge (Hermle)
- Balance (Diethelm & Co., Ltd)
- pH meter (Beckman, 360)
- Laminar flow (Hotpack, 527044)
- Vortex mixer (Lab-Line, 1297)

- Microscope (Olympus, CX31)
- Stereo zoom microscope (Olympus, SZ40)
- Water bath (Memmert, W350)

2.2 Methods

2.2.1 Sample Collection (adapted from Parungao *et al*., 2007)

Soil samples were collected from ten different natural locations such as mountain areas, waterfalls, mangroves etc. From each location, five soil samples were collected from 10 to 15 cm below the surface. Each sample was placed in a small pre-labeled plastic bag which was tightly sealed. Soil samples were air dried for one week at the Mycology Laboratory, Department of Microbiology, Faculty of Science, Prince of Songkla University before isolation.

2.2.2 Isolation of Actinomycetes

Soil samples were air-dried and ground into powder. One gram of each sample was suspended in 9 ml sterile distilled water and serially diluted to 10^{-4}. Then 0.1 ml of the dilutions 10^{-2} to 10^{-4} were spread onto Actinomycete Isolation Agar (AIA) and incubated at room temperature (RT) for one week. After incubation, actinomycete isolates were distinguished from other microbial colonies by their morphological characteristics such as tough, leathery colonies which are partially submerged into the agar (Jensen *et al.,* 1991). The pure isolates were maintained on yeast extract-malt extract (YME) agar slants at RT (Parungao *et al*., 2007).

2.2.3 Antimicrobial testing of actinomycetes by cross streak technique

One hundred isolates of actinomycetes were tested for antimicrobial activity against bacteria and yeasts by cross streak technique. Each actinomycete isolate was cultured in YME broth on a rotary shaker at 120 rpm at 30°C for 1 week. One loop of each isolate was streaked along the center of YME agar and incubated at RT for 4 weeks or until sporulation occured. Then one loop of each tested strain was streaked on YME agar at the edge of the actinomycetes streak as shown in Figure 12. Plates were incubated at 35°C for 24 h for bacteria and *C. albicans*. After measuring the inhibition zones for bacteria and *C. albicans*, plates were further incubated at room temperature for 48 h and measured the inhibition zones for *C. neoformans* (Sawasdee, 2008).

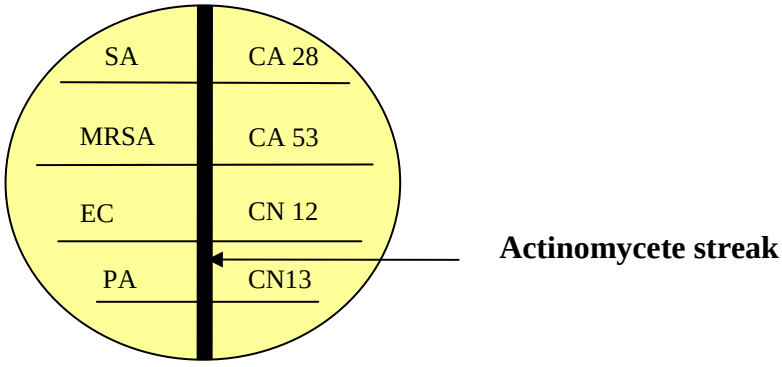


Figure 12 Diagram of antimicrobial testing of actinomycetes by cross streak technique

SA	=	*Staphylococcus aureus* ATCC 25923	CA53	=	*Candida albicans* NCPF 3153
MRSA	=	methicillin-resistant *S. aureus* SK1	CN12	=	*Cryptococcus neoformans* ATCC 90112
EC	=	*Escherichia coli* ATCC 25922	CN13	=	*Cryptococcus neoformans* ATCC 90113
PA	=	*Pseudomonas aeruginosa* ATCC 27853	CA28	=	*Candida albicans* ATCC 90028

2.2.4 Hyphal inhibition by actinomycetes (adapted from Jimenez-Esquilin and Roane, 2005)

Each actinomycete isolate was streaked onto one half of YME agar plate and incubated at RT for 4 weeks or until sporulation occurred. Then a mycelial plug from an actively growing fungal colony (*M. gypseum* or *P. marneffei*) was placed about 0.5 cm from the edge of actinomycete streak (Figure 13) and incubated at RT for 7 days. Fungal inhibition was observed everyday. The radii of the fungal colony on the test and control plates were then measured. The percentage of hyphal growth inhibition was calculated using the following formula:

$$\% \text{ inhibition} = 100-(\frac{R^2 \quad 100}{r^2}) \quad \text{(Gamliel } et\ al.\text{, 1989)}$$

R = radius of treated colony, r = radius of the control colony

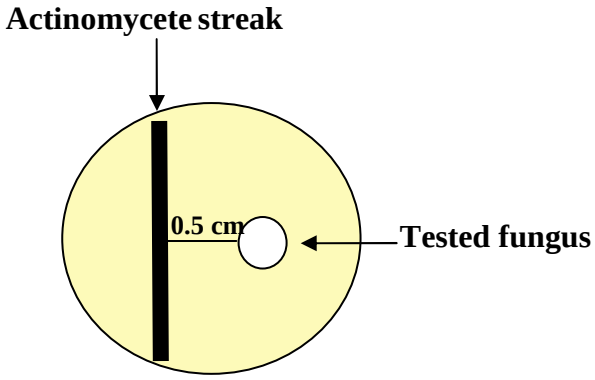


Figure 13 Diagram of fungal inhibition test by actinomycetes

2.2.5 Actinomycete fermentation and culture filtrate extraction (adapted from Duangsook, 2010)

Actinomycete isolates that showed inhibition zone over 25 mm and hyphal growth inhibition over 80% were selected and cultured in YME broth for extraction of bioactive compounds. Each active actinomycete isolate was cultured in ISP-2 broth on a rotary shaker at 120 rpm at 30°C for 1 week. Then 10^8 spore/ml of the actinomycete suspension was added to 100 ml ISP-2 broth in flat bottles and incubated at RT for 6 weeks.

The culture filtrate of actinomycetes was extracted three times with an equal volume of ethyl acetate (EtOAc) in a separating funnel. The EtOAc layer was dried over anhydrous sodium sulfate (Na_2SO_4) and evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to obtain a crude BE extract. The mycelia were extracted with 500 ml of methanol (MeOH) for 2 days. The aqueous MeOH layer was concentrated under reduced pressure. H_2O (50 ml) was added to the extract and the mixture was then mixed with hexane (100 ml). The aqueous layer was then extracted three times with an equal volume of EtOAc. The hexane extract and the combined EtOAc extracts were dried over Na_2SO_4 and evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to give CH and CE extracts, respectively (Figure 14).

Figure 14 Actinomycete fermentation and crude extracts from actinomycete

A : Actinomycete fermentation in flat bottles

B : Crude extracts

2.2.6 Screening for antimicrobial activities

2.2.6.1 Inoculum preparation

Tested bacteria (*S. aureus* ATCC 25923, MRSA-SK1, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) were streaked onto nutrient agar (NA) and incubated at 35^{o}C for 18-24 h *C. albicans* and *C. neoformans* were streaked onto Sabouraud dextrose agar (SDA) and incubated at 35^{o}C for 18-24 h and at RT for 48 h, respectively. Three to five single colonies of bacteria were picked into nutrient broth (NB), of *C. albicans* and *C. neoformans* were picked into RPMI-1640, and both incubated at 35^{o}C for 3-5 h while shaking at 150 rpm. After incubation, sterile normal saline solution (NSS) was used to adjust the turbidity to equal the 0.5 McFarland standard (MF) for bacteria and the 2.0 MF for yeasts. Agar plugs of *M. gypseum* and *P. marneffei* were placed on SDA and incubated at 25^{o}C for 2-3 weeks or until it produced spores. The spores were collected by scraping with a sterile glass beads and were suspended with NSS. The spore suspension was adjusted to 4 x 10^{3} – 5 x 10^{4} CFU/ml using a hemacytometer.

2.2.6.2 Testing for antibacterial activity (modification of CLSI M7-A4, 2002a)

Actinomycete crude extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions of 100 mg/ml, and stored at -20^{o}C until used. DMSO was used to dilute to 1:10 and Mueller-Hinton broth (MHB) for further dilutions of 1:25 to obtain concentrations of 400 µg/ml. Triplicate 50 µl samples of crude extracts (400 µg/ml) were placed into sterile 96-well microtiter plates. The bacterial inocula (0.5 MF) were diluted 1:200 (~10^{6} CFU/ml) using MHB and 50 µl added to the top well in each row so that the final concentration of crude extract was then 200 µg/ml. Plates were incubated at 35^{o}C for 15 h, then 10 µl of resazurin indicator (0.18%) was added to each well and examined after incubation for 2-3 h at 35^{o}C for the completed incubation time (Sarker *et al.*, 2007).

Vancomycin and gentamicin at final concentrations of 10 µg/ml were used as standard antibacterial agents for positive inhibitory controls against

Gram-positive and Gram-negative bacteria, respectively and for comparisons with the extracts.

2.2.6.3 Testing for antifungal activity (yeasts) (modification of CLSI M27-A2, 2002b)

Yeasts were tested in a similar way to bacteria but using RPMI-1640 medium. Microtiter plates were incubated at 35°C for 24 h for *C. albicans* and 48 h at RT for *C. neoformans*, then 10 µl resazurin indicator (0.18%) was added to each well and examined after incubation for 5 h at 35°C (adapted from Sarker *et al.*, 2007).

Amphotericin B at a final concentration of 10 µg/ml was used as a positive inhibitory control and for comparison with the extracts.

2.2.6.4 Testing for antifungal activity (filamentous fungi) (modification of CLSI M38-A, 2002c)

Filamentous fungi (*M. gypseum* and *P. marneffei*) were tested in a similar way to bacteria but using RPMI-1640 medium. Microtiter plates were incubated at 25°C for 6 days, then 10 µl resazurin indicator (0.18%) was added to each well and incubated for one day at 25°C.

Miconazole (32 µg/ml) was used as the standard antifungal drug for *M. gypseum* and for comparison with the extracts.

Amphotericin B (10 µg/ml) was used as a positive inhibitory control for *P. marneffei* and for comparison with the extracts.

Interpretation of the screening results

After incubation, a blue or purple color of the wells indicated inhibition of growth (positive result) and a pink color meant growth had occurred (negative result).

Crude extracts shown to have antimicrobial activity were assayed further for their minimal inhibitory concentrations (MIC), minimal bactericidal concentrations (MBC) and minimal fungicidal concentrations (MFC).

2.2.6.5 Determination of minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) of actinomycete crude extracts

The MICs of crude extracts were determined by a broth microdilution method according to a modification of CLSI M7-A4 (CLSI, 2002a) against bacteria, CLSI M27-A2 (CLSI, 2002b) against yeasts and CLSI M38-A (CLSI, 2002c) against filamentous fungi. Crude extracts were diluted using the serial dilution method starting with final concentrations of 128, and diluting to 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 µg/ml, each tested in triplicate.

After incubation under appropriate conditions, the lowest concentration of extract that inhibited growth (blue or purple color) was recorded as the MIC.

The MBCs and the MFCs of actinomycete crude extracts were determined by the streaking method. Concentrations of crude extract less dilute than the MIC and the MIC were streaked onto NA plate for bacteria and SDA plates for yeasts and filamentous fungi. Plates were incubated under appropriate conditions and the lowest concentration of extract that killed organisms (no growth) was recorded as the MBC or MFC.

2.2.7 Effect of crude extract on target cells detected by scanning electron microscopy (SEM)

Tested bacteria (*S. aureus* ATCC 25923 and MRSA-SK1) were streaked onto MHA and incubated at 35^{o}C for 24 h. Three to five single colonies of bacteria were picked into MHB and incubated at 35^{o}C for 24 h and centrifuged at 5,000 rpm, 5 min. Cells of bacteria were resuspended in MHB (10 ml) and treated with the extracts at various concentrations (MIC, 2MIC and 4MIC), incubated at 35^{o}C for 18 h. Cells of bacteria treated with 1% DMSO were used as control. The cells were collected by centrifugation and washed with the phosphate buffer saline (PBS) 3 times. The samples were fixed in 2.5% glutaraldehyde in PBS for 1 h, then washed with PBS 3 times, post fixed in 1% osmium tetroxide for 1 h, washed with sterile

water 3 times and dehydrated with alcohol series (50%, 70%, 80%, 90% and 100% of ethanol) before processing for electron microscopy at The Scientific Equipment Center, Prince of Songkla University.

2.2.8 Optimization of antifungal metabolite production (modification of Augustine *et al*., 2005)

2.2.8.1 Agitation: comparison between shaking and static conditions.

A spore suspension of actinomycetes was prepared in distilled water from cultures grown on ISP-2 medium. The suspension was added to 100 ml ISP-2 broth in flat bottles to obtain about 10^8 spores/ml of the liquid medium and incubated at RT for 5 weeks. Actinomycete inocula were added into flasks containing 100 ml of ISP-2, pH7 broth and incubated statically and on a rotary shaker at 200 rpm at 30^{o}C for 5 weeks. Culture filtrates were harvested every week for antimicrobial activity detection by the agar-well diffusion and the biomass measurement

2.2.8.1.1 The agar-well diffusion

Tested bacterial suspensions (0.5 MF) were spread onto MHA. Holes (diameter of 6 mm) were then punched in the agar and filled with 80 µl of culture filtrates. The plates were incubated at 37°C for 18-24 h. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter (Pandey *et al*., 2004).

2.2.8.1.2 The biomass measurement

The biomass of actinomycetes was separated from the culture filtrate by means of centrifugation. The biomass was transferred to a pre-weighed dry filter paper using a clean spatula and then placed in an oven at 55^{o}C overnight to reach a fixed weight (Singh *et al*., 2009).

2.2.8.2 Temperature

Actinomycete inocula (described in 2.2.8.1) were added to flat bottles containing 100 ml of ISP-2 broth, pH7 and incubated at different temperatures (25, 30 and 35^{o}C) under static condition for 5 weeks and culture filtrates were harvested every week to check for antimicrobial activity by the agar-well diffusion and the biomass measurement (described in 2.2.8.1.1 and 2.2.8.1.2).

2.2.8.3 pH

The initial pH of the ISP-2 media was adjusted to 6, 7 and 8. Actinomycete inocula (described in 2.2.8.1) were added to flat bottles containing 100 ml of ISP-2 broth and incubated under static conditions, temperature described in 2.2.8.2 for 5 weeks and culture filtrates were harvested every week to check for antimicrobial activity by the agar-well diffusion and the biomass measurement (described in 2.2.8.1.1 and 2.2.8.1.2).

2.2.9. Identification of actinomycetes

Actinomycetes were identified by morphological characteristics and molecular technique (16S rDNA).

2.2.9.1 Morphological characteristics

All morphological characters were observed on ISP-2 agar according to Taddei *et al*. (2006) as follows:

2.2.9.1.1 Macroscopic morphology

The mass color of mature sporulating aerial mycelium was observed following growth on ISP-2 plates. The aerial mass color was classified according to the Bergey's Manual of Systematic Bacteriology (Locci, 1989) in the following color series: gray, white, red, yellow, green, blue, and violet. Distinctive colors of the substrate mycelium were also recorded. The observed colors were: beige, black, blue, biscuit, brown, ivory, olive, orange, purple, pink, red, red-violet, tan, violet-purple, yellow, and yellow-greenish.

2.2.9.1.2 Microscopic morphology

The microscopic characterization was done by cover slip culture method in YME medium incubated at 28°C and observed after 30 days. According to the shape of the spore chains observed under light microscopy, the isolates were grouped as follows: Rectus-Flexibilis (RF), spores in straight or flexuous chains, and Spira (S), spore chains in the form of short gnarled or compact coils or extended, long and open coils.

2.2.9.2 Molecular identification

Selected potential isolates were identified based on the analysis of 16S rDNA sequences. DNA extraction, PCR amplification and DNA sequencing were done by the KU Vector, Kasetsart University as the followings:

2.2.9.2.1 Actinomycete DNA extraction

Actinomycetes were grown on an ISP-2 plate at RT for 3 to 4 weeks. The DNA of each isolate was extracted by suspending some colonies of actinomycetes in 400 µl of TE buffer, and 8 µl of lysozyme (50 mg/ml) in a microtube. The mixture was agitated and incubated at 37^{o}C for 30 min. Then 4 µl of proteinase K (20 mg/ml),

20 µl of 10%SDS and 4 µl of RNase A (100 mg/ml) were added. The mixture was mixed together and incubated at 37^{o}C for 30 min. After that, 70 µl of 5M NaCl, 55 µl of 10%CTAB (10%CTAB/0.7M NaCl) were added and incubated at 65 ^{o}C for 10 min. Then an equal volume of chloroform was added and centrifuged at 15,000 rpm, RT for 5 min. This step was repeated twice. The supernatant was transferred to a new microtube, added an equal volume of phenol/chloroform and centrifuged at 15,000 rpm, RT for 5 min. The supernatant was transferred to a new microtube, then isopropanal was added and centrifuged at 8,000 rpm, RT for 2 min. DNA pellet was washed twice with 1 ml of 70% ethanol and centrifuged at 8,000 rpm for 1 min. After drying DNA pellet was resuspended in 20 µl of water or TE buffer for PCR amplification.

2.2.9.2.2 PCR amplification

Target region of the 16S rDNA was amplified using 27F and 1389 primers listed in Table 11.

Table 11 Primers used for the polymerase chain reaction (PCR) and DNA sequencing

Primers		Sequence (5'-3')
PCR	27F	AGA GTT TGA TCM TGG CTC AG
PCR	1389R	ACG GGC GGT GTG TAC AAG
DNA sequencing	520F	GT GCC AGC MGC CGC GG

Note: M represents A or C

PCR mixture

10 X *Ex-Taq* Buffer	1.5	µl
25 mM dNTP mix	1.2	µl
10 pmol/µl Forward primer (27F)	1.5	µl
10 pmol/µl Reverse primer (1398R)	1.5	µl
Ex-Taq polymerase	0.075	µl
Nanopure water	8.225	µl
DNA template (100 ng/µl)	1.0	µl
Total volume	15.0	l

PCR profiles for amplification:

The PCR profile for primers 27F and 1389R

95 ^{o}C	1 min	
95 ^{o}C	20 s	} 35 cycles
50 ^{o}C	30 s	
72 ^{o}C	2 min	
72 ^{o}C	4 min	

2.2.9.2.3 DNA sequencing and sequence alignment

Sequencing mixture

Nanopure water	9.0	µl
5X sequencing buffer	2.25	µl
520F primer	0.75	µl
Ready reaction mix	1.5	µl
DNA template	1.5	µl

The PCR profile for primer 520F

96 ^{o}C	1 min	
96 ^{o}C	10 s	} 25 cycles
50 ^{o}C	5 s	
60 ^{o}C	4 min	

The 16S rDNA sequences were then compared with GenBank using BLASTN program. Sequences of the selected actinomycetes and other sequences obtained from the GenBank database were aligned by ClustalW (Thompson *et al*., 1994). Manual gap adjustments were made to improve the alignments. The tree construction procedure was performed in PAUP* 4.0b10 in Window versions (Swofford, 2002). Maximum Parsimony (MP) was conducted using heuristic searches as implemented in PAUP* 4.0b10, with the default options method. Clade stability was assessed in a bootstrap analysis with 1,000 replicates, random sequence additions with maxtrees set to 1,000 and other default parameters as implemented in PAUP* 4.0b10. Neighbor joining (NJ) tree was constructed based on the total character differences and bootstrap values were calculated from 1,000 replicates using PAUP* 4.0b10.

CHAPTER 3

RESULTS

3.1. Actinomycetes isolation

A total of 100 actinomycetes isolates were included in this study. Forty isolates (ACK1-55) with different morphotypes were isolated from soils from ten locations in the south of Thailand (Table 12) and 60 isolates (ACK56-120) having antifungal activity against plant pathogenic fungi were provided by Assoc. Prof. Dr. Vasun Petcharat, Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University.

Table 12 Numbers of actinomycetes isolated from soils from various locations in the south of Thailand

Locations	No. of isolates	Code
Pak Panang Basin Region, Nakhon Si Thammarat	12	ACK1-21
Karom Waterfall, Nakhon Si Thammarat	6	ACK22-28
Khao Maha Chai, Nakhon Si Thammarat	1	ACK29
Lam Talumphuk, Nakhon Si Thammarat	1	ACK30
Ton Nga Chang Waterfall**,** Songkhla	5	ACK31-37
Songkhla Lake, Songkhla	4	ACK39-42
Khao Chaison, Phattalung	1	ACK43
Kanghurae, Phattalung	-	
Klong Palean, Trang	7	ACK44-51
Khao Phap Pha, Trang	3	ACK53-55
Total	**40**	

3.2. Primary antimicrobial testing of actinomycetes by cross streak technique and hyphal growth inhibition

A total of 100 isolates of actinomycetes were tested for antimicrobial activity against bacteria and yeasts by cross streak technique against human pathogenic bacteria: *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *E. coli*, *P. aeruginosa*, two species of yeasts: *C. neoformans* and *C. albicans* (Figure 15) and hyphal growth inhibition against two species of human pathogenic filamentous fungi: *M. gypseum* and *P. marneffei* (Figure 16). Eighty percents of the isolates showed antimicrobial activity against at least one test microorganism, 48 isolates were from the active isolates against plant pathogenic fungi and 32 from the new isolates (Table 13). Among them, 8% inhibited only bacteria, 12% inhibited only yeasts, 11% inhibited only filamentous fungi, 9% inhibited both yeasts and filamentous fungi and 40% had both antibacterial and antifungal activities (Table 14).

The percentage of active actinomycetes against each test strain and the top 46 active actinomycetes were shown in Figure 17 and Table 15, respectively. For antibacterial activity, 40% of soil actinomycetes inhibited both strains of *S. aureus*. Isolate ACK18 showed the best inhibitory activity against *S. aureus* ATCC 25923 (inhibition zone 30.95 mm) and ACK84 against MRSA SK1 (inhibition zone 35.17 mm) (Table 15). Only 9% and 15% inhibited *E. coli* and *P. aeruginosa*, respectively. ACK108 had the best activity against *E. coli* (inhibition zone 32.87 mm) and ACK117 against *P. aeruginosa* (inhibition zone 33.62 mm).

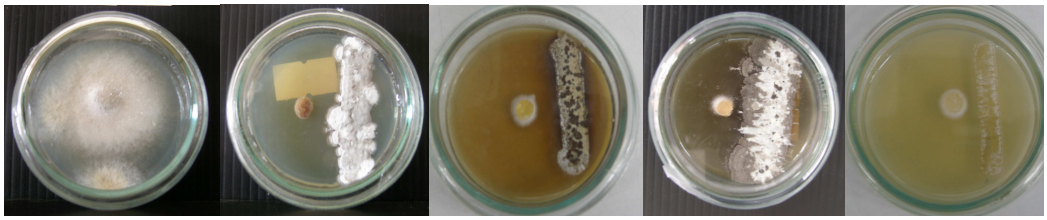
For anti-yeast activity, 30% of actinomycetes inhibited *C. albicans* and ACK26 had the best activity against both strains of *C. albicans* (inhibition zones 29.27 and 32.45 mm, respectively). 49% inhibited *C. neoformans* and actinomycetes ACK60 and ACK7 had the best activity against *C. neoformans* ATCC90112 and ATCC 90113 (inhibition zones 34.37 and 28.87 mm, respectively).

For antifungal activity against filamentous fungi, *M. gypseum* and *P. marneffei,* 21% inhibited *M. gypseum* and 41 % inhibited *P. marneffei*. In addition, 11 and 30 isolates showed high inhibitory activity >80% hyphal growth inhibition and 9 isolates strongly inhibited both fungi.

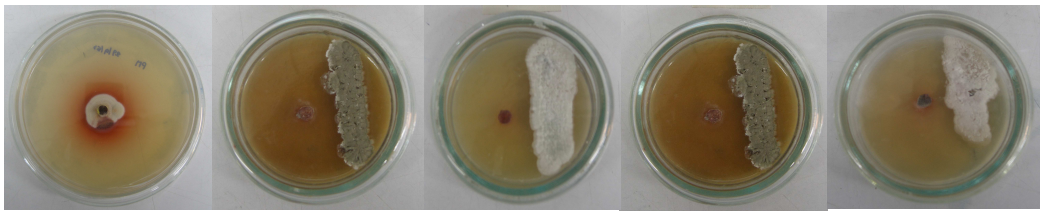
Control **ACK 26** **ACK 79** **ACK 84** **ACK 98**

Figure 15 Primary antimicrobial testing of actinomycetes by cross streak technique against bacteria and yeasts

SA	=	*Staphylococcus aureus* ATCC 25923	CA28	=	*Candida albicans* ATCC 90028
MRSA	=	methicillin-resistant *S. aureus* SK1	CA53	=	*Candida albicans* NCPF 3153
EC	=	*Escherichia coli* ATCC 25922	CN12	=	*Cryptococcus neoformans* ATCC 90112
PA	=	*Pseudomonas aeruginosa* ATCC 27853	CN13	=	*Cryptococcus neoformans* ATCC 90113



Control **ACK 9** **ACK 34** **ACK 58** **ACK 94**



Control **ACK 34** **ACK 110** **ACK 116** **ACK 117**

Figure 16 Primary antifungal testing of actinomycetes by hyphal inhibition test against filamentous fungi. Upper row: *Microsporum gypseum* and lower row: *Penicillium marneffei*

Table 13 Distribution of actinomycetes included in this study according to their antimicrobial activity by cross streak technique and hyphal growth inhibition

Origin of actinomycetes	**Active isolates/Total isolates tested (%)**
New isolates from soil	32/40 (80%)
Isolates having antifungal activity against plant pathogenic fungi (From Mr. Sawai Boukaew)	48/60 (80%)
Total	**80/100 (80%)**

Table 14 Distribution of antimicrobial spectrum of 80 active actinomycetes

% Active actinomycetes	**Activity**		
	Antibacterial	**Anti-yeast**	**Anti-filamentous fungi**
8	↔		
12		↔	
11			↔
9		↔	↔
18	↔	↔	
9	↔		↔
13	↔	↔	↔

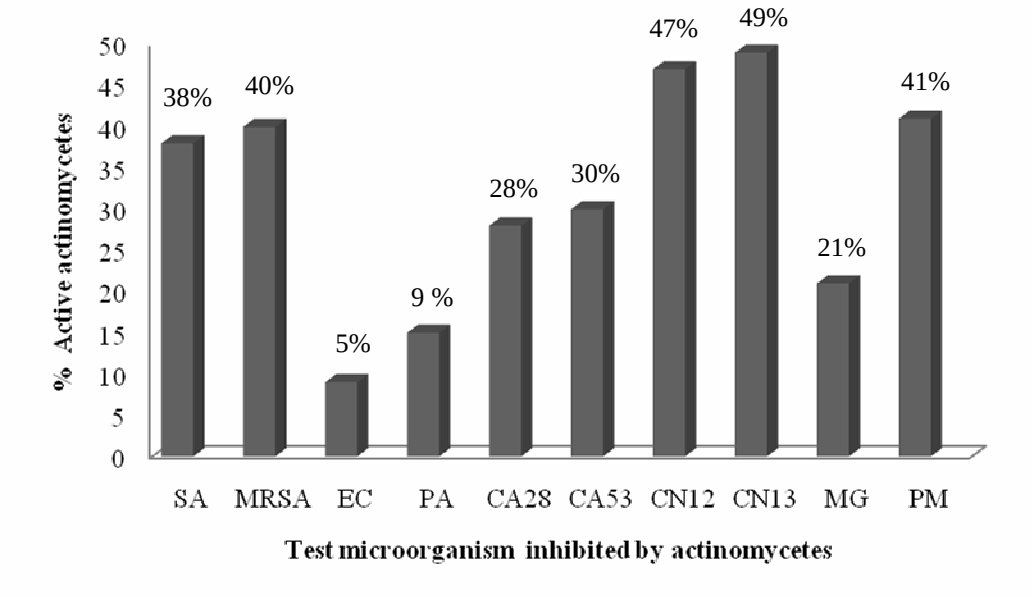


Figure 17 Percentages of active actinomycetes against each test microorganism

SA	=	*Staphylococcus aureus* ATCC 25923	CA53	=	*Candida albicans* NCPF 3153
MRSA	=	methicillin-resistant *S. aureus* SK1	CN12	=	*Cryptococcus neoformans* ATCC 90112
EC	=	*Escherichia coli* ATCC 25922	CN13	=	*Cryptococcus neoformans* ATCC 90113
PA	=	*Pseudomonas aeruginosa* ATCC 27853	MG	=	*Microsporum gypseum* SH- MU 4
CA28	=	*Candida albicans* ATCC 90028	PM	=	*Penicillium marneffei*

Table 15 Top 46 actinomycetes having antimicrobial activity against ten tested microorganisms by cross streak technique and hyphal growth inhibition test

Actino-mycetes Code	Inhibition zone (mm)								% inhibition	
	SA	MRSA	EC	PA	CA 28	CA 53	CN 12	CN 13	MG	PM
ACK 7	6.57	6.47	10.35	0.00	18.87	21.17	**25.77**	**28.87**	0.00	**100.00**
ACK 8	24.90	**33.27**	0.00	0.00	23.80	**28.17**	18.85	21.40	0.00	**100.00**
ACK 9	5.80	3.62	0.00	0.00	24.87	**26.67**	24.00	18.82	0.00	0.00
ACK 18	**30.95**	**30.87**	0.00	0.00	24.32	24.00	**26.75**	20.97	0.00	**100.00**
ACK 20	**25.62**	**26.87**	0.00	0.00	0.00	0.00	0.00	0.00	**99.00**	0.00
ACK 21	**30.00**	**34.37**	0.00	0.00	0.00	5.72	0.00	0.00	0.00	0.00
ACK 26	**27.40**	**30.95**	0.00	0.00	**29.27**	**32.45**	**31.62**	24.87	0.00	0.00
ACK 36	0.00	0.00	0.00	0.00	0.00	0.00	16.75	10.75	0.00	**100.00**
ACK 39	**25.87**	**31.65**	0.00	0.00	20.45	18.05	**25.30**	19.20	0.00	**100.00**
ACK 40	0.00	0.00	0.00	0.00	0.00	0.00	**27.63**	23.28	0.00	0.00
ACK 41	0.00	13.20	0.00	**27.75**	0.00	0.00	0.00	0.00	0.00	**100.00**
ACK 43	**25.37**	**26.77**	0.00	0.00	**26.65**	**25.60**	24.05	19.55	0.00	0.00
ACK 44	20.62	24.25	0.00	0.00	**27.32**	**29.20**	**27.07**	24.87	0.00	0.00
ACK 47	19.02	**25.30**	0.00	0.00	8.00	11.62	14.25	10.87	**100.00**	**100.00**
ACK 49	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	69.75	**90.00**
ACK 50	0.00	0.00	0.00	0.00	0.00	0.00	23.50	17.50	0.00	**100.00**
ACK 51	23.82	**27.25**	**27.27**	0.00	0.00	0.00	0.00	22.00	0.00	**100.00**
ACK 53	10.50	18.00	0.00	0.00	11.17	13.72	15.80	10.25	**91.00**	**100.00**

SA = *Staphylococcus aureus* ATCC 25923
MRSA = methicillin-resistant *S. aureus* SK1
EC = *Escherichia coli* ATCC 25922
PA = *Pseudomonas aeruginosa* ATCC 27853
CA28 = *Candida albicans* ATCC 90028
CA53 = *Candida albicans* NCPF 3153
CN12 = *Cryptococcus neoformans* ATCC 90112
CN13 = *Cryptococcus neoformans* ATCC 90113
MG = *Microsporum gypseum* SH- MU 4
PM = *Penicillium marneffei*

Table 15 (Cont.) Top 46 actinomycetes having antimicrobial activity against ten tested microorganisms by cross streak technique and hyphal growth inhibition test

Actino-mycetes Code	Inhibition zone (mm)								% inhibition	
	SA	MRSA	EC	PA	CA 28	CA 53	CN 12	CN 13	MG	PM
ACK 56	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**100.00**	**87.75**
ACK 57	11.72	10.97	20.72	0.00	9.45	13.22	**27.82**	19.55	0.00	**100.00**
ACK 58	0.00	0.00	0.00	0.00	0.00	0.00	23.65	20.43	0.00	**100.00**
ACK 60	0.00	7.72	14.25	16.37	0.00	0.00	**34.37**	**28.12**	0.00	0.00
ACK 62	0.00	0.00	0.00	0.00	0.00	0.00	24.50	17.25	0.00	**90.00**
ACK 64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**100.00**	**100.00**
ACK 65	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**100.00**	**100.00**
ACK 66	12.32	18.62	14.70	13.30	10.85	15.05	12.97	8.37	**84.00**	**100.00**
ACK 67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**90.00**
ACK 70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**100.00**
ACK 71	9.73	**34.82**	0.00	**25.50**	0.00	0.00	4.50	0.00	0.00	0.00
ACK 73	0.00	0.00	0.00	19.37	0.00	0.00	0.00	0.00	**100.00**	**100.00**
ACK 74	0.00	0.00	0.00	0.00	10.87	14.72	15.22	8.75	**96.00**	**100.00**
ACK 76	0.00	0.00	0.00	0.00	20.33	17.68	**29.88**	**26.25**	0.00	0.00
ACK 83	22.25	**25.70**	0.00	0.00	0.00	0.00	**26.32**	21.75	0.00	0.00
ACK 84	22.05	**35.17**	0.00	27.10	0.00	0.00	0.00	0.00	0.00	**100.00**
ACK 87	10.37	0.00	0.00	0.00	9.50	12.25	16.87	13.00	0.00	**100.00**
ACK 90	4.35	6.72	0.00	0.00	13.07	11.80	15.92	12.50	**84.00**	**100.00**

SA = *Staphylococcus aureus* ATCC 25923
MRSA = methicillin-resistant *S. aureus* SK1
EC = *Escherichia coli* ATCC 25922
PA = *Pseudomonas aeruginosa* ATCC 27853
CA28 = *Candida albicans* ATCC 90028
CA53 = *Candida albicans* NCPF 3153
CN12 = *Cryptococcus neoformans* ATCC 90112
CN13 = *Cryptococcus neoformans* ATCC 90113
MG = *Microsporum gypseum* SH- MU 4
PM = *Penicillium marneffei*

Table 15 (Cont.) Top 46 actinomycetes having antimicrobial activity against ten tested microorganisms by cross streak technique and hyphal growth inhibition test

Actino-mycetes Code	Inhibition zone (mm)								% inhibition	
	SA	**MRSA**	**EC**	**PA**	**CA 28**	**CA 53**	**CN 12**	**CN 13**	**MG**	**PM**
ACK 91	12.97	16.12	0.00	0.00	0.00	8.75	11.25	5.50	0.00	**100.00**
ACK 102	0.00	0.00	0.00	0.00	0.00	0.00	**29.10**	**27.00**	0.00	**100.00**
ACK 103	14.75	14.37	0.00	0.00	4.97	6.50	18.42	12.40	69.75	**100.00**
ACK 104	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**100.00**
ACK 108	0.00	**28.55**	**32.87**	24.87	0.00	0.00	0.00	0.00	0.00	0.00
ACK 110	0.00	0.00	0.00	**28.00**	0.00	0.00	0.00	0.00	**82.60**	78.09
ACK 112	13.12	**32.00**	0.00	**27.62**	0.00	0.00	0.00	0.00	0.00	0.00
ACK 116	0.00	0.00	0.00	0.00	0.00	0.00	**29.25**	**25.62**	0.00	**100.00**
ACK 117	0.00	0.00	0.00	**33.62**	0.00	0.00	0.00	0.00	64.87	54.23
ACK 119	0.00	0.00	0.00	**28.00**	0.00	0.00	0.00	0.00	0.00	0.00

SA = *Staphylococcus aureus* ATCC 25923
MRSA = methicillin-resistant *S. aureus* SK1
EC = *Escherichia coli* ATCC 25922
PA = *Pseudomonas aeruginosa* ATCC 27853
CA28 = *Candida albicans* ATCC 90028
CA53 = *Candida albicans* NCPF 3153
CN12 = *Cryptococcus neoformans* ATCC 90112
CN13 = *Cryptococcus neoformans* ATCC 90113
MG = *Microsporum gypseum* SH- MU 4
PM = *Penicillium marneffei*

Top 46 actinomycetes that can inhibit each group of the tested bacteria and yeast from cross streak technique with inhibition zone over 25 mm and hyphal growth inhibition over 80% were selected for further study (Table 15). Eighteen isolates were from the new isolates group and 28 isolates from the active group against plant pathogenic fungi. Among the 18 new active isolates from soil, the highest number was from Pak Panang Basin Region (66.67%) followed by Karom Waterfall (16.67%) and Klong Palean (16.67%).

According to the spectrum of 80 active actinomycetes, it was found that most of actinomycetes inhibited three tested microorganisms (18 isolates) followed by 17, 13, 12, 7 and 7 isolates that inhibited 2, 6, 4, 1 and 8 tested microorganisms, respectively (Figure 18). Only actinomycete ACK66 inhibited all the tested microorganisms with high potential inhibitory activity against both filamentous fungi (Table 15).

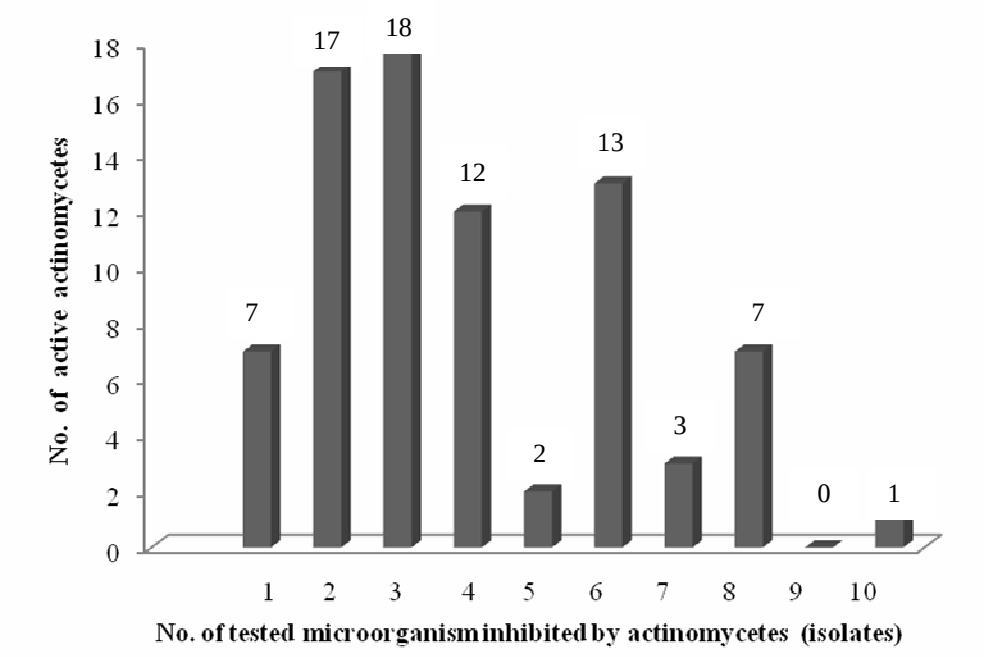


Figure 18 Number of active actinomycetes that inhibited tested microorganisms

3.3 Screening of actinomycetes crude extracts for antimicrobial activity

The top 46 active actinomycetes were grown in ISP-2 for 6 weeks and their culture broths and mycelia were extracted. In total, 138 crude extracts comprising crude ethyl acetate extracts from culture broths (BE, 46), crude ethyl acetate extracts of mycelia (CE, 46) and crude hexane extracts of mycelia (CH, 46) were obtained for antimicrobial assay.

Actinomycete crude extracts at a concentration of 200 µg/ml were primarily tested for antimicrobial activity against the tested microorganisms by the colorimetric microdilution method (Figure 19). The result showed that 90 extracts (65.22%) from 46 active actinomycete isolates were inhibitory.

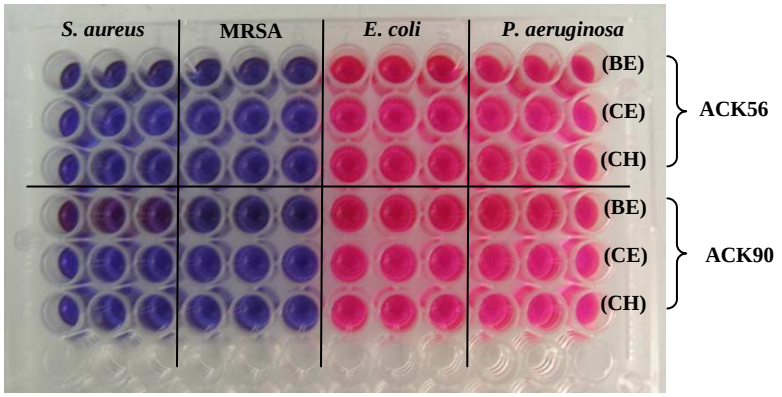


Figure 19 Primary antibacterial assay by a colorimetric microdilution method at 200 µg/ml (blue or violet color indicates an inhibitory result and pink color indicates growth of the test strain)

According to the types of crude extract CH provided the highest active extracts (34/46, 73.91%) followed by CE (31/46, 67.39%), and BE (25/46, 54.34%), respectively (Figure 20). CH extracts exhibited the most activity against *S. aureus* (34) and MRSA (29), followed by *C. neoformans* ATCC 90112 (4), *P. marneffei* (3), both strains of *C. albicans* (1) and *P. aeruginosa* (1). CE extracts exhibited the most activity against *S. aureus* (27) followed by MRSA (22), *C. neoformans* ATCC 90112 (14), *C. neoformans* ATCC 90113 (10), both strains of *C. albicans* (4) and *P. marneffei* (2). BE extracts exhibited the most activity against *S. aureus* (21) followed by MRSA (19), *C. neoformans* ATCC 90112 (12), *C. neoformans* ATCC 90113 (11), *C. albicans* ATCC 90028 (5), *C. albicans* NCPF 3153 (4) and *M. gypseum* (1) (Table 16).

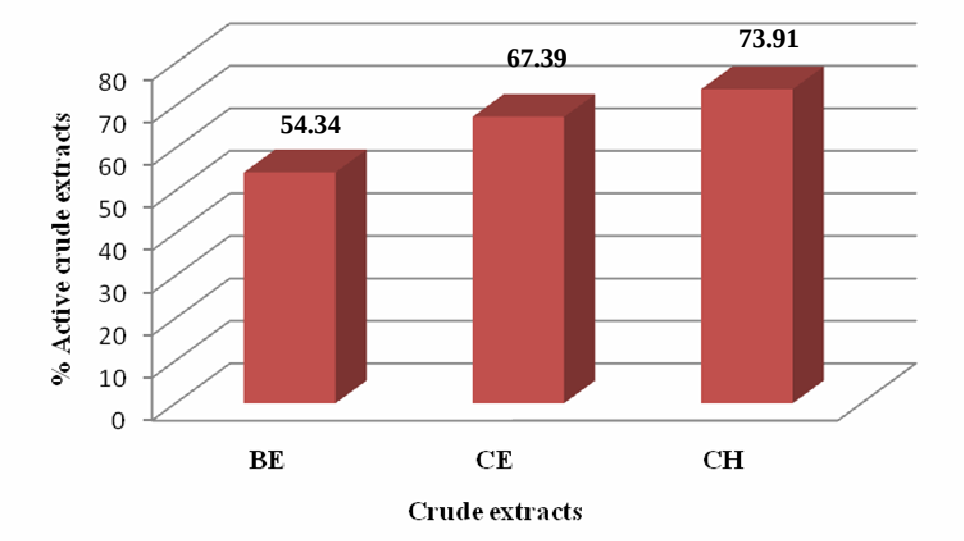


Figure 20 Percentage of types of active crude extracts from actinomycetes tested at 200 µg/ml (BE = Broth EtOAc, CE = Cell EtOAc, CH = Cell Hexane)

Table 16 Antimicrobial activity of each type of crude extracts against test microorganisms

Type of crude extract	Test microorganisms									
	Bacteria				Yeasts				Filamentous fungi	
	SA	MRSA	EC	PA	CA 28	CA 53	CN 12	CN 13	MG	PM
BE (n=46)	21	19	0	0	5	4	12	11	1	0
CE (n=46)	27	22	0	0	4	4	14	10	0	2
CH (n=46)	34	29	0	1	1	1	4	0	0	3

SA = *Staphylococcus aureus* ATCC 25923
MRSA = methicillin-resistant *S. aureus* SK1
EC = *Escherichia coli* ATCC 25922
PA = *Pseudomonas aeruginosa* ATCC 27853
CA28 = *Candida albicans* ATCC 90028
BE = Broth EtOAc
CH = Cell Hexane
CA53 = *Candida albicans* NCPF 3153
CN12 = *Cryptococcus neoformans* ATCC 90112
CN13 = *Cryptococcus neoformans* ATCC 90113
MG = *Microsporum gypseum* SH-MU 4
PM = *Penicillium marneffei*
CE = Cell EtOAc

Extracts of active actinomycetes were highly active against both strains of *S. aureus* (50.72-59.42%), followed by *C. neoformans* (15.11-21.58%), *C. albicans* (6.47-7.19%) and *P. marneffei* (5%). Only each one extract (0.72%) inhibited *P. aeruginosa* and *M. gypseum*. None of the extracts inhibited *E. coli* (Figure 21).

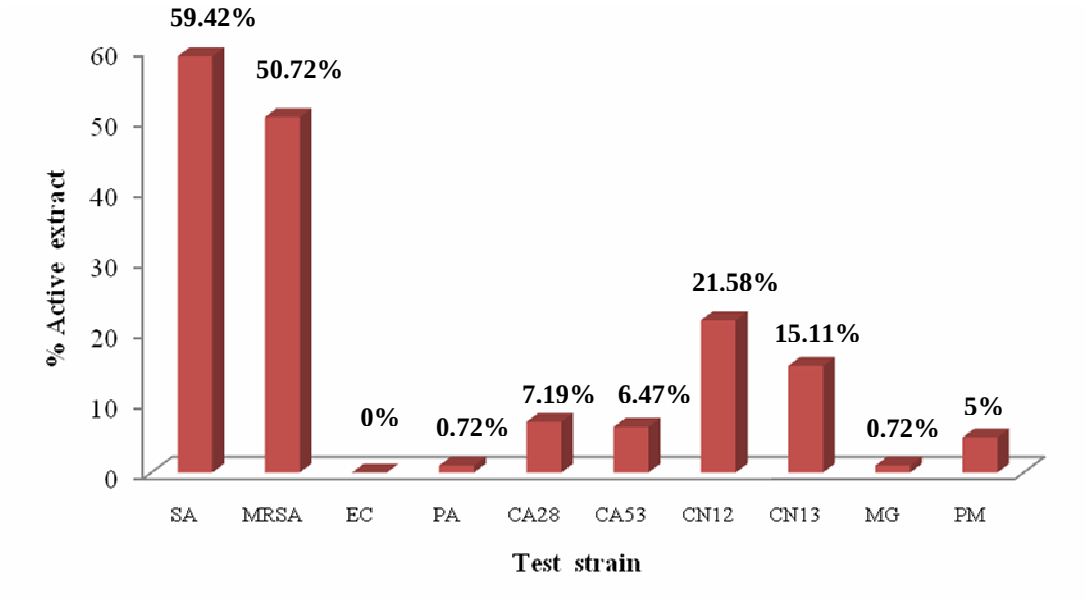
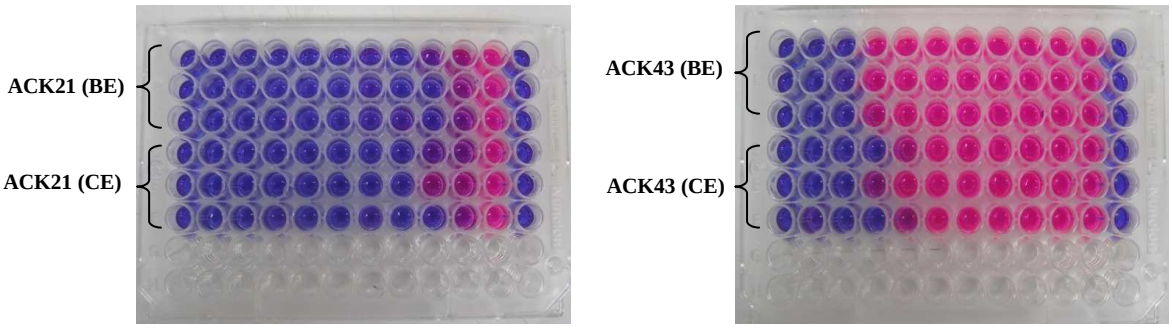


Figure 21 Antimicrobial activity of actinomycete crude extracts against various microbes tested at 200 µg/ml

SA	=	*Staphylococcus aureus* ATCC 25923	CA53	=	*Candida albicans* NCPF 3153
MRSA	=	methicillin-resistant *S. aureus* SK1	CN12	=	*Cryptococcus neoformans* ATCC 90112
EC	=	*Escherichia coli* ATCC 25922	CN13	=	*Cryptococcus neoformans* ATCC 90113
PA	=	*Pseudomonas aeruginosa* ATCC 27853	MG	=	*Microsporum gypseum* SH- MU 4
CA28	=	*Candida albicans* ATCC 90028	PM	=	*Penicillium marneffei*

3.4 Determination of MIC and MBC or MFC

All extracts that showed inhibitory activity at 200 µg/ml were further assayed for their MICs (Figure 22), MBCs or MFCs by the colorimetric microdilution method. The results are shown in Table 17.



a) *S. aureus* ATCC 25923 b) MRSA-SK1

Figure 22 Determination of MIC of crude extracts by colorimetric microdilution method

3.4.1 Antibacterial activity

Of the 138 crude extracts from actinomycete isolates, 59.42% (82/138) and 50.72% (70/138) of extracts were active against *S. aureus* and MRSA (Figure 21, Table 16) with MIC/MBC values that ranged from 0.5-200/4->200 and 0.5-200/2->200 µg/ml, respectively. None of the extracts inhibited *E. coli* (MIC and MBC>200 µg/ml). One extract was active against *P. aeruginosa* with MIC/MBC values of 200/>200 µg/ml. Crude CH extract from ACK21 (ACK21CH) exhibited the strongest antibacterial activity against *S. aureus* and MRSA with MIC/MBC 0.5/4 and 0.5/8 µg/ml, respectively followed by ACK20CE against *S. aureus* and MRSA with MIC/MBC 2/8 µg/ml, 0.5/2 µg/ml, respectively. This was comparable to vancomycin

(MIC 0.5-1 µg/ml). ACK20CE and ACK21CH at concentrations of 4 times MIC strongly destroyed *S. aureus* ATCC25923 and MRSA cells causing the leakage of cytoplasm and cell death as shown in the SEM in Figures 23 and 24.

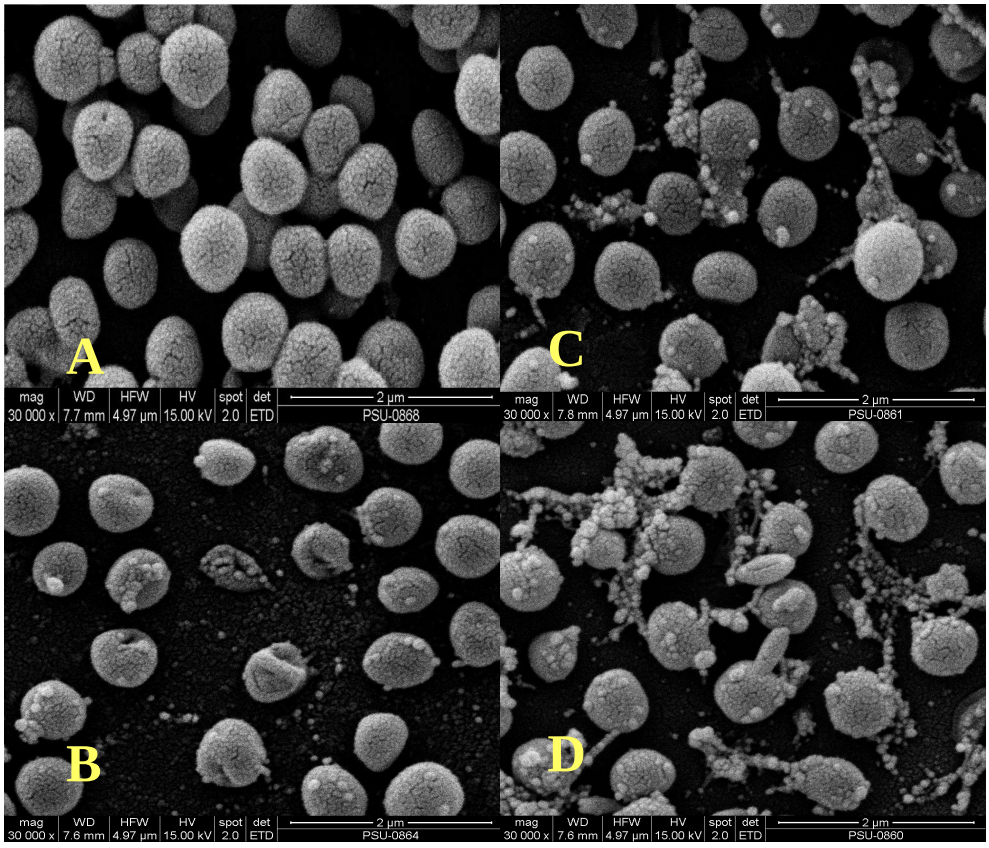


Figure 23 Scanning electron micrographs of the treated *S. aureus* ATCC 28923 (SA) after incubation at 35^{o}C for 18 h

A : Control SA+ 1%DMSO
B : SA+ Vancomycin (4MIC)
C : SA+ ACK 20CE (4MIC)
D : SA+ ACK 21CH (4MIC)

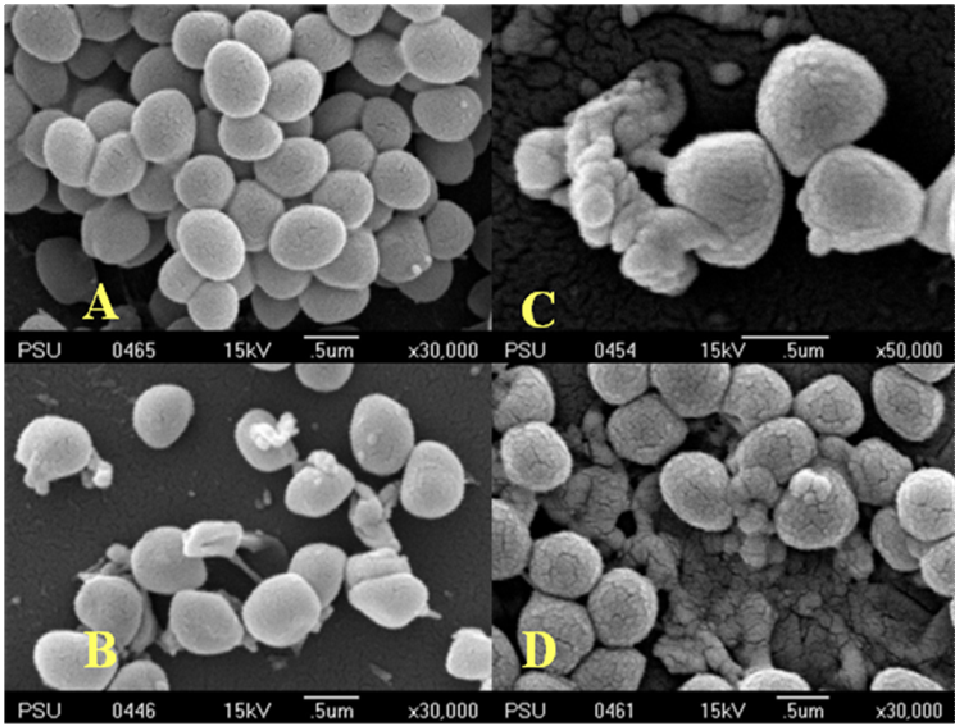


Figure 24 Scanning electron micrographs of the treated methicillin-resistant *S. aureus* SK1 (MRSA) after incubation at 35°C for 18 h

A : Control MRSA+ 1%DMSO
B : MRSA+ Vancomycin (4MIC)
C : MRSA+ ACK 20CE (4MIC)
D : MRSA+ ACK 21CH (4MIC)

3.4.2 Antifungal activity

Thirty nine out of 138 extracts (28.26%) showed antifungal activity. The results are shown in Figure 21 and Table 16. The most active extracts (30/138, 21.74%) were active against *C. neoformans* ATCC90112 with MIC/MFC values that ranged from 16-200/>128 µg/ml, respectively, followed by 15.22% (21/138) of the extracts against *C. neoformans* ATCC90113 with MIC and MFC value of 16–200 and 64->200 µg/ml, respectively, and 5% against *P. marneffei*. Only one extract was active against *M. gypseum* (MIC/MFC 200/>200 µg/ml). Crude CH extract from ACK21 (ACK21CH) exhibited the strongest antifungal activity against *C. albicans* NCPF3153 with MIC/MFC 4/128 µg/ml. The antifungal drug amphotericin B had MIC value of 0.0625 µg/ml and MFC 2 µg/ml against *C. albicans*.

Table 17 MICs, MBCs or MFCs (µg/ml) of selected active crude extracts from actinomycetes against human pathogenic microorganisms

Test organisms / Actinomycetes		Bacteria						Yeasts								Filamentous fungus			
Code	Extract	SA		MRSA		PA		CA28		CA53		CN12		CN13		MG		PM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ACK 7	BE											200	>200	200	>200			200	>200
	CE											200	>200	200	>200				
	CH	200	>200	128	>128														
ACK 8	BE							200	>200										
	CE	200	>200																
	CH	64	>128	128	>128														
ACK 18	BE	200	>200					200	>200	200	>200	200	>200	200	>200				
	CE	200	>200	200	>200														
	CH	128	>128	200	>200														
ACK 20	BE	**4**	**16**	**1**	**4**			200	>200	128	>128	200	>200	200	>200				
	CE	**2**	**8**	**0.5**	**2**			128	>128	32	64	200	>200	200	>200				
	CH	128	>128	128	>128	200	>200												

Table 17 (cont.) MICs, MBCs or MFCs (µg/ml) of selected active crude extracts from actinomycetes against human pathogenic microorganisms

Test organisms / Actinomycetes		Bacteria						Yeasts								Filamentous fungus			
Code	Extract	SA		MRSA		PA		CA28		CA53		CN12		CN13		MG		PM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ACK 21	BE	**0.5**	**128**	**1**	**16**			200	>200	200	>200								
	CE	**0.5**	**>128**	**0.5**	**16**			200	>200	200	>200								
	CH	**0.5**	**4**	**0.5**	**8**			200	>200	4	128								
ACK 26	BE	64	>128	64	>128									200	>200				
	CH	64	>128									200	>200						
ACK 36	CE	128	>128																
	CH	64	>128	64	>128														
ACK39	BE							200	>200	200	>200	200	>200	128	>128				
ACK40	CE	128	>128	64	>128														
	CH	64	>128	64	>128														
ACK 43	BE	32	>128	32	>128														
	CE	16	128	16	>128														
	CH	64	128	128	>128														

Table 17 (cont.) MICs, MBCs or MFCs (µg/ml) of selected active crude extracts from actinomycetes against human pathogenic microorganisms

Test organisms / Actinomycetes		Bacteria						Yeasts								Filamentous fungus			
Code	Extract	SA		MRSA		PA		CA28		CA53		CN12		CN13		MG		PM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ACK 44	CE											200	>200						
	CH	200	>200																
ACK 47	BE	200	>200									128	>128	64	>128				
	CE									200	>200	64	>128	64	>128				
	CH	200	>200	200	>200							200	>200						
ACK 50	CE	64	>128	64	>128							200	>200						
	CH	64	>128	64	>128														
ACK 53	BE	200	>200	200	>200														
	CE	200	>200																
	CH	200	>200																
ACK 56	BE	64	>128	128	>128														
	CE	128	>128	200	>200														
	CH	64	>128	64	>128														

Table 17 (cont.) MICs, MBCs or MFCs (µg/ml) of selected active crude extracts from actinomycetes against human pathogenic microorganisms

Test organisms / Actinomycetes		Bacteria						Yeasts								Filamentous fungus			
Code	Extract	SA		MRSA		PA		CA28		CA53		CN12		CN13		MG		PM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ACK 57	BE	128	>128	200	>200							200	>200	200	>200				
	CE	128	>128	200	>200														
	CH	32	>128	64	>128							200	>200						
ACK 58	CE							128	>128	200	>200	64	>128	64	64				
	CH	128	>128	128	>128														
ACK 60	CH	128	>128																
ACK 62	BE	200	>200	200	>200														
	CE	200	>200	200	>200														
	CH	128	>128	200	>200														
ACK 64	CE	128	>128	200	>200							200	>200						
	CH	32	>128	64	>128														

Table 17 (cont.) MICs, MBCs or MFCs (µg/ml) of selected active crude extracts from actinomycetes against human pathogenic microorganisms

Test organisms / Actinomycetes		Bacteria						Yeasts								Filamentous fungus			
Code	Extract	SA		MRSA		PA		CA28		CA53		CN12		CN13		MG		PM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ACK 65	BE	128	>128	200	>200							16	>128	16	128				
	CE	128	>128	200	>200							64	>128	64	>128				
	CH	64	>128	64	>128														
ACK 66	BE	128	>128	64	>128														
	CE	64	>128	64	>128														
	CH	32	>128	32	>128														
ACK 67	CH	128	>128	200	>200														
ACK 70	CH	200	>200																
ACK 73	BE	128	>128	128	>128														
	CE	64	>128	64	>128							200	>200						
	CH	128	>128	128	>128														

Table 17 (cont.) MICs, MBCs or MFCs (µg/ml) of selected active crude extracts from actinomycetes against human pathogenic microorganisms

Test organisms / Actinomycetes		Bacteria						Yeasts								Filamentous fungus			
Code	Extract	SA		MRSA		PA		CA28		CA53		CN12		CN13		MG		PM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ACK 74	BE	32	>128	32	>128														
	CE	200	>200	200	>200			200	>200			200	>200	200	>200			200	>200
	CH	200	>200	200	>200													200	>200
ACK 76	BE	16	>128	64	>128														
	CE	16	>128	16	>128														
	CH	2	>128	4	>128														
ACK 83	CE	200	>200									200	>200	200	>200				
	CH	32	>128	64	>128														
ACK 87	BE	32	>128	64	>128							32	>128	32	>128	200	>200		
	CE	128	>128	200	>200							200	>200						
	CH	64	>128	64	>128														
ACK 90	BE	200	>200	200	>200														
	CE	128	>128	128	>128													200	>200
	CH	128	>128	128	>128													200	>200

Table 17 (cont.) MICs, MBCs or MFCs (µg/ml) of selected active crude extracts from actinomycetes against human pathogenic microorganisms

Test organisms / Actinomycetes		Bacteria						Yeasts								Filamentous fungus			
Code	Extract	SA		MRSA		PA		CA28		CA53		CN12		CN13		MG		PM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ACK 91	BE	64	>128	200	>200							64	>128	64	>128				
	CE	64	>128	200	>200							200	>200	200	>200				
	CH	64	>128	128	>128														
ACK 102	BE	200	>200	200	>200							200	>200	200	>200				
	CE	128	>128	128	>128														
	CH	32	>128	64	>128														
ACK 104	CE	200	>200																
	CH	64	>128	64	>128														
ACK 112	CE	200	>200	200	>200														
	CH	64	>128	128	>128														
ACK 116	BE	32	>128	128	>128							128	>128	200	>200				
	CE	32	>128	64	>128														
	CH	64	>128	64	>128							200	>200						

Table 17 (cont.) MICs, MBCs or MFCs (µg/ml) of selected active crude extracts from actinomycetes against human pathogenic microorganisms

Test organisms / Actinomycetes		Bacteria						Yeasts								Filamentous fungus			
Code	Extract	SA		MRSA		PA		CA28		CA53		CN12		CN13		MG		PM	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ACK 117	BE											200	>200						
	CE																		
	CH																		
ACK 119	BE	200	>200	200	>200														
	CE	64	>128	64	>128							200	>200	200	>200				
	CH																		
Vancomycin		1	2	1	2														
Gentamicin						1	2												
Miconazole																0.5	2		
Amphotericin B								0.0625	2	0.0625	2	0.125	2	0.25	1			2	4

SA = *Staphylococcus aureus* ATCC25923
MRSA = methicillin-resistant *S. aureus* SK1
EC = *Escherichia coli* ATCC25922
PA = *Pseudomonas aeruginosa* ATCC27853
CA28 = *Candida albicans* ATCC90028
CA53 = *Candida albicans* NCPF3153
CN12 = *Cryptococcus neoformans* ATCC90112
CN13 = *Cryptococcus neoformans* ATCC90113
MG = *Microsporum gypseum* SH-MU 4
PM = *Penicillium marneffei*
BE = Broth EtOAc
CE = Cell EtOAc
CH = Cell Hexan
MIC = Minimal Inhibitory Concentration
MBC = Minimal Bactericidal Concentration
MFC = Minimal Fungicidal Concentration
Strong activity : MIC ≤ 8 µg/ml
Moderate activity : MIC 16-64 µg/ml
Weak activity : MIC 128-200 µg/ml

3.5 Optimization of culturing conditions of actinomycetes for the production of antimicrobial metabolite

3.5.1 Effect of agitation

In this study the secondary metabolite productions of selected actinomycetes ACK 20 and ACK21 were cultured under shaking (200 rpm) and static conditions in ISP-2 medium pH7 at 30^{o}C. The culture broths were incubated for 5 weeks to investigate the inhibition activity against *S. aureus* and MRSA and to measure cell dry weight. Under the shaken condition as shown in Figure 25A and D, the growth of both actinomycetes increased at the first week of cultivation and the maximum growth was obtained at the second week, then gradually decreased, whereas the maximum growth under the static condition was found after 3 weeks of cultivation. Antibacterial activity was measured in terms of diameter of inhibition zone (mm) by agar well assay. It was found that the antibacterial activity was detected starting from the first and second week of cultivation of ACK20 and ACK21 under static condition, respectively and reaching its maximum activity in the fourth week of incubation against both *S. aureus* and MRSA. Culture filtrates of both isolates under static condition showed better antibacterial activity than those from the shaking condition (Figure 25B-F).

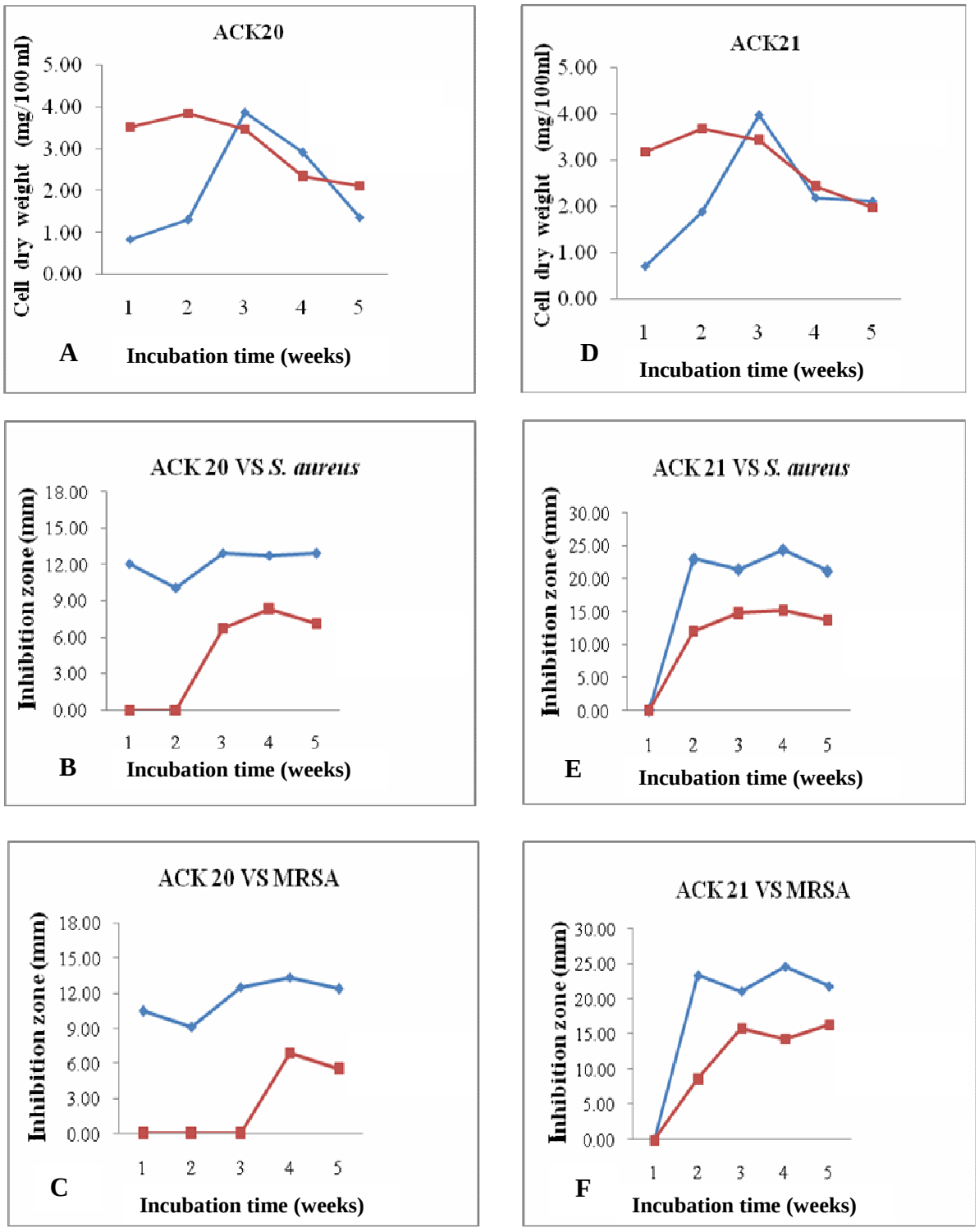


Figure 25 Effect of culture conditions on growth and production of antibacterial metabolites by actinomycetes ACK20 (A-C) and ACK21 (D-F) in ISP-2 medium pH7 at 30°C. Symbols: ◆, static condition; ■, shaking condition (200 rpm).

3.5.2 Effect of temperature

The temperature for growth and antibacterial production of actinomycetes in this study was carried out at 25, 30 and 35^{o}C in ISP-2 medium pH 7 under static condition for 5 weeks. The growth of both actinomycetes cultivated at 25 and 35^{o}C as shown in Figure 26A and D increased at the first week of cultivation and the maximum growth was obtained at the third week, then gradually decreased, whereas the maximum growth under 30°C was found at the fifth week for ACK20. It was found that the antibacterial activities were detected starting from the first week of cultivation of ACK20 and ACK21, except the activity of ACK21 at 35°C was detected starting from the third and fourth weeks against *S. aureus* and MRSA, respectively. The maximum activities of ACK20 and ACK21 against *S. aureus* and MRSA were found in the fourth week at 25 and 30°C of incubations, respectively (Figure 26).

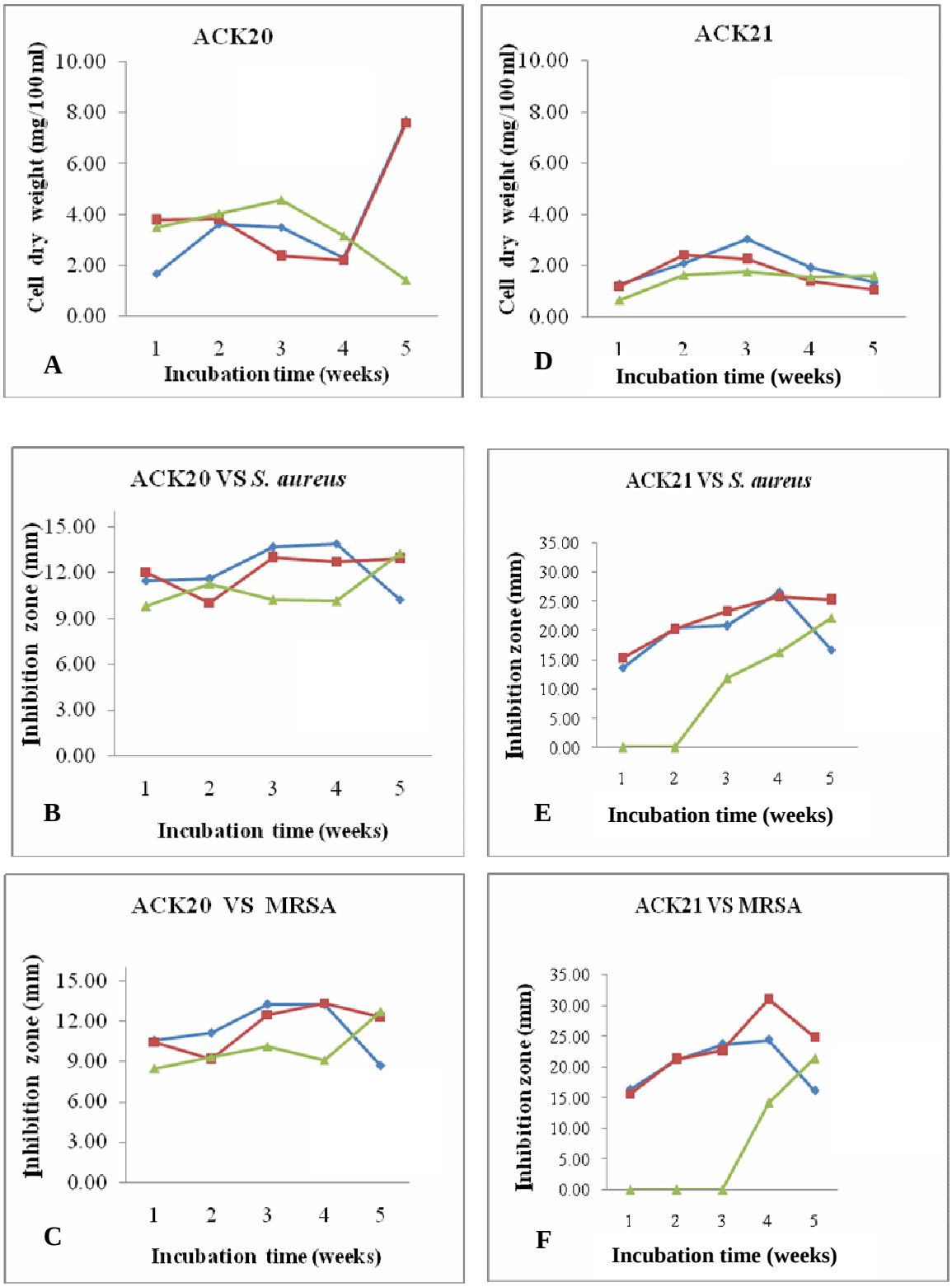


Figure 26 Effect of incubation temperature on growth and production of antibacterial metabolites by actinomycetes ACK20 (A-C) and ACK21 (D-F) in ISP-2 medium pH7 under static condition. Symbols: ◆, 25°C; ■, 30°C; ▲, 35°C.

3.5.3 Effect of the initial pH of ISP-2 medium

The initial pH of medium is important for growth and antibacterial production of actinomycetes. In this study actinomycetes ACK20 and ACK21 were inoculated in ISP-2 medium at different initial pHs (6, 7 and 8) at 25^{o}C for ACK 20 and at 30^{o}C for ACK 21 under static condition for 5 weeks. Under pH6, 7 and 8 of medium as shown in Figure 27A and D, the growth of both actinomycetes increased at the first week of cultivation and the maximum growth was achieved at the second week, then gradually decreased, except under cultivation at pH7 the growth of ACK20 increased at the first and second weeks of cultivation, then gradually decreased and the maximum growth was achieved at the fifth week. The best activity of ACK20 was observed in the medium with initial pH6 and 7 against both *S. aureus* ATCC 25923 and MRSA (Figure 27B and C) whereas the best activity of ACK21 was obtained from the medium with initial pH 7 (Figure 27E and F).

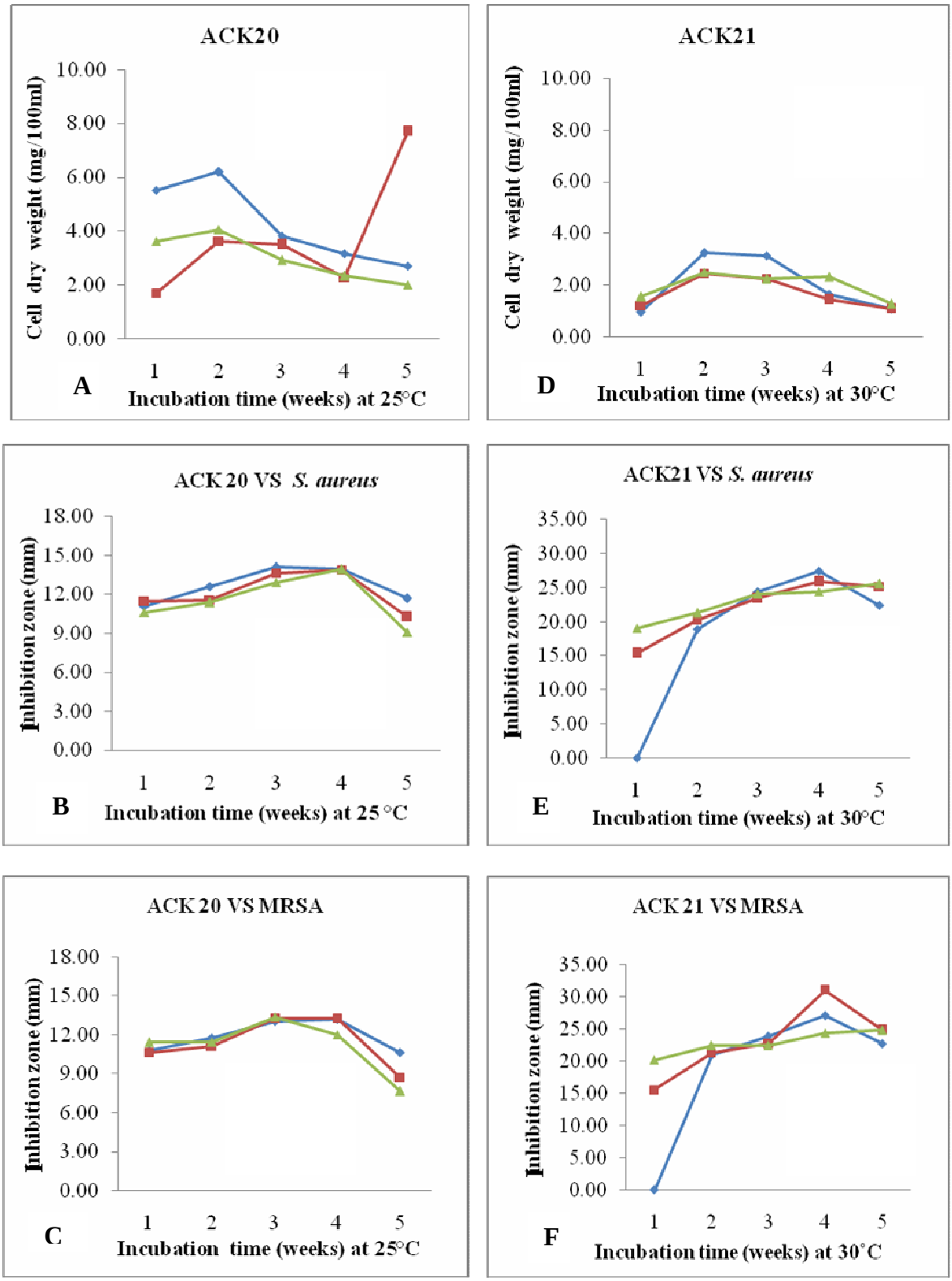


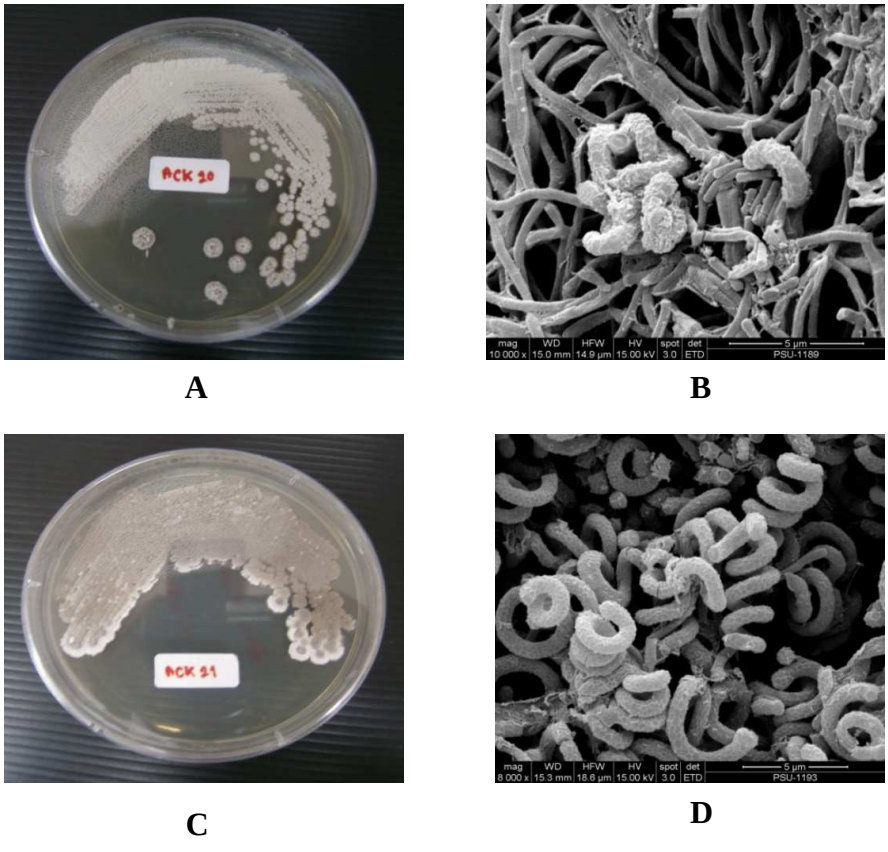
Figure 27 Effect of different initial pH of ISP-2 medium on growth and production of antibacterial metabolites by actinomycetes ACK20 (A-C) and ACK21 (D-F). Symbols: ◆, pH 6; ■, pH 7; ▲, pH 8.

3.6 Identification of actinomycetes

The top 2 actinomycetes, ACK20 and ACK21 were identified by morphological characteristics and molecular technique (16S rDNA).

3.6.1 Morphological characteristics

The two best active actinomycetes that grew on YME agar were slow growing, aerobic, glabrous or chalky and with gray aerial mycelia (Figure 28A and C) and possessed an earthy odour. The microscopic examination of ACK21 under scanning electron microscope showed that the spore chains are in spiral form (Figure 28D) which is the characteristic of the genus *Streptomyces*. The scanning electron micrograph of ACK20 revealed that this strain produced curved spore (Figure 28B).



A **B**

C **D**

Figure 28 Morphological characteristics of ACK 20 (A and B) and ACK 21 (C and D)
A and C, colonial morphology on YME agar at 30°C for 6 weeks
B and D, Scanning electron micrographs showing curved spore and spiral type of spore chains, respectively

3.6.2 Molecular technique (16S DNA)

The 16S DNA aligment of actinomycete ACK20 consisted of 17 taxa in *Amycolatopsis*, with *Amycolatopsis alba* as an outgroup. The dataset comprised 900 characters, 875 of which were constant charecters, 9 were uninformative characters and 16 were informative characters. Maximum parsimony analysis yieled 1 MPT. CI and RI of 100 steps, 1.0000 and 1.0000 respectively. The tree from NJ analysis had an identical topology to the tree from the MP analysis. Actinomycetes ACK20 was placed in subclade A and was closely related to *Amycolatopsis echigonensis* (AB546313, NR041404 and AB248535) with short branch length and supported with 60% and 62% bootstrap values from the MP (Figure 29) and NJ analyses respectively. The nucleotide identity were 99.6, 99.3 and 99.3 %, respectively which indicated that ACK20 had two base difference with AB546313. ACK 20 was then identified as *Amycolatopsis echigonensis*.

The 16S DNA aligment of Actinomycetes ACK21 consisted of 30 taxa in *Streptomyces*, with *Streptomyces aureofaciens* as an outgroup. The dataset comprised 1124 characters, 1034 of which were constant charecters, 58 were uninformative characters and 32 were informative characters. Maximum parsimony analysis yieled 1 MPT. CI and RI of 10 steps, 0.7544 and 0.9327 respectively. The tree from NJ analysis had an identical topology to the tree from the MP analysis. Actinomycete ACK21 was placed in a species complex subclade A comprising *Streptomyces indonesiensis* (HQ244467, FJ406119), *Streptomyces rhizosphaericus* (NR041415, FJ406121), *Streptomyces asiaticus* (NR041418, HQ244468) *Streptomyces cangkringensis* (HQ244471, FJ406120) and *Streptomyces hygroscopicus* (FJ968105, EU016370) with short branch length and supported with 99% and 98% bootstrap values from the MP (Figure 30) and NJ analyses respectively, which the nucleotide identity difference between 99.3-99.7% and have 2-7 base difference, Therefore ACK21 was identified as *Streptomyces* sp.

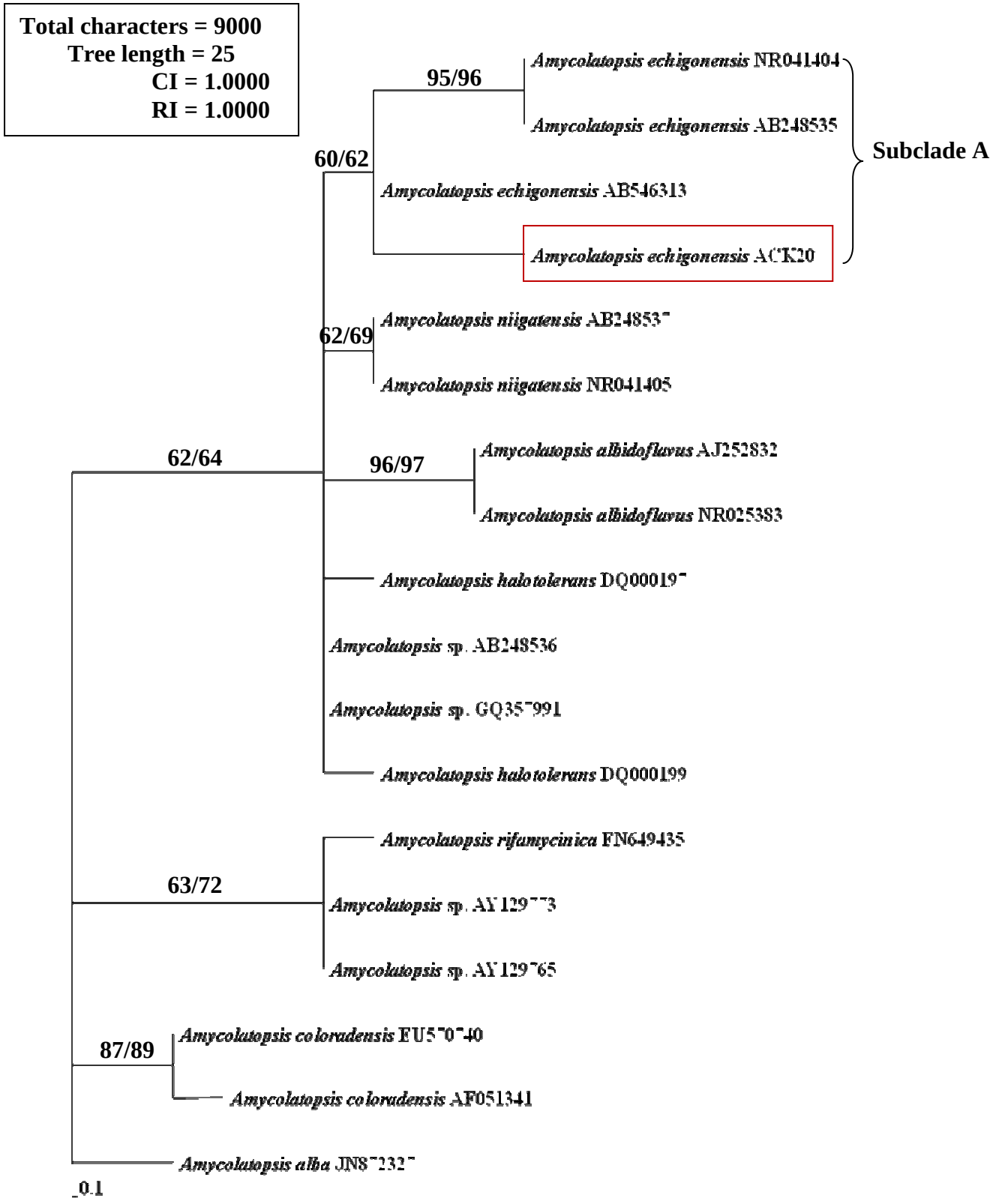


Figure 29 Phylogram obtained from 16S rDNA sequence analysis of actinomycetes ACK20 and related *Amycolatopsis echigonensis* from Maximum parsimony analysis. Scale bar represents 0.1 base pair changes.

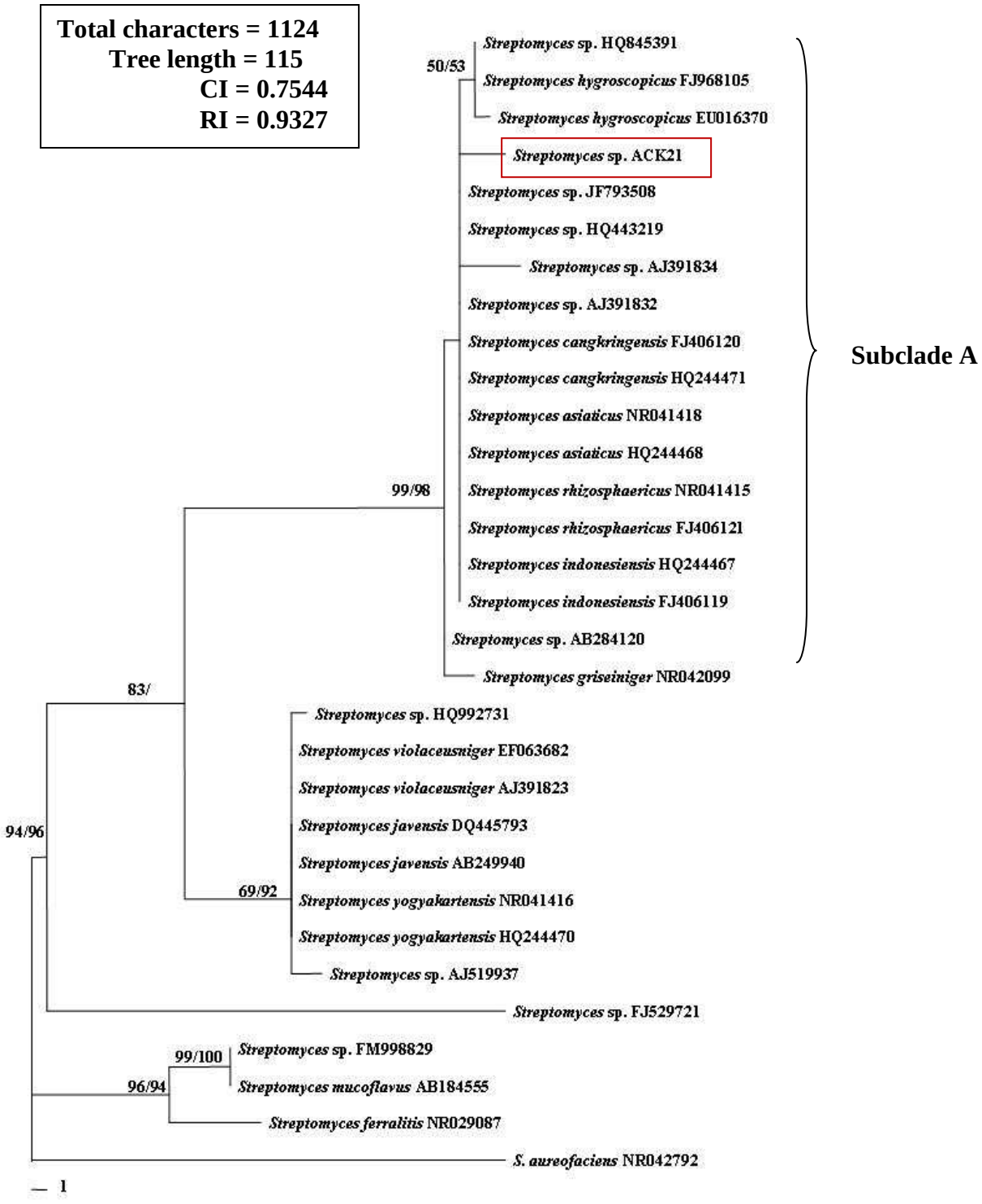


Figure 30 Phylogram obtained from 16S rDNA sequence analysis of actinomycetes ACK21 and related *Streptomyces* sp. from Maximum parsimony analysis. Scale bar represents 1 base pair changes.

CHAPTER 4

DISCUSSION

4.1. Antimicrobial activity

It has been known for a long time that actinomycetes are a good source of antibiotics. The majority of antibiotics commonly used to treat infectious diseases are from actinomycetes. It has been extensively studied in the past. However, the searches for antibiotic producing actinomycetes are continuing because of a high demand for new types of antibiotics against drug resistant pathogens. Scientists are trying to screen actinomycetes that can produce antimicrobial substances from different sources continuously (Pandey *et al.*, 2004; Oskay *et al.*, 2004; Nedialkova and Naidenova, 2004-2005; Anansiriwattana *et al.*, 2006; Singh *et al.*, 2006; Parungao *et al.*, 2007; Arasu *et al.*, 2009; Dharmaraj and Sumantha, 2009; Ningthoujam *et al.*, 2009; Arifuzzaman *et al.*, 2010; Dehnad *et al.*, 2010; Hozzein *et al.*, 2011). In this study, 100 selected actinomycetes isolated from soils in southern Thailand were screened for their ability to inhibit human pathogens. From the initial screening, 80% of actinomycetes showed inhibitory activity against at least one tested microorganism, from these 8% had antibacterial activity, 32% had antifungal activity and 40% had both antibacterial and antifungal activities. The percentage of active isolates obtained from this study is markedly high when compared with the reports of Parungao *et al.* (2007) in the Philippines (26%), Oskay *et al.* (2004) in Turkey (34%), Ningthoujam *et al.* (2009) and Singh *et al.* (2006) in India (37% and 57%, respectively). In Thailand, Anansiriwattana *et al*. (2006) isolated actinomycetes from soil and sand samples in Koh Samed, Rayong province, Thailand and reported that 69% of their isolates were active against *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633 and *C. albicans* ATCC

10231. Our result is consistent with the study of de Oliveira *et al*. (2010) that 88.6% of endophytic actinobacteria tested showed antimicrobial activity against at least one phytopathogen. Duangsook (2010) also found that 85% of her actinomycete isolates have antifungal activity against fungi contaminated on pararubber sheet. The most active actinomycetes from this study showed antifungal activity against human pathogenic fungi: *C. neoformans* (49%), *P. marneffei* (41%), *C. albicans* (30%) and *M. gypseum* (21%), respectively. It is possible that 60% of the actinomycete strains in this study were known to have antifungal activity against plant fungal pathogens (Boukaew *et al.,* 2010). Isolate ACK60 was most active against *C. neoformans* with the largest inhibition zone of 34.37 mm and ACK26 against *C. albicans* (32.45 mm) compared with the results from other studies. Cho *et al.* (2001) found that 3-hydroxy- -butyrolactones (50 g/disk) from *Streptomyces* sp. can inhibit the growth of *C. albicans* with inhibition zone of 19.0 mm. Chatujinda *et al.* (2007) indicated that actinomycetes strain CB5-3 isolated from soil showed antifungal activity against *C. albicans* ATCC 10231 with an inhibition zone of 21.3 mm in the primary screening test. In addition, 11 and 30 isolates showed high inhibitory activity >80% hyphal growth inhibition and 9 isolates strongly inhibited both *P. marneffei* and *M. gypseum*. Candidiasis and cryptococcosis have emerged as major problems worldwide in cancer patients, transplant recipients, and other immunocompromised individuals, including those with AIDS (Pitisuttithum *et al.,* 2001; Umeh and Umeakanne, 2010). Penicilliosis marneffei is an opportunistic fungal infection in AIDS patients with a high incidence in Southeast Asia, in particular in the northern part of Thailand (Vanittanakom *et al.,* 2006; Hai *et al.,* 2010). Despite advances in antifungal therapy in the last decade and the increasing numbers of options available for treating fungal infections, a high prevalence of antifungal resistance and failures of clinical treatments have been reported (Bueid *et al.,* 2010; Pfaller *et al.,* 2010, 2011). The results from this study showed that soil actinomycetes are a good source of antifungal compounds against those pathogens.

For antibacterial activity, 40% of our soil actinomycetes inhibited both strains of *S. aureus* and only 9% and 15% inhibited *E. coli* and *P. aeruginosa*, respectively. In general, Gram-negative bacteria are more resistant to chemical agents than the Gram-positive bacteria due to their outer membrane structures that act as a

permeability barrier (Denyer *et al.,* 2004; Bansal *et al.,* 2010). ACK18 showed the best inhibitory activity against *S. aureus* with an inhibition zone of 30.95 mm and ACK84 against MRSA SK1 (inhibition zone 35.17 mm) whereas ACK108 had the best activity against *E. coli* (inhibition zone 32.87 mm) and ACK117 against *P. aeruginosa* (inhibition zone 33.62 mm). Schumacher *et al*. (2003) reported that bonactin from *Streptomyces* sp. strain BD21-2 can inhibit the growth of *B. megaterium, M. luteus, Klebsiella pneumoniae, S. aureus, Alcaligenes faecalis, E. coli* and *Saccharomyces cerevisiae* with inhibition zones in the range of only 7-10 mm. Chatujinda *et al.* (2007) reported that actinomycetes strain CB5-3 had antibacterial activity against *S. aureus* and *E. coli* with inhibition zones of 24.2 and 15.7 mm, respectively. In addition, many investigators also found that metabolites from *Streptomyces* spp. have antibacterial activities against various bacteria including *S. aureus*, MRSA, VRSA, *B. subtilis, S. epidermidis, Enterococcus faecalis, Micrococcus luteus, E. coli, P. aeruginosa,* and *Klebsiella* sp. (Arasu *et al.,* 2008; Selvameenal *et al.,* 2009; Yadav *et al.,* 2009; Duraipandiyan *et al.,* 2010; Aouiche *et al.,* 2012; Dasari *et al.,* 2012).

The top 46 actinomycetes that can inhibit each group of the tested bacteria and yeasts from cross streak technique with inhibition zones over 25 mm and hyphal growth inhibition over 80% were selected for chemical extraction. Three types of crude extracts were obtained; the ethyl acetate extracts from the culture broth (BE), the ethyl acetate extracts from the cells (CE), and the hexane extracts from the cells (CH). Among these, CH extracts gave positive results more than the CE and BE extracts. This indicated that the active constituents are cell-bound and nonpolar. Holloway (2006) reported that moderate degree of polarity is a characteristic of most antibiotics.

Of the 138 crude extracts from 46 actinomycetes isolates, 59.42% and 50.72% of extracts were active against *S. aureus* and MRSA while only 28.26% showed antifungal activity. This result is in contrast to the primary screening. The cross streak technique can detect the water soluble substances that are diffused through the agar medium whereas the crude extracts are less polar. Different compounds can inhibit different microorganisms. Crude CH extract from ACK21 (ACK21CH) exhibited the strongest antibacterial activity against *S. aureus* and MRSA

with MIC/MBC 0.5/4 and 0.5/8 µg/ml, respectively followed by ACK20CE against *S. aureus* and MRSA with MIC/MBC 2/8 µg/ml, 0.5/2 µg/ml, respectively. Thess results are comparable to the standard drug vancomycin (MIC/MBC 1/2 µg/ml). None of the extracts inhibited *E. coli* (MIC and MBC>200 µg/ml) and only one extract (ACK20CH) was active against *P. aeruginosa* with MIC/MBC values 200/>200 µg/ml. Compared with the results of Duraipandiyan *et al*. (2010), actinomycetes ERIH-44 showed both antibacterial and antifungal activity with MICs against *B. subtilis* (<15.62 µg/ml), *S. aureus* (<15.62 µg/ml), *E. coli* (125 µg/ml) and *P. aeruginosa* (500 µg/ml), *Botrytis cinerea* (500 µg/ml) and *Trichophyton mentagrophytes* (1000 µg/ml). Aouiche *et al.* (2012) reported that isolate PAL111 showed a strong activity against *C. albicans*, filamentous fungi, and Gram-positive and Gram-negative bacteria with MICs between 2 and 20 µg/ml for yeast, 10 and 50 µg/ml for filamentous fungi, 2 and 10 µg/ml for Gram-positive bacteria and 20 and 75 µg/ml for Gram-negative bacteria. In addition, the effects of ACK20CE and ACK21CH at 4 times MIC were also observed by SEM along with the vancomycin. Both extracts had an effect on the morphological structure of the cells which was indicated by the presence of pores and shrinkage in the cell wall. Some cells had undergone cell lysis. This may be due to the imperfect synthesis of the cell wall. The cells in contact with actinomycete extracts had more damaged cells (Figures 23C, 23D, 24C, 24D) than those treated with vancomycin (at 4MIC) (Figures 23B and 24B). Vancomycin inhibits the bacterial cell wall synthesis by strongly binding to the end of the D-Ala-D-Ala, therefore it inhibits peptidoglycan synthesis and also inhibits the cross linkage. The cell becomes vulnerable and easily lysed causing the cell wall shrunk. Besides that the cell membrane also becomes damaged leading to cell death (Anam *et al.,* 2010). The SEM result indicated that ACK20CE and ACK21CH interfere the cell wall synthesis. Active compounds from these two isolates are under investigation.

For antifungal activity, crude CH extract from ACK21 (ACK21CH) exhibited the strongest antifungal activity against *C. albicans* NCPF3153 with MIC/MFC 4/128 µg/ml which is better than the results from other investigations. Arasu *et al*. (2008) reported that extract from actinomycetes isolate ERI-26 showed the MIC of 500 µg/ml against *C. albicans* while Gandhimathi *et al*. (2008) found that

extract from *Streptomyces* sp. strain CPI 13 inhibited *C. tropicalis* with MIC and MFC values of 10 and 12.5 μg/ml, respectively. The extracts had less activity against *C. neoformans*, *M. gypseum* and *P. marneffei*. However, this is the first study of actinomycetes against *P. marneffei*. Although only 5% of the extracts were active against *P. marneffei* with MIC of 200 μg/ml, but it is worth noting that many actinomycetes could completely inhibit the hyphal growth of this fungus. The active actinomycetes may produce polar antifungal compounds into the agar medium.

4.2 Optimization of the antimicrobial production by actinomycetes ACK20 and ACK21

As isolates ACK20 and ACK21 showed the best antimicrobial activity, they were then selected for the optimization study. Effects of agitation, pH of initial medium and incubation temperature on the production of antimicrobial compounds by ACK20 and ACK21 were investigated. The cell mass obtained from each condition was measured and antibacterial activity of culture broth against *S. aureus* ATCC 25923 and MRSA were detected by agar well diffusion.

The production of antibacterial agents by ACK20 and ACK21 was carried out in ISP-2 broth. ISP media were developed by Difco Laboratories for the International *Streptomyces* Project (ISP). ISP-2 is also referred to as Yeast Extract-Malt Extract agar and contains 0.4% yeast extract, 1% malt extract and 0.4% dextrose. Yeast extract and malt extract provide nitrogen, amino acids and vitamins, and dextrose is the main carbon source (Duangsook, 2010). ISP-2 is the traditional medium used to determine the cultural characteristics of actinomycetes. However, it has also been used by many investigators for production of antimicrobial agents by various actinomycetes (Augustine *et al.,* 2005; Boudjella *et al.,* 2006; Thakur *et al*., 2009; Duangsook, 2010; Nookao, 2011).

Cultivation conditions play an important role in the antibiotic production by actinomycetes. In this study, the production of antibacterial compounds by ACK20 and ACK21 was carried out in static and shaking cultures. Both isolates grew well under shaking condition but the antibacterial activity obtained from static cultures was higher than those from the shaking cultures. Antibiotics are secondary

metabolites which microorganisms produce in the stationary phase. A high degree of oxygen transfer during the exponential growth phase may ultimately help to improve antibiotic production. Different strains require different cultivation conditions. Many investigators have found that their actinomycetes produce high antibiotic yields in shake flask condition (Boudjella *et al.*, 2006; Singh *et al*., 2009; Thakur *et al*., 2009). Thakur *et al*. (2009) reported that the optimum production of the antimicrobial agent by *Streptomyces* sp. 201 in laboratory-scale fermentation could be achieved in culture medium supplemented with mannitol at a concentration of 1.5 g/l as carbon and asparagine concentration 0.9 g/l as nitrogen sources, temperature at 30^{o}C, initial pH of the medium 7.5, inoculum size equal to 2x10^{9} spore/ml and incubation period of 60 days under shaking condition. In the contrary, other studies by Hassan *et al*. (2001), El-sersy and Abou-Elela (2006), Al-Zahrani (2007) and Duangsook (2010) have shown that the static condition is good for their isolates. Therefore, each isolate must be checked for its optimum culture condition.

The biosynthesis of secondary metabolites by microorganisms is regulated by the growth temperature. In this study, the maximum activity of ACK20 and ACK21 against *S. aureus* and MRSA was found in the fourth week at 25 and 30°C of incubation, respectively. Boudjella *et al.* (2006) studied the antimicrobial production of *Streptosporangium* sp. Sg 10 cultured in ISP 2 broth, pH 7.2 in a rotary shaker at 250 rpm, at 30^{o}C for 17 days and tested against *M. luteus* and *Mucor ramannianus* by agar diffusion method. The antimicrobial activity was detected on day 4 until day 11 of incubation and the antibacterial was stronger than the antifungal activity. In 2010, de Oliveira *et al.* reported that the metabolite produced by *Streptomyces* sp. R18(6) grown in SC broth at 30^{o}C and pH 7.0 showed the best inhibition zone observed with the diffusion-well method. Nookao (2011) indicated that actinomycetes strain KM 1.1-7.9 grown in GMP liquid medium on a rotary shaker (180 rpm) at 30^{o}C for nine days produced antibacterial metabolites against *Ralstonia solanacearum*.

Another important factor affecting antibiotic production is the initial pH of medium. The activity of several major enzymes that catalyze metabolic reactions of cell growth and antibiotic formation are affected by pH (Guimaraes *et al*., 2004; Liang *et al*., 2008). In general, the growth pH range of actinomycetes is 6.5-8.0

(Locci, 1989) and the optimum pH is 7.0 (Jensen, 1991). ACK20 and ACK21 grew well in all pHs studied and the cell mass increased at the first week of cultivation and the maximum growth was achieved at the second week, then gradually decreased, except for ACK20 under cultivation at pH7. The best activity of ACK20 and ACK21 was observed in the medium with initial pH6-7 and pH 7, respectively**.** Kontro *et al*. (2005) studied the pH effects on growth and sporulation of ten *Streptomyces* spp. The growth pH ranges and pH ranges for the optimal growth of those *Streptomyces* spp. were strongly dependent on the nutrient composition of the media but the ability to sporulate was independent of the pH and medium composition.

The optimum culture conditions for the production of antimicrobial metabolites from many studies, have varied. In 2005, Augustine *et al*. reported that the optimum conditions for antifungal metabolite production of *Streptomyces rochei* AK 39 were starch casein medium pH 7 containing glycerol 1.2%, agitation at 200 rpm, temperature at 37^{o}C. Oskay (2009) reported that the optimum conditions for antimicrobial production by *Streptomyces* sp. KEH 23 were an initial pH of 7.5, a temperature of 30^{o}C under shaking condition. The optimum conditions for *Streptomyces tanashiensis* strain A2D were 28^{o}C, at a pH of 8 under shaking condition and this strain grew upto pH 9, and indicated that this strain was part of the alkaliphilic actinomycetes group (Singh *et al*., 2009). In addition, Duangsook (2010) found that the optimum conditions for the production of antifungal metabolites by *Streptomyces* spp. AC41 and AC51 against fungi contaminated on rubber sheet were under the static condition, a pH of 7 at 30^{o}C for 6 weeks. In this study, the optimum conditions for the production of antimicrobial metabolites by isolates ACK20 were pH 6-7 at 25^{o}C under static condition for 4 weeks and by ACK21 were pH 7 at 30^{o}C for 4 weeks. Both isolates are mesophilic and neutrophilic.

4.3 Identification of actinomycetes

In this study, the two best antimicrobial producing isolates ACK20 and ACK 21 were identified based on morphological and molecular characteristics as *Amycolatopsis echigonensis* and *Streptomyces* sp., respectively. *A. echigonensis* was first isolated from a filtration substrate made from Japanese volcanic soil and described as a new species based on morphological, physiological and genotypic characteristics by Ding *et al*. in 2007. The genus is known to contain numerous antibiotic producing strains, with the glycopeptide (e.g. vancomycin) and ansamycin (e.g. rifamycin) producers being the most important to medicine. PCR screening for antibiotic biosynthetic potential revealed the presence of antibiotic biosynthetic genes in all the *Amycolatopsis* type strains (Everest and Meyers, 2011). Recently, Igarashi *et al*. (2008) reported that *Amycolatopsis* ML1-hF4 produced pargamicin A, a novel cyclic peptide antibiotic with a potent antibacterial activity against *S. aureus* strains including MRSA and *Enterococcus faecalis*/*faecium* strains including vancomycin-resistant enterococci (VRE). Extracts from *A. echigonensis* ACK20 showed both antibacterial and antifungal activities but the antibacterial activity is much stronger than the antifungal one. To our knowledge this is the first report on antimicrobial metabolites produced by *A. echigonensis*. The chemical contituents produced by this strain is under investigation.

Streptomyces sp. ACK21 was grouped together with *S. cangkringensis*, *S. asiaticus*, *S. rhizosphaericus*, *S. indonesiensis*, *S. hygroscopicus* and *Streptomyces* sp. 02-32. The first four species are members of *Streptomyces violaceusniger* clade which is known to be antagonistic to various plant pathogenic fungi (Sembiring *et al.*, 2000) and a source of antibacterial and antifungal metabolites (Goodfellow *et al.*, 2007). *S. cangkringensis*, *S. asiaticus* and *S. indonesiensis* were described as new species by Sembiring *et al.* in 2000. They shared 16S rRNA gene similarities within the range of 99.2 – 99.7 values that correspond to between 4 and 6 nucleotide differences (Goodfellow *et al.*, 2007). *S. asiaticus* and *S. indonesiensis* were first isolated from rhizosphere soils whereas *S. cangkringensis* was from the non-rhizosphere soil adjacent to a stand of the tropical legume, *Paraserianthes falcataria* in Indonesia. They produce spiral spore chains and the spore surface is rugose. Our

Streptomyces sp. ACK21 produces spiral spore chain too. Like *A. echigonensis* there is no detailed reports on the production of antibiotics from these three species. *Streptomyces hygroscopicus* and related species are the most well known candidate producers of antibiotics and many other industrially and agronomically important secondary metabolites in the genus *Streptomyces*. Currently more than 650 kinds of bioactive substances have been produced by *S. hygroscopicus* and related species (Rong and Huang, 2012). The examples of antibiotics produced by *S. hygroscopicus* are such as geldamycin (antitumor), hygromycin B (anthelmintic), nigericin (against Gram-positive bacteria) and validamycin (fungicide). All three extracts from our isolate *Streptomyces* sp. ACK21 exhibited the strongest antibacterial activity against *S. aureus* strains including MRSA with MICs lower than vancomycin. The chemical contituents produced by ACK21 is under investigation.

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

In this study, total of 100 actinomycetes isolated from soils from four provinces in southern Thailand were screened for their ability to produce antimicrobial substances by cross streak and hyphal growth inhibition tests against ten human pathogens. Eighty percents of the isolates showed antimicrobial activity against at least one test microorganism. Among them, 8% inhibited only bacteria, 32% inhibited only yeasts and/or fungi and 40% had both antibacterial and antifungal activities. For antibacterial activity, 40% of soil actinomycetes inhibited both strains of *S. aureus* and only 9% and 15% inhibited *E. coli* and *P. aeruginosa*, respectively. For antifungal activity, 21, 30, 41, and 49% inhibited *M. gypseum, C. albicans, P. marneffei* and *C. neoformans*, respectively.

Forty-six active isolates that showed inhibition zone over 25 mm and hyphal growth inhibition over 80% were selected and cultured in Yeast Malt Extract broth for extraction of bioactive compound. Three types of crude extracts, BE, CH and CE were obtained. CH provided the highest active extracts (34/46, 73.91%) followed by CE (31/46, 67.39%), and BE (25/46, 54.34%), respectively. Crude extracts were then tested for their MICs, MBCs and MFCs by broth microdilution methods according to Clinical and Laboratory Standards Institute (CLSI). Ninety extracts out of 138 total extracts from 46 actinomycete isolates were inhibitory. They can inhibit both strains of *S. aureus* with MIC values of 0.5-200 µg/ml and MBC values of 2->200 µg/ml, respectively. Crude CH extracts from ACK21 (ACK21CH) exhibited the strongest antibacterial activity against *S. aureus* and MRSA with MIC/MBC 0.5/4 and

0.5/8 µg/ml, respectively followed by ACK20CE against *S. aureus* and MRSA with MIC/MBC 2/8 µg/ml, 0.5/2 µg/ml, respectively. In addition, scanning electron microscopic study showed that extracts from ACK21CH and ACK20CE strongly destroyed *S. aureus* cells causing cytoplasm leakage and cell death.

Three factors including agitation, temperature and initial pH of the medium on the production of antimicrobial metabolites by the isolates ACK21 and ACK20 were investigated. The optimum condition for ACK21 was observed at the static condition, pH7 and temperature 30^{o}C and ACK20 was observed at the static condition, pH6 and 7 and temperature 25^{o}C for 4 weeks for the production of antibacterial metabolites against both *S. aureus* ATCC25923 and MRSA.

Based on morphological characteristics and 16S DNA analysis, ACK21 was identified as *Streptomyces* sp. and ACK20 as *Amycolatopsis echigonensis*.

5.2 Suggestion for future work

1) Purification and structural identification of the bioactive compounds from the most active *Streptomyces* sp. ACK21 and *Amycolatopsis echigonensis* ACK20

2) Study on mechanisms of action of bioactive compounds from these two best active isolates

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APPENDIX

Actinomycete Isolation Agar (AIA)	**1 litre**
Sodium caseinate	2.0 g
Asparagine	0.1 g
Sodium propionate	4.0 g
Dipotassium phosphate	0.5 g
Magnesium sulfate	0.1 g
Ferrous sulfate	0.001 g
Agar	15.0 g

Nutrient Agar (NA)	**1 litre**
Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g

Yeast extract-malt extract broth (ISP-2)	**1 litre**
Malt extract	10.0 g
Yeast extract	4.0 g
Glucose	4.0 g
pH	7

1.8% resazurin

Add 1.8 g of resazurin dye to 100 ml of distilled water and mix thoroughly. Filter resarurin dye solution with membrane 0.45 µm and store in eppendorf wrapping with foil at 4 ^{o}C. Dilute 1.8% resazurin with sterile distilled water to 1:10 and mix thoroughly before using for antimicrobial test.

Neighbour-joining: Neighbor-joining is based on the minimum-evolution criterion for phylogenetic trees, i.e. the topology that gives the least total branch length is preferred at each step of the algorithm. However, neighbor-joining may not find the true tree topology with least total branch length because it is a greedy algorithm that constructs the tree in a step-wise fashion. Even though it is sub-optimal in this sense, it has been extensively tested and usually finds a tree that is quite close to the optimal tree. Nevertheless, it has been largely superseded in phylogenetics by methods that do not rely on distance measures and offer superior accuracy under most conditions (Duangsook, 2010)

Maximum parsimony: Maximum parsimony is a character-based method that infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given set of data, or in other words by minimizing the total tree length. The trees requiring the fewest character state changes are considered optimal. Individual characters are termed “informative” in maximum parsimony analysis when they require different numbers of changes on different trees. To be informative, an unpolarized character must have at least two character states and each state must be present in at least two taxa. Character state changes required for the tree is the length of the tree. As the number of taxa increases, the number of possible trees increases even faster, finding the most parsimonious trees (MPTs) for a given data set can be a computationally intensive task to choose the tree that result in fewest character state changes. (Duangsook, 2010)

Bootstrapping (Simpson, 2006)**:** Bootstrapping is a resampling tree evaluation method that works with distance, parsimony, likelihood and just about any other tree derivation method. The result of bootstrap analysis is typically a number associated with a particular branch in the phylogenetic tree that gives the proportion of bootstrap replicates that supports the monophyly of the clade. Bootstrap values greater than 70% correspond to a probability of greater than 95% that the true phylogeny has been found and greater than 50% will be an overestimate of accuracy. A high bootstrap value can make the right phylogeny.

Consistency index (CI) (Simpson, 2006)**:** One measure of the relative amount of homoplasy in the cladogram is the consistency index. The consistency index is equal to the ratio of minimum changes of character state or minimum possible tree length that must occur and the actual number of changes or tree length that do occur. A consistency index close to 1 indicates little to no homoplasy; a CI close to 0 is indicative of considerable homoplasy.

$$CI = \frac{\text{Minimum possible tree length}}{\text{Tree length}}$$

Retention index (RI) (Simpson, 2006)**:** The retention index is calculated as the ratio (g-s)/(g-m), where g is the maximum possible tree length that could occur on any conceivable tree, s and m are tree length and minimum possible tree length, respectively. Thus, the RI is influenced by the number of taxon in the study. A consistency index close to 1 indicates little to no homoplasy; a RI close to 0 is indicative of considerable homoplasy.

$$RI = \frac{\text{Maximum possible tree length - tree length}}{\text{Maximum possible tree length - minimum possible tree length}}$$

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Sawasdee, S., Phongpaichit, S., Rukachaisirikul, V. and Sukhoom, A. 2011. Screening for Antimicrobial Substance Producing Actinomycetes from Soil. The International Congress for Innovation in Chemistry (PECH-CIC Congress VIII) “Chemistry, Environment and Society”, 4-7th May 2011, Jomtien Plam Beach Hotel and Resort, Pattaya, Thailand.

Sawasdee, S., Phongpaichit, S., Rukachaisirikul, V. and Sukhoom, A. 2011. Screening for Antimicrobial Substance Producing Actinomycetes from Soil. The International Congress on Natural Products (I), 17-18th October 2011, Phang Nga, Thailand.