การพิสูจน์เอกลักษณ์และฤทธิ์ต้านจุลชีพของแอคติโนมัยสีทเอนโดไฟท์
ที่แยกได้จากพืชสมุนไพรในประเทศไทย

นางสาวณัฐพร คล้ายคลึง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM MEDICINAL PLANTS IN THAILAND

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Microbiology
Department of Biochemistry and Microbiology
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Thesis Title	IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM MEDICINAL PLANTS IN THAILAND
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Field of Study	Microbiology
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แอคติโนมัยสีทจำนวน 47 ไอโซเลต ซึ่งแยกได้จากใบ ลำต้น และรากของพืชสมุนไพร มีแพงพวยฝรั่ง หนอนตายหยาก ลูกใต้ใบ ร่องไม้ ว่านธรณีสาร หญ้าดอกขาว และ *Ophiorrhiza* sp. โดยใช้อาหาร วุ้น starch-casein, humic acid-vitamin และ water agar จากการศึกษาลักษณะทางฟีโนไทป์ และจีโนไทป์ สามารถพิสูจน์เอกลักษณ์แอคติโนมัยสีทเหล่านี้ได้เป็นสกุล *Streptomyces* 14 ไอโซเลต, *Amycolatopsis* 1 ไอโซเลต, *Nocardia* 5 ไอโซเลต , *Micromonospora* 6 ไอโซเลต, *Microbispora* 15 ไอโซเลต และ *Nonomuraea* 6 ไอโซเลต จากผลการวิเคราะห์ลำดับเบสในช่วง 16S rRNA gene เป็นสกุล *Streptomyces* 2 ไอโซเลต ได้แก่ PA1-07 คล้ายคลึงกับ *Streptomyces curacoi* JCM 4219^{T} (98.75%) และ VC1-01 คล้ายคลึงกับ *S. coelurescens* JCM 4360^{T} (99.04%) สกุล *Amycolatopsis* 1 ไอโซเลต ได้แก่ ST1-08 คล้ายคลึงกับ *Amycolatopsis pretoriensis* JCM 12673^{T} (99.17%) สกุล *Nocardia* 1 ไอโซเลต ได้แก่ ST1-06 คล้ายคลึงกับ *Nocardia araoensis* JCM 12118^{T} (99.03%) สกุล *Microbispora* 7 ไอโซเลต ได้แก่ CR1-01, CR1-04, CR1-07, CR1-08 และ CR1-11 คล้ายคลึงกับ *Microbispora rosea* subsp. *rosea* JCM 3006^{T} (98.83-99.17%) ในขณะที่ CR1-09 และ OH1-01 คล้ายคลึงกับ *M. hainanensis* JCM 19666^{T} (98.98%) และ *M. corallina* JCM 10267^{T} (98.96%) ตามลำดับ และสกุล *Nonomuraea* 1 ไอโซเลต ได้แก่ PA1-10 คล้ายคลึงกับ *Nonomuraea candida* JCM 15928^{T} (98.31%) จากการศึกษาฤทธิ์ต้านจุลชีพของสารสกัดเอทิลอะซิเตทของไอโซเลตเหล่านี้ โดยวิธี disc diffusion พบว่าสารสกัดจากไอโซเลต CR1-01 และ CR1-08 มีฤทธิ์ยับยั้ง *Staphylococcus aureus* ATCC 6538 สารสกัดจาก CR1-01 และ CR1-05 มีฤทธิ์ยับยั้ง *Kocuria rhizophila* ATCC 9341 ส่วนสารสกัดจาก ST1-02 กับ ST1-05 มีฤทธิ์ยับยั้ง *Candida albicans* ATCC 10231

ภาควิชา	ชีวเคมีและจุลชีววิทยา	ลายมือชื่อนิสิต	.....
สาขาวิชา	จุลชีววิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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NATTAPORN KLYKLEUNG: IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM MEDICINAL PLANTS IN THAILAND. ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., CO-ADVISOR: PATTAMA PITTAYAKHAJONWUT, Ph.D., 122 pp.

Forty-seven actinomycetes were isolated from leaves, stems and roots of seven medicinal plants, *Catharanthus roseus*, *Stemona* sp., *Phyllanthus amarus, Pseuderanthemum graciliflorum*, *Phyllanthuspulcher, Vernoniacinerea* and *Ophiorrhiza* sp. by using starch-casein, humic acid-vitamin and water agar. They were identified as *Streptomyces* (14 isolates), *Amycolatopsis* (1 isolate), *Nocardia* (5 isolates), *Micromonospora* (6 isolates), *Microbispora* (15 isolates) and *Nonomuraea* (6 isolates) based on phenotypic and genotypic characteristics. On the basis of 16S rRNA gene sequences analysis, *Streptomyces* (2 isolates), PA1-07 was similar to *Streptomyces curacoi* JCM 4219^{T} (98.75%) while VC1-01 was similar to *S. coelurescens* JCM 4360^{T} (99.04%). *Amycolatopsis* (1 isolate), ST1-08 was similar to *Amycolatopsis pretoriensis* JCM 12673^{T} (99.17%). *Nocardia* (1 isolate), ST1-06 was similar to *Nocardia araoensis* JCM 12118^{T} (99.03%). *Microbispora* (7 isolates), CR1-01, CR1-04, CR1-07, CR1-08 and CR1-11 were similar to *Microbispora rosea* subsp. *rosea* JCM 3006^{T} (98.83-99.17%) while CR1-09 was similar to *M. hainanensis* JCM 19666^{T} (98.98-99.93%) and OH1-01 was similar to *M. corallina* JCM 10267^{T} (98.96%). *Nonomuraea* (1 isolate), PA1-10 was similar to *Nonomuraea candida* JCM 15928^{T} (98.31%). The ethyl acetate extract of these isolates were screened for antimicrobial activity based on disc diffusion method. The crude extract of CR1-01 and CR1-08 showed antibacterial activity against *Staphylococcus aureus* ATCC 6538. The crude extract of CR1-01 and CR1-05 showed against *Kocuria rhizophila* ATCC 9341. Whereas, the crude extract of ST1-02 and ST1-05 exhibited against *Candida albicans* ATCC 10231.

Department:	Biochemistry and Microbiology	Student's Signature	.....
Field of Study:	Microbiology	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....

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LIST OF ABBREVATIONS

α	=	Alpha
β	=	Beta
ω	=	Omega
ATCC	=	American Type Culture Collection, Maryland, U.S.A.
$Ba(OH)_2$	=	Bariumhydroxide
cm	=	Centimeter
Czk	=	Czapek's sucrose
°C	=	Degree of celsius
Ca^{2+}	=	Calcium ion
MeOH	=	Methanol
$CHCl_3$	=	Chloroform
DAP, A_2pm	=	Diaminopimelic acid
DDBJ	=	DNA Data Bank of Japan
DNase	=	Deoxyribonuclease
dNTP	=	Deoxyribonucleotide triphosphate
DNA	=	Deoxyribonucleic acid
EDTA	=	Disodiumethylenediaminetetraacetate
g	=	Gram
µg	=	Microgram
mg	=	Milligram
G+C	=	Guanine-plus-cytosine
GenBank	=	National Institute of Health genetic sequence database
GlyA	=	Glycerol-asparagine agar
GluA	=	Glucose-asparagine agar
h	=	Hour
HV	=	Humic-vitamin agar
HCl	=	Hydrochloric acid
H_2O	=	Water

HPLC	=	High performance liquid chromatography
H_2SO_4	=	Sulfuric acid
ISP	=	International Streptomyces Project
JCM	=	Japan Collection of Microorganisms
K_2HPO_4	=	Potassium phosphate
KNO_3	=	Potassium nitrate
KOH	=	Potassium hydroxide
L	=	Liter
LL-DAP	=	*LL*-Diaminopimelic acid
mL	=	Milliliter
µL	=	Microliter
mm	=	Millimeter
µm	=	Micrometer
mM	=	Millimole
M	=	Molar
Max	=	Maximum
MEGA	=	Molecular Evolutionary Genetics Analysis
MK	=	Menaquinone
meso-DAP	=	*meso*-Diaminopimelic acid
min	=	Minute
Methyl-PE	=	Methylphosphatidylethanolamine
nt	=	nucleotide
N	=	Normal
NA	=	Nutrient agar
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide
NPG	=	Ninhydrin-positive glycophospholipids
nm	=	Nanometer
nov.	=	Novel
OD	=	Optical density
OH-PE	=	Hydroxyphosphatidylethanolamine

%	=	Percent
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
PC	=	phosphatidylcholine
PE	=	Phosphatidylethanolamine
PG	=	Phosphatidylglycerol
PI	=	Phosphatidylinositol
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
sec	=	Second
SEM	=	Scanning electron microscope
SDS	=	Sodium dodecyl sulfate
sp.	=	Species
SSC	=	Standard sodium citrate
TAE	=	Tris-acetate EDTA
Tm	=	Melting temperature
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
YM	=	Yeast extract-malt extract agar

CHAPTER I
INTRODUCTION

Actinomycetes are group of Gram-positive bacteria that have high guanine and cytosine content in their DNA and mycelium formation with asexual spore on aerial or vegetative mycelia (H. A. Lechevalier & Lechevalier, 1967). They inhabit in various environments such as in soil, aquatic environment, marine environment, extreme environment and plant tissue. Actinomycetes are saprophytes that decompose organic matter or humus in soil. In plant tissue, they are called endophytic actinomycetes which are endosymbiosis to live within host plant but not be the phytopathogens (Hasegawa, Meguro, Shimizu, Nishimura, & Kunoh, 2006). Numerous studies exhibited that endophytic actinomycetes were more useful for the host plant. They draw nutrition from rhizospheric soil as nitrogen-fixing and produce various bioactive metabolites that protect as antibiotics and stimulate to host plant growth as plant growth promoters (Janso & Carter, 2010). In general, *Streptomyces* are a major group of endophytic actinomycetes. *Micromonospora, Microbispora, Nocardiopsis, Pseudonocardia* and *Streptosporangium* are also common genera of actinomycetes in plant (Qin *et al.,* 2009).

Actinomycetes have been well-known as major group of microorganisms that produce antibiotics such as streptomycin, aminoglycosides, macrolides, tetracyclines, glycopeptides, β-lactams, polyenes, peptides, and quinones (Okami & Hotta, 1988). In 2005, Berdy reported that almost 50 percent of microbial metabolites (more than 20,000 compounds) were reported from actinomycetes, especially from the genus *Streptomyces* (Bérdy, 2005). Moreover, their secondary metabolites are discovered more utilities in pharmaceutical industry, agriculture and environment such as antibiotics (streptomycin, neomycin, chloramphenicol, erythromycin, tetracycline, kanamycin, vancomycin, gentamicin, lincomycin, maklamicin, etc.), antifungal (amphotericin B, nystatin, etc.), antimalarial (salinosporamide A), anticancers (paclitaxel, actinomycin, anthracyclines, elsamicin A, chartreusin, proximicins, lupinacidin A, lupinacidin B, L-asparaginase, catechoserine, etc.), anticholesterol,

pesticide, plant growth hormone (phytohormone, indole-3-acetic acid (IAA)), enzymes (cellulase, hemi-cellulase, xylanase, chitinase, amylase), etc. Many studies showed that plants were potential resources for the discovery of new actinomycetes, which led to new bioactive compound isolation (Qin, Xing, Jiang, Xu, & Li, 2011). In 2013, the reviews from Kitasato university exhibited that plants are resources of new actinomycetes and new drug discovery e.g. *Actinoallomurus liliacearum* sp. nov. and *Actinoallomurus vinaceus* sp. nov. were isolated from roots of *Ophiopogon japonicas* (Koyama, Matsumoto, Inahashi, ōmura, & Takahashi, 2012), *Phytohabitans suffuscus* gen. nov., sp. nov. and *Phytohabitans flavus* sp. nov. were isolated from roots of an orchid (Inahashi, Matsumoto, Danbara, Omura, & Takahashi, 2010).

There are nearly 300,000 species of plant on the earth that each individual plant has many endophytes (Strobel & Daisy, 2003). In addition, it was estimated that less than 1% of bacterial species have recently been identified, therefore millions of microbial species remain to be discovered (Davis, Joseph, & Janssen, 2005). This study is aimed to search for the biodiversity of endophytic actinomycetes isolated from medicinal plants in Thailand, to screen for antimicrobial activity, and to evaluate production of their secondary metabolites from chemical profiles.

The main objectives of this investigation are as follows:

1. To isolate and identify the endophytic actinomycetes from medicinal plants in Thailand by phenotypic and genotypic characterization.

2. To evaluate antimicrobial activity of crude extracts of the isolates by paper disc diffusion method.

3. To evaluate the production of secondary metabolites extracted from the selected isolates by using HPLC analysis.

CHAPTER II
LITERATURE REVIEW

Actinomycetes are group of Gram-positive bacteria with high guanine and cytosine contents in their DNA (about more than 50 mol %) that belong to the phylum and class *Actinobacteria*. In general, they are aerobic or facultative anaerobic bacteria that form branching filaments and asexual spores on substrate or aerial mycelium, as shown in Figure 2.1. They look like fungi that are maybe called filamentous bacteria. Their hypha diameter (ranges from 0.4 to 1.2 μm) is smaller than fungi filament diameter (ranges from 3 to 8 μm) and no cell nucleus that are different from fungi.

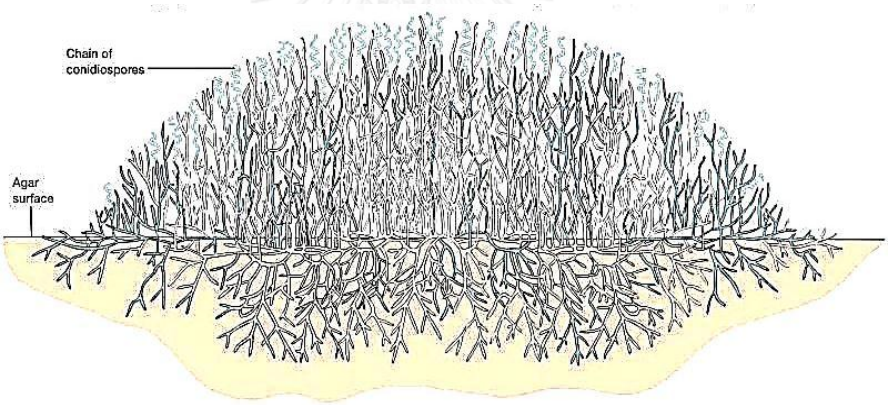


Figure 2.1 Morphology of actinomycete (Prescott, 2002).

Life cycle of actinomycetes begins with spore germination and growth of substrate mycelium. The colony of actinomycete responses to nutrition and some stress signals by producing reproductive aerial hyphae, and then the hypha cell differentiate to spores. The secondary metabolites will be produced while hyphae differentiation, as shown in Figure 2.2. Most of actinomycetes are saprophytes that decompose organic matters for growth. They contribute to degradation of lignin, organic matter and chitin, formation and stabilization of compost piles and humus, and production of bioactive secondary metabolites as antibiotics.

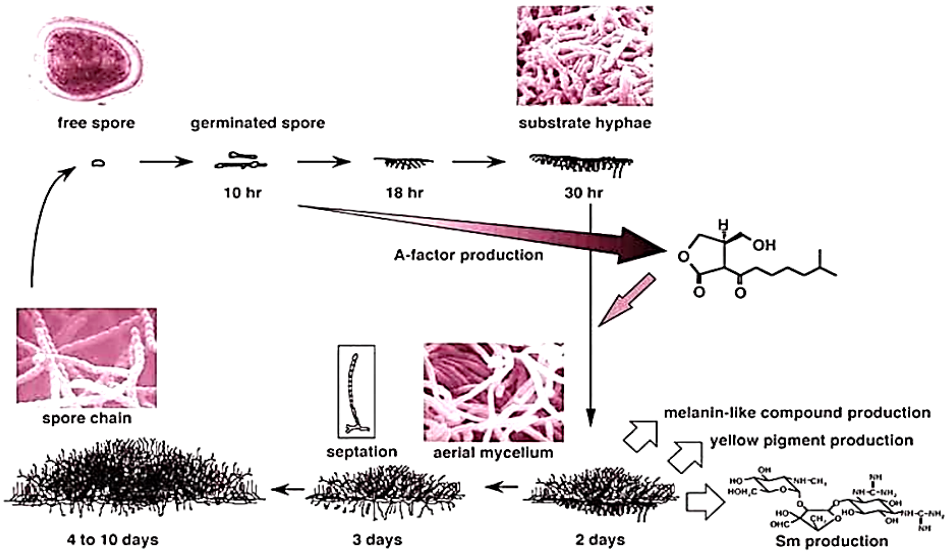


Figure 2.2 Actinomycete life cycle that shows growth and differentiation of actinomycete (Flärdh & Buttner, 2009).

The actinomycetes inhabit in various environments such as in soil, aquatic environment, marine environment, extreme environment and plant tissue. They are usually harmless and particularly beneficial microorganisms in pharmaceutical industry, agriculture and environment such as antibiotics, antifungals, antimalarial, anticancers, pesticide, plant growth hormone, etc. (Shimizu, 2011) However, some actinomycetes are human, animal or plant pathogens such as various *Nocardia species* are human pathogens that cause an opportunistic infection (Murray, Baron, Jorgensen, Landry, & Pfaller, 2007), *Streptomyces scabies* that causes scab disease in potatoes while the closely related species is used for its control (Emmert & Handelsman, 1989), etc.

2.1 Taxonomy of actinomycetes

Actinomycetes belong to the phylum and class *Actinobacteria*. Class *Actinobacteria* consists of fifteen orders and an order *incertae sedis* (Ludwig, Euzeby, & Whitman, 2012) as shown in Figure 2.3.

Phylum *Actinobacteria*

Class *Actinobacteria*

Order *Actinomycetales*

Family *Actinomycetaceae*

Order *Actinopolysporales*

Family *Actinopolysporaceae*

Order *Bifidobacteriales*

Family *Bifidobacteriaceae*

Order *Catenulisporales*

Family *Catenulisporaceae*

Family *Actinospica*

Order *Corynebacteriales*

Family *Corynebacteriaceae*

Family *Dietziaceae*

Family *Mycobacteriaceae*

Family *Nocardiaceae*

Family *Segniliparaceae*

Family *Tsukamurellaceae*

Order *Frankiales*

Family *Frankiaceae*

Family *Acidothermaceae*

Family Cryptasporangiaceae

Family *Geodermatophilaceae*

Order *Pseudonocardiales*

Family *Pseudonocardiaceae*

Order *Streptomycetales*

Family *Streptomycetaceae*

Order *Jiangellales*

Family *Jiangellaceae*

Order *Kineosporiales*

Family *Kineosporiaceae*

Order *Micrococcales*

Family *Micrococcaceae*

Family *Beutenbergiaceae*

Family *Bogoriellaceae*

Family *Brevibacteriaceae*

Family *Cellulomonadaceae*

Family *Dermabacteraceae*

Family *Dermatophilaceae*

Family *Intrasporangiaceae*

Family *Jonesiaceae*

Family *Microbacteriaceae*

Family *Promicromonosporaceae*

Family *Rarobacteraceae*

Family *Ruaniaceae*

Family *Sanguibacteraceae*

Order *Micromonosporales*

Family *Micromonosporaceae*

Order *Streptosporangiales*

Family *Streptosporangiaceae*

Family *Nocardiopsaceae*

Family *Thermomonosporaceae*

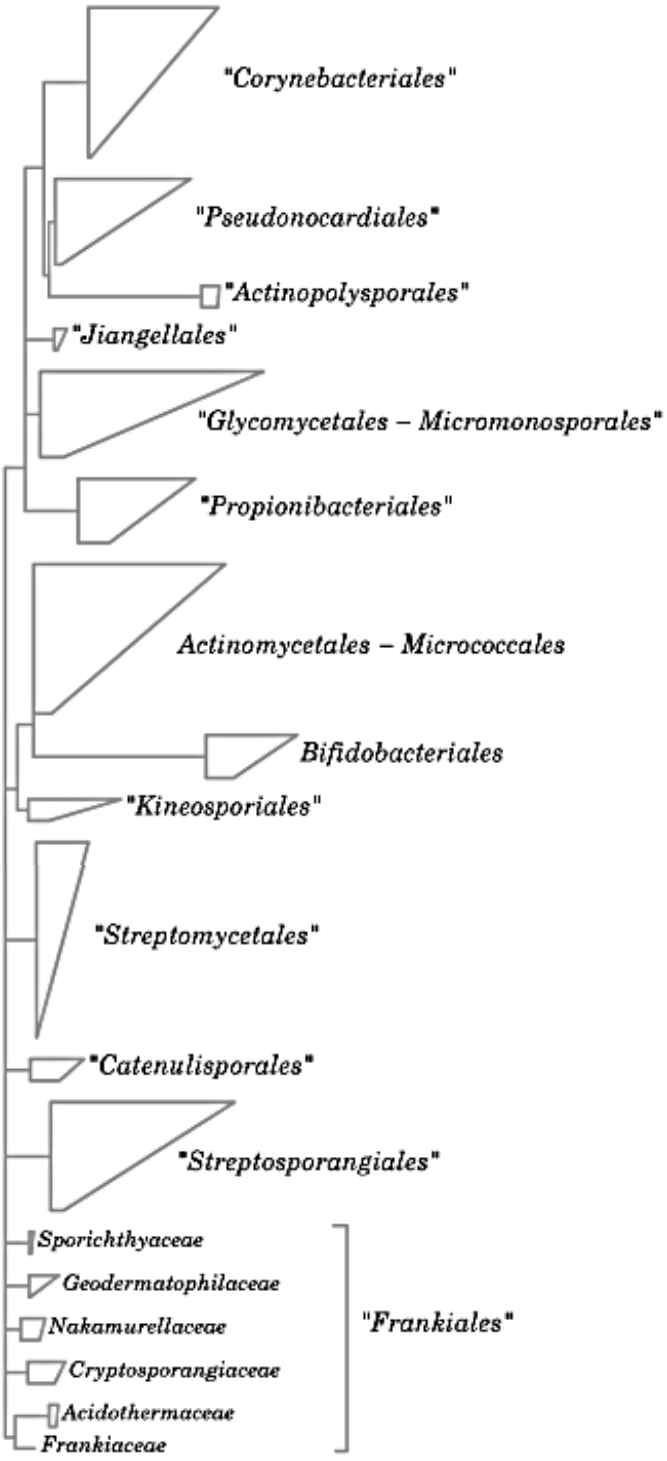


Figure 2.3 The road map of orders of class *Actinobacteria* (Ludwig et al., 2012).

Streptomyces

The genus *Streptomyces* belongs to the family *Streptomycetaeae* and *Streptomyces albus* is type species of this genus. At present, *Streptomyces* contains about 668 species and 38 subspecies with validly published names in the *List of Prokaryotic names* that distributed in soil, plant, sediment of freshwater and marine (Euzéby, 2014). They form smooth, warty, rugose, spiny or hairy type spore chains (sporophores) on extensively branched substrate and aerial mycelia. The differentiated characteristics between *Strepyomyces* and genera belonging to this family are shown in Table 2.1.

Table 2.1 Differential characteristics of genera in family *Streptomycetaceae* (P. Kämpfer, 2012)

Genus	G+C (mol%)	Fatty acid Type[a]	Major menaquinone (s)	Polar lipid Type[b]	Whole cell sugar Type[c]	DAP Type
Streptomyces	69-78	2	MK-9(H_6,H_8)	PII	-	LL-DAP
Kitasatospora	69-73	2	MK-9(H_6,H_8)	PII	C, E	LL/*meso*-DAP
Streptacidiphilus	70-72	2	MK-9(H_6,H_8)	PII	E	LL-DAP

[a] According to the classification of fatty acids (Kroppenstedt, 1985)

[b] According to the classification of polar lipids (M. P. Lechevalier, DeBievre, & Lechevalier, 1977)

[c] According to the classification of whole cell sugar (Goodfellow, 1988)

Amycolatopsis

The genus *Amycolatopsis* belongs to the family *Pseudonocardiaceae* and *Amycolatopsis orientalis* is type species of this genus. At present, *Amycolatopsis* comprises of 65 species and 4 subspecies with validly published names in the *List of Prokaryotic names* (Euzéby, 2014). They distribute in various sources as equine placenta (D. P. Labeda, Donahue, Williams, Sells, & Henton, 2003), human (Huang, Pas´ciak, Liu, Xie, & Gamian, 2004), volcanic soil (Ding, Hirose, & Yokota, 2007), rhizospheric soil (Lee, 2009), polluted sediment (Albarraci´n, Alonso-Vega, Trujillo, Amoroso, & Abate, 2010), ocean sediment (Bian et al., 2009) and plants (Duangmal et al., 2011). The key to the genera of the family *Pseudonocardiaceae* describe

Amycolatopsis as no produced sporangia or pseudosporangia and motile spores, arabinose as diagnostic whole-cell sugar, MK-9(H_4) as predominant menaquinone, irregular rod-shaped fragmented substrate mycelium and fragmented spore chains on aerial mycelium. The differentiated characteristics between *Amycolatopsis* and some genera belonging to this family are shown in Table 2.2.

Table 2.2 Differential characteristics of genera in family *Pseudonocardiaceae* (D.P. Labeda & Goodfellow, 2012)

Genus	G+C (mol%)	Fatty acid Type[a]	Major menaquinone (s)	Polar lipid Type[b]	whole cell sugar Type[c]	DAP Type
Pseudonocardia	68-79	2	MK-8(H_4)	PII,III	A	*meso*-DAP
Amycolatopsis	66-75	1a, 2	MK-9(H_2,H_4,H_6)	PII	A	*meso*-DAP
Saccharomonospora	68-74	2	MK-9(H_2,H_4)	PII,IV	A	*meso*-DAP
Saccharopolyspora	66-77	2	MK-9(H_4,H_6,H_8)	PIII	A	*meso*-DAP
Prauserella	66-70	1a, 2	MK-9(H_4)	PII,III	A	*meso*-DAP

[a] According to the classification of fatty acids (Kroppenstedt, 1985)

[b] According to the classification of polar lipids (M. P. Lechevalier et al., 1977)

[c] According to the classification of whole cell sugar (Goodfellow, 1988)

Nocardia

The genus *Nocardia* belongs to the family *Nocardiaceae* and *Nocardia asteroids* is type species of this genus. At present, *Nocardia* contains 104 species with validly published names in the *List of Prokaryotic names* (Euzéby, 2014). In Thailand, *N. thailandica* was isolated from clinical specimens (Kageyama et al., 2004). They form rod-shaped to cocci and non-motile fragment on extensively branched substrate and aerial hyphae. They usually form carotenoid-like pigments. Morphological colonies are variable, smooth, irregular or wrinkled rod-shape. The differentiated characteristics between *Nocardia* and the genera belonging to this family are shown in Table 2.3.

Table 2.3 Differential characteristics of genera in family *Nocardiaceae* (Goodfellow, 2012)

Genus	G+C (mol%)	Fatty acid Type[a]	Major menaquinone (s)	Polar lipid Type[b]	whole cell sugar Type[c]	DAP Type
Nocardia	64-72	1b	MK-9($H_{4cyclic}$)	PII	A	*meso*-DAP
Rhodococcus	67-73	1b	MK-8(H_2)	PII	A	*meso*-DAP

[a] According to the classification of fatty acids (Kroppenstedt, 1985)

[b] According to the classification of polar lipids (M. P. Lechevalier et al., 1977)

[c] According to the classification of whole cell sugar (Goodfellow, 1988)

Micromonospora

The genus *Micromonospora* belongs to the family *Micromonosporaceae* and *Microbispora chalcea* is type species of this genus. At present, *Micromonospora* contains 60 species and 7 subspecies with validly published names in the *List of Prokaryotic names* (Euzéby, 2014). *M. aurantionigra, M. eburnea, M. siamensis, M. narathiwatensis, M. pattaloongensis, M. chaiyaphumensis, M. krabiensis* and *M. marina* were reported in Thailand (Thawai, Tanasupawat, Itoh, Suwanborirux, & Kudo, 2004, 2005; Thawai, Tanasupawat, & Kudo, 2008; Thawai, Tanasupawat, Suwanborirux, Itoh, & Kudo, 2007). Some species were reported to produce antibiotics as gentamicin produced by *M. purpurea* (Weinstein et al., 1963), etc. They form single conidial spore, branched and substrate mycelium but lack aerial mycelium. The black smooth and non-motile spores are formed at surface of the dark yellow-orange to orange-red colonies as become viscid or mucoid. The differentiated characteristics of some genera belonging to this family are shown in Table 2.4.

Table 2.4 Differential characteristics of genera in family *Micromonosporaceae* (Genilloud, 2012)

Genus	G+C (mol%)	Fatty acid Type[a]	Major menaquinone (s)	Polar lipid Type[b]	whole cell sugar Type[c]	DAP Type
Micromonospora	71-73	3b	MK-9(H_4,H_6), MK-10(H_4,H_6	PII	D	*meso*-DAP
Actinocatenispor	72	3b	MK-9(H_4,H_6)	PII	D,A	*meso*-DAP
a Actinoplanes	72-73	2d	MK-9(H_4), MK -10 (H_4,H_6)	PII	D	*meso*-DAP
Catellatospora	71-72	3b	MK-9(H_4,H_6), MK-10(H_6,H_8	PII	D	*meso*-DAP
Dactylosporangiu	72-73	3b	MK-9(H_4,H_6, H_8)	PII	D	*meso*-DAP
mKrasilnikovia	71	2d	MK-9(H_6)	PII	D,A	*meso*-DAP
Longispora	70	2d	MK-10(H_4,H_6)	PII	D,A	*meso*-DAP
Pillimelia	70-72	2d	MK-9(H_2,H_4)	PII	D	*meso*-DAP
Polymorphospora	70-71	2a	MK-9(H_4,H_6), MK-10(H_4,H_6	PII	D	*meso*-DAP
Verrucosispora	70	2b	MK-9(H_4)	PII	D	*meso*-DAP
Virgisporangium	71-72	2d	MK-10(H_4,H_6)	PII	D,A	*meso*-DAP

[a] According to the classification of fatty acids (Kroppenstedt, 1985)

[b] According to the classification of polar lipids (M. P. Lechevalier et al., 1977)

[c] According to the classification of whole cell sugar (Goodfellow, 1988)

Microbispora

The genus *Microbispora* belongs to family *Streptosporangiaceae* and *Microbispora rosea* is type species of this genus. In the past, *Microbispora* were cited about 18 species in the *List of Prokaryotic names* but the seven species as *Mb. parva, Mb. amethystogenes, Mb. chromogenes, Mb. diastatica, Mb. viridis, Mb. karnatakensis*, and *Mb. indica* were proposed to combine into the subspecies, *Mb. rosea* subsp. *rosea* while three species as *Mb. aerate, Mb. thermorosea, Mb. thermodiastatica* were proposed to combine into the subspecies, *Mb. rosea* subsp. *aerate* based on DNA-DNA hybridization experiment (Euzéby, 2014; Miyadoh, Amano, Tohyama, & Shomura, 1990). *Mb. corallina* and *Mb. siamensis* were reported in Thailand (Boondaeng, Ishida, Tamura, Tokuyama, & Kitpreechavanich, 2009; Nakajima, Kitpreechavanich, Suzuki, & Kudo, 1999). They form non-motiled smooth oval to ellipsoidal paired spores on aerial mycelium. Some species produce soluble

pigments and antibiotic secondary metabolites. The differentiated characteristics of all genera belonging to this family are shown in Table 2.5.

Nonomuraea

The genus *Nonomuraea* belongs to family *Streptosporangiaceae* and *Nonomuraea pusilla* is type species of this genus. *Nonomuraea* comprises of 36 species and 2 subspecies with validly published names in the *List of Prokaryotic names* (Euzéby, 2014). They form straight, hooked or spiral spore chains that their surface are smooth, spiny or warty on aerial mycelium and extensively branching substrate. The differentiated characteristics of all genera belonging to this family are shown in Table 2.5.

Table 2.5 Differential characteristics of genera in family *Streptosporangeaceae* (P. Kämpfer, 2012)

Genus	G+C (mol%)	Fatty acid Type[a]	Major menaquinone(s)	Polar lipid Type[b]	Whole cell sugar Type[c]	DAP Type
Streptosporangium	69-71	3c	MK-9(H_2, H_4)	IV	B	*meso*-DAP
Acrocapospora	68-69	3c	MK-9(H_2, H_4, H_6)	IV, II	B,C	*meso*-DAP
Herbidospora	69-71	3c	MK-10(H_4, H_6, H_8)	IV	B	*meso*-DAP
Microbispora	71-73	3c	MK-9(H_0, H_2, H_4)	IV	B,C	*meso*-DAP
Microtetraspora	69-71	3c	MK-9(H_2, H_4, H_6)	IV	B,C	*meso*-DAP
Nonomuraea	64-69	3c	MK-9(H_0, H_2, H_4)	IV	B,C	*meso*-DAP
Planobispora	70-71	3c	MK-9(H_0, H_2, H_4)	IV	B	*meso*-DAP
Planomonospora	72	3c	MK-9(H_0, H_2, H_4)	IV	B	*meso*-DAP
Planotetraspora	71	3d	MK-9(H_2, H_4)	IV	B	*meso*-DAP
Sphaerisporangium	70-72	3c	MK-9(H_0, H_2, H_4, H_6)	IV	B	*meso*-DAP

[a] According to the classification of fatty acids (Kroppenstedt, 1985)

[b] According to the classification of polar lipids (M. P. Lechevalier et al., 1977)

[c] According to the classification of whole cell sugar (Goodfellow, 1988)

2.2 Distribution of endophytic actinomycetes

Numerous novel endophytic actinomycetes have been discovered since the genus *Frankia* was isolated from non-legume root nodules, indicating that actinomycetes were closely associated with plants in early 1886 (Okazaki, 2003). In Thailand, many endophytic actinomycetes were isolated from Thai agricultural plants and medicinal plants such as *Streptomyces*, *Micromonospora, Microbispora, Nocardiopsis*, *Pseudonocardia* and *Streptosporangium*. For examples; *Rhinacanthus communisa, Amaranthus gracilisa, Brassica junceaa, Brassica oleraceaa, Cyperus difformisa, Cyperus iriaa, Cyperus kyllingiaa, Cyperus malaccensisa, Cyperus rotundusa, Chloris barbataa, Cymbopogon citratusa, Cymbopogon nardusa, Echinochloa colonaa, Echinochloa crusgallia, Imperata cylindricaa, Eleutherine palmifoliaa, Ocimum tenuifloruma, Coffea arabicab, Citrus hystrixb*, *Tacca chantrieria, Apium graveolensa, Coriandrum sativuma, Alpinia blepharocalyxa, Alpinia galangaa, Amomum siamensea, Boesenbergia pandurataa, Curcuma domesticaa, Curcuma longaa, Etlingera elatiora, Zingiber cassumunara, Zingiber officinal* (Taechowisan *et al.,* 2003); *Alpinia galangal*, *Azadirachta indica*, *Boesenbergia rotunda*, *Cymbopogon citrates, Curcuma longa*, *Eryngium foetidum, Kaemferia galangal, Melissa officinalis*, *Ocimum tenuiflorum, Houttuynia cordata* (Pukclai, 2006); *Kineococcus gynurae* sp. nov. isolated from roots of *Gynura pseudochina, Pseudonocardia acaciae* sp. nov. isolated from roots of *Acacia auriculiformis* (Duangmal, Thamchaipenet, Ara, Matsumoto, & Takahashi, 2008; Duangmal, Thamchaipenet, Matsumoto, & Takahashi, 2009); *Actinoallomurus oryzae* sp. nov. isolated from roots of Thai jasmine rice plant (Indananda *et al.,* 2011); *Actinoallomurus acaciae* sp. nov. isolated from *Acacia auriculiformis* (Thamchaipenet *et al.*, 2010). Table 2.6 shows the distribution of endophytic actinomycetes based on 16S rRNA gene sequences (Qin *et al.,* 2009).

Table 2.6 The distribution of novel endophytic actinomycetes

Name of the endophyte	Host plant	Closest species (% similarity)	Reference
Micromonospora coriariae	*Coriaria myrtifolia*	*M. endolithica* (98.94%)	Trujillo *et al.* 2006
M. lupini	*Lupinus angustifolius*	*M. mirobrigensis* (98.5%)	Trujillo *et al.* 2007
M. saelicesensis	*Lupinus angustifolius*	*M. purpureochromogenes* (98.7%)	Trujillo *et al.* 2007
M. pisi	*Pisum sativum*	*M. pattaloongensis* (98.7%)	Garcia *et al.* 2010
Pseudonocardia oroxyli	*Oroxylum indicum*	*P. halophobica* (97.8%)	Gu *et al.* 2006
P. acaciae	*Acacia auriculiformis*	*P. spinosispora* (96.2%)	Duangmal *et al.* 2009
P. endophytica	*Lobelia clavata*	*P. kongjuensis* (98.5%)	Chen *et al.* 2009
P. tropica	*Maytenus austroyunnanensis*	*P. alni* (99.5%)	Qin *et al.* 2009
P. adelaidensis	*Eucalyptus microcarpa*	*P. zijingensis* (98.7%)	Kaewkla and Franco 2010
P. eucalypti	*Eucalyptus microcarpa*	*P. spinosispora* (96.3%)	Kaewkla and Franco 2010
P. artemisiae	*Artemisia annua*	*P. saturnea* (96.6%)	Zhao *et al.* 2010
P. sichuanensis	*Jatropha curcas*	*P. zijingensis* (98.6%)	Qin *et al.* 2010
Dietzia schimae	*Schima* sp.	*D. maris* (99.8%)	Li *et al.* 2008
D. cercidiphylli	*Cercidiphyllum Japonicum*	*D. natronolimnaea* (99.5%)	Li *et al.* 2008
Glycomyces endophyticus	*Carex baccans*	*G. algeriensis* (99.0%)	Qin *et al.* 2008
G. sambucus	*Sambucus adnata*	*G. lechevalierae* (97.2%)	Gu *et al.* 2007
G. scopariae	*Scoparia dulcis*	*G. algeriensis* (97.4%)	Qin *et al.* 2009
G. mayteni	*Maytenus austroyunnanensis*	*G. algeriensis* (97.1%)	Qin *et al.* 2009
Streptomyces alni	*Alnus nepalensis*	*S. hebeiensis* (97.6%)	Liu *et al.* 2009
S. artemisiae	*Artemisia annua* L.	*S. armeniacus* (99.9%)	Zhao *et al.* 2010
S. sedi	*Sedum* sp.	*S. specialis* (97.5%)	Li *et al.* 2009
S. mayteni	*Maytenus austroyunnanensis*	*S. phaeopurpureus* (99.5%)	Chen *et al.* 2009
Nonomuraea endophytica	*Artemisia annua*	*N. candida* (98.8%)	Li *et al.* 2010
N. antimicrobica	*Maytenus austroyunnanensis*	*N. candida* (98.2%)	Qin *et al.* 2009
S. gloriosae	*Gloriosa superba*	*S. gregorii* (99.1%)	Qin *et al.* 2010
S. tripterygii	*Tripterygium hypoglaucum*	*S. flava* (97.6%)	Li *et al.* 2009
Saccharopolyspora	*Maytenus austroyunnanensis*	*S. flava* (97.7%)	Qin *et al.* 2008
Actinoallomurus acaciae	*Acacia auriculiformis*	*A. caesius* (99.3%)	Thamchaipenet *et al.* 2010
A. oryzae	*Oryza sativa*	*A. iriomotensis* (99.2%)	Indananda *et al.* 2010
Micrococcus endophyticus	*Aquilaria sinensis*	*M. luteus* (99.06%)	Chen *et al.* 2009
M. yunnanensis	*Polyspora axillaris*	*M. luteus* (99.7%)	Zhao *et al.* 2009
Nocardia callitridis	*Callitris preissii*	*N. nova* (97.4%)	Kaewkla & Franco 2010
N. endophytica	*Jatropha curcas*	*N. nova* (97.5%)	Xing *et al.* 2010
N. caricicola	*Carex scabrifolia*	*N. pyridinolyticus* (97.0%)	Song *et al.* 2010
Actinomadura flavalba	*Maytenus austroyunnanensis*	*A. atramentaria* (97.4%)	Qin *et al.* 2009
Actinophytocola oryzae	*Oryza sativa*	*Kibdelosporangium aridum* (95.5%)	Indananda *et al.* 2010
Plantactinospora mayteni	*Maytenus austroyunnanensis*	*M. endolithica* (98.1%)	Qin *et al.* 2009
Phytohabitans suffuscus	Orchid	*M. pattaloongensis* (97.7%)	Inahashi *et al.* 2009
Jiangella alba	*Maytenus austroyunnanensis*	*J. alkaliphila* (98.8%)	Qin *et al.* 2009
Jishengella endophytica	*Acanthus illicifolius*	*M. olivasterospora* (98.7%)	Xie *et al.* 2010
Herbidospora osyris	*Osyris wightiana*	*H. cretacea* (99.9%)	Li *et al.* 2009
Kineosporia mesophila	*Tripterygium wilfordii*	*K. mikuniensis* (98.2%)	Li *et al.* 2009
Leifsonia ginsengi	Ginseng	*L. poae* (97.6%)	Qiu *et al.* 2007

2.3 Secondary metabolites of endophytic actinomycetes

Nowadays, approximately 32,500 bioactive natural products produced from microorganisms and over two-thirds of the antibiotics produced from actinomycetes. In recently, endophytic actinomycetes are discovered many new actinomycetes and new bioactive products with diverse biological activities such as paclitaxel, an anticancer produced from *Kitasatospora* sp. that was isolated from *Taxus baccata* (Caruso et al., 2000). The novel bioactive metabolites of endophytic actinomycetes were listed in Table 2.7.

Table 2.7 The novel secondary metabolites of endophytic actinomycetes

Endophytic actinomycetes	Host plants	Secondary metabolites	Activity	Reference
Streptomyces sp. MaB-QuH-8	*Maytenus aquifolia*	Celastramycins A and B	antimicrobial	Pullen *et al.* 2002
Streptomyces sp. NRRL30562	*Kennedia nigriscans*	Munumbicins A-D	antibiotic	Castillo *et al.* 2002
Streptomyces sp. NRRL30566	*Grevillea pteridifolia*	Kakadumycins	antibiotic	Castillo *et al.* 2003
Streptomyces sp.CS	*Maytenus hookeri*	24-demethyl-bafilomycin C1	antimicrobial,	Lu & Shen 2003
Streptomyces sp.CS	*M. hookeri*	24-demethyl-bafilomycin A2	anticancer	Lu & Shen 2004
Streptomyces sp. TP-A0456	*Cryptomeria japonica*	Cedarmycins A and B	antifungal	Igarashi 2004
Streptomyces sp.TP-A0595	*Allium tuberosum*	6-Prenylindole	antifungal	Igarashi 2004
Streptomyces sp.TP-A0556	*Aucuba japonica*	Demethylnovobiocins	antimicrobial	Igarashi 2004
Streptomyces sp.MSU-2110	*Monstera sp.*	Coronamycins	antibiotic	Ezra *et al.* 2004
Streptomyces sp.ls9131	*M. hookeri*	Dinactin, Nonactin	antitumor	Zhao *et al.* 2005
S. aureofaciens CMUAc130	*Zingiber officinale*	Phenylcoumarin	antifungal, antitumor, anti-inflammatory	Taechowisan *et al.* 2005, 2007
Streptomyces sp. NRRL30562	*K.nigriscans*	Munumbicins E-4, E-5	antibiotic	Castillo *et al.* 2006
S. laceyi	*Ricinus communis*	Salaceyins A, B	antitumor	Kim *et al.* 2006
S. hygroscopicus TP-A0451	*Pteridium aquilinum*	Pterocidin	antitumor	Igarashi *et al.* 2006
Streptomyces sp. CS	*M. hookeri*	Naphthomycin K	antitumor	Lu & Shen 2007
Micromonospora lupini	*Lupinus angustifolius*	Lupinacidins A and B	antitumor	Igarashi *et al.* 2007
Streptomyces sp. SUC1	Ficus benjamina	Lansai B and C	anticancer, anti- inflammatory	Tuntiwachwuttikul *et al.* 2008; Taechowisana *et al.* 2009
S. albidoflavus	*Bruguiera gymnorrhiza*	Antimycin A18	antifungal	Yan et al. 2010
Streptomyces sp.CS	*M. hookeri*	Bafilomycin A1, D	antitumor	Li et al. 2010
Micromonospora sp.	*L. angustifolius*	Maklamicin	antimicrobial	Igarashi *et al.* 2011

2.4 Identification techniques of actinomycetes

The polyphasic approaches by using morphological, cultural, physiological, biochemical characteristics, chemotaxonomic analyses, comparative analysis of 16S rRNA gene sequence and phylogenetic pattern have been used for identification and classification at the genus and family level of actinomycetes (Stackebrandt and Schumann, 2006) as described below.

2.4.1. Phenotypic characteristics

Phenotypic characteristics comprise of morphological, cultural, biochemical and physiological characteristics that use the characterization methods of Shirling & Gottieb (Shirling & Gottlieb, 1966). The morphological characteristics are spore germination, substrate mycelium, aerial mycelium and spore formation. For spore formation, the aerial mycelium develops to spore is useful for actinomycete identification at the genus level. While cultural, biochemical and physiological characteristics as the color of colony, soluble pigment production, carbon and nitrogen utilization, growth at different pH, temperature, NaCl (%) and acid production are also useful characteristics at the species level (Arai, 1975).

2.4.2. Chemotaxonomic characteristics

Chemotaxonomic characteristics such as G+C content of genomic DNA, the components of cell wall and cell membrane, including diaminopimelic acid, whole cell sugar, fatty acid, phospholipids and menaquinone are important in the identification of actinomycetes.

Cell wall composition

Peptidoglycan in bacterial cell wall contains polymer of *N*-acetylglucosamine acid (NAG), *N*-acetylmuramic acid (NAM) and peptide moiety linked chain that type is one of the most important for Gram-positive bacterial classification, as in Figure 2.4. The 2,6-diaminopimelic acid (DAP or A_2pm) is majority peptide linked chain of actinomycetes that has three stereoisomers (*LL-, DD-* and *meso*-A_2pm) and hydroxyl diaminopimelic acid (3-OH-A_2pm) as the key of

chemotaxonomic analysis. *LL*-DAP always found in the hydrolysated cell wall of *Streptomyces* and *Nocardioides* while *meso*-DAP found in rare actinomycetes. DAP isomers in cell wall peptidoglycan are analyzed by using cellulose TLC (Staneck & Roberts, 1974).

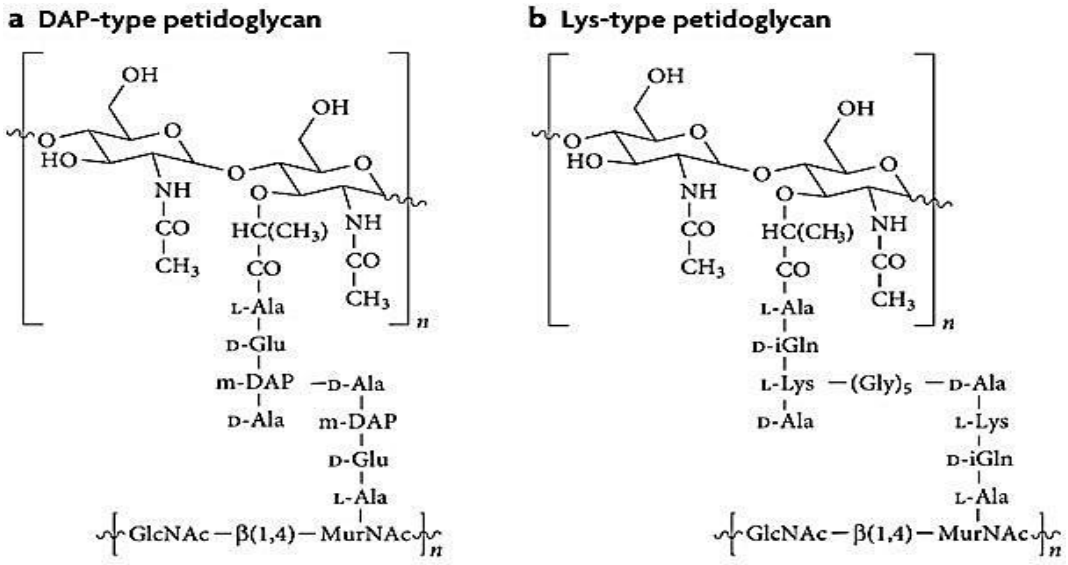


Figure 2.4 The structure of peptidoglycan in bacterial cell wall (J. & R. Dziarski, 2007)

The pattern of whole cell sugar composition is used to identify the sporulated actinomycetes which have *meso*-DAP in the cell wall, as shown in Table 2.8 (M. P. Lechevalier & Lechevalier, 1970). Whole cell sugars are analyzed by using cellulose TLC (Staneck & Roberts, 1974) or HPLC (Mikami & Ishiday, 1983).

Table 2.8 Whole cell sugar patterns (M. P. Lechevalier & Lechevalier, 1970)

Patterns	Diagnotic sugars					Genera related
	Arabinose	Fucose	Galactose	Madurose	Xylose	
A	+		+			*Nocardia, Mycobacterium, Pseudonocardia, Micropolyspora*
B				+		*Actinomadura, Microbispora, Streptosporangium*
C	No diagnostic sugar					*Nocardiopsis, Saccharothrix*
D	+				+	*Micromonospora, Actinoplanes, Dactylosporangium*
E		+				*Frankia*

Cell membrane composition

Isoprenoid quinones concern the electron transport system in respiration and oxidative phosphorylation at bacterial cell membrane that are devided into 2 types as menaquinone (2-methyl-3-polyprenyl-I,4-naphthoquinones) and ubiquinone (2,3-dimethoxy-5-methyl-6-polyprenyl-I,4-benzoquinones). The predominant isoprenoid quinone in actinomycete cell membrane was menaquinone (Collins, Pirouz, Goodfellow, & Minnikin, 1977). The number of isoprene units and the degree of hydrogenation of double bonds in the isoprenyl chain are the key for identification (Komagata & Suzuki, 1987). Menaquinones are analyzed by using HPLC (Collins *et al.,* 1977).

The polar lipids (phospholipids) in the lipid bilayer of bacterial cell membrane relate to permeability and regulation at the membrane that are a heterogeneous group of molecules, as shown in the Figure 2.5. The phospholipid patterns of actinomycetes are shown in Table 2.9 (M. P. Lechevalier & Lechevalier, 1970). Diphosphatidylglycerols (DPG) and phosphatidylinositiol (PI) widely distribute in the member of actinomycetes that not be useful for classification and identification. While phosphatidylinositol-mannosides (PIMs), glycophospholipids, unknown phospholipids containing glucosamine (GluNU), phosphatidylethanolamine (PE), methylphosphatidyl-ethanolamine (PME) and phosphatidylcholine (PC) are the

phospholipid marker to be useful for identification (Komagata & Suzuki, 1987). Phospholipid types of actinomycetes relate to the genus level as shown in Table 2.10 (M. P. Lechevalier et al., 1977). Polar lipid composition is analyzed by using two dimensional TLC (D. E. Minnikin et al., 1984).

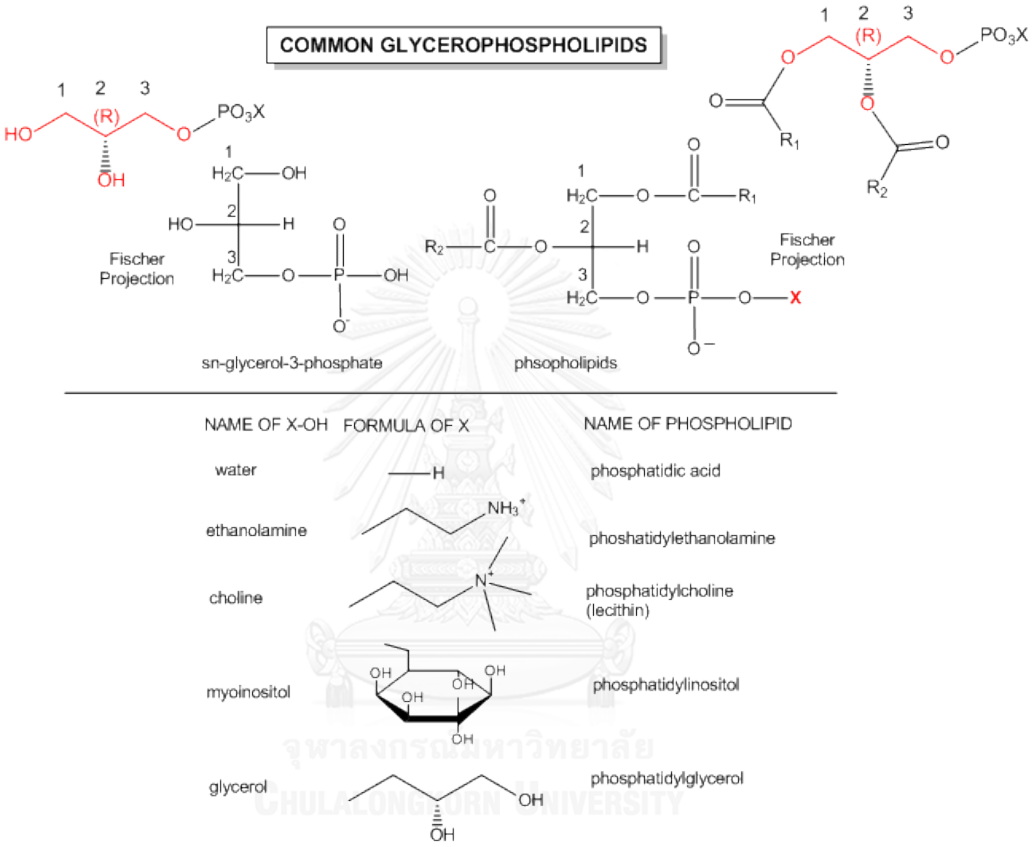


Figure 2.5 The structure of some phospholipids (Mescar & Koshland, 2000)

Table 2.9 Phospholipid types (M. P. Lechevalier et al., 1977)

Types	Phospholipids								
	PIMs	PI	PC	PG	PE	PME	GluNU	APG	DPG
I	+	+		v				v	v
II	+	+		v	+			v	+
III	v	+	+	v	v	+		v	v
IV	ND	+			v	v	+		+
V	ND	+		+	v		+	v	+

v, variable; ND, no data

Table 2.10 Phospholipid types of related genera (M. P. Lechevalier et al., 1977)

Types	Phospholipids	Genera related
I	No nitrogenous phospholipids	*Actinomadura, Microtetraspora, Nocardioides*
II	Phosphatidylethanolamine	*Micromonospora, Actinoplanes, Nocardia, Streptomyces, Amycolatopsis*
III	Phosphatidylcholine	*Nocardia, Nocardiopsis, Pseudonocardia, Micropolyspora, Saccharopolyspora*
IV	GluNU*	*Actinomadura, Microbispora, Nonomuraea, Streptosporangium*
V	GluNU and phosphatidyl glycerol	*Oerskovia, Promicromonospora*

*, Unknown phospholipids containing glucosamine

The long chain fatty acids form the lipid bilayer of bacterial cell membrane. For actinomycetes, fatty acids may be separated into two groups as the between 12 and 20 carbon atoms and the 20 up to 80 carbons (e.g. mycolic acid). The carbon chain length, the branching location of methyl groups (e.g. iso- or anteiso-) and the position of double bond were used as the key for bacterial characterization (D.E. Minnikin & Goodfellow, 1980). Fatty acid types are shown in Table 2.11 (Kroppenstedt, 1985). Cellular fatty acids are analyzed by using GC (Sasser, 1990). The mycolic acid is analyzed by TLC (Tomiyasu, 1982)

Table 2.11 Fatty acid types (Kroppenstedt, 1985)

Types I	**Pathway to straight-chain fatty acid**
Ia	Saturated and unsaturated straight chain
Ib	10-Methyl branched fatty acids
Ic	Cyclopropane fatty acids
Type II	**Pathway to terminally branched fatty acids**
II	*iso*- and *anteiso*-Fatty acid
Type III	**Complex branched fatty acids**
III	*iso*- and *anteiso*- fatty acids and 10-methyl branched

DNA base compositions

DNA is a double helix of two strands of polydeoxyribonucleotides. Purine and pyrimidine base of each nucleotide forms hydrogen bond as adenine (A) paired with thymine (T) while cytosine (C) paired with guanine (G). The molar ratio, [(G+C)/(G+C+A+T)x100] is useful for classification and identification of actinomycetes that express the values of DNA base composition as G+C content (%mol). The DNA base compositions are analyzed by using HPLC (J. Tamaoka & Komagata, 1984).

2.4.3. Genotypic characteristics

Genotypic characterization is the determination of the sequence of bacterial genome by using 16S rRNA gene sequences on ribosomal RNA that indicate the phylogenetic relationships in comparing microorganisms.

16S rRNA gene sequence

16S rRNA gene sequencing is a primary screening technique for determining the phylogenetic position of bacterial classification due to having in almost bacteria, no changing of the function of 16S rRNA gene, and large enough for informatic propose (Kim *et al.,* 2012). The 16S rRNA gene sequence is analyzed by using % similarity with closely related species and phylogenetic tree construction that depicts all possible topological line of evolutionary descent of different species

(Saitou & Nei, 1987). However, 16S rRNA gene sequencing is only used to identify into the genus level (Janda & Abbott, 2007)

DNA-DNA hybridization

DNA-DNA hybridization is the method for bacterial classification that DNA-DNA relatedness values are the key parameter in the species delineation. The values of DNA-DNA relatedness are determined by using colorimetry (Ezaki, Hashimoto, & Yabuuchi, 1989). A species as a group of strains including the type strain have the values at least 70% total genome DNA–DNA hybridization (Ezaki et al., 1989).

CHAPTER III

RESEARCH METHODOLOGY

This study was to isolate and identify the endophytic actinomycetes isolated from medicinal plants in Thailand. The methods for identification were based on phenotypic, chemotaxonomic, and genotypic characterization. The secondary metabolites of all isolates were screened for antimicrobial activity by paper disc diffusion method. The isolates, that showed effective antimicrobial activity, were studied on the chemical profiles of their crude extracts by using HPLC analysis.

3.1 Plant sample collection

Healthy leaf, stem and root of plant samples collected from Faculty of Pharmaceutical sciences at Chulalongkorn University were kept in plastic bags and stored at 4 °C until isolation.

3.2 Isolation of endophytic actinomycetes

Plant samples were washed to remove soil particles by running tap water for 1-2 minutes. Surface samples were sterilized by soaking in 95% ethanol for 10 minutes, followed by 1% (w/v) sodium hypochlorite for 15 minutes, and 10% (w/v) $NaHCO_3$ for 10 minutes before rinsed with sterilized water for three times based on the method of Indananda *et al* (Indananda et al., 2011). After that, the surface-sterilized samples were aseptically cut into small pieces (0.5 x 0.5 cm) and incubated onto starch casein agar (Küster & Williams, 1964), Humic acid-vitamin agar medium (Hayakawa & Nonomura, 1987) and 2.5% water agar medium, containing 25 µg/ml nalidixic acid and 50 µg/ml cyclohexamide to suppress the growth of gram-negative bacteria and fungi. The endophytic actinomycetes were observed and picked up to streak for purification on yeast extract-malt extract agar (YMA, ISP no.2) after incubation at 28-30 °C for 1-4 weeks. The pure cultures were preserved as colony in

ISP no.2 slant for short-term period and as lyophilized cells in 10% skim milk for long- term preservation.

3.3 Identification methods

3.3.1 Phenotypic characterization

Morphological, cultural, physiological and biochemical characteristics of all isolates were determined by the methods as described in the International *Streptomyces* Project (Shirling & Gottlieb, 1966).

3.3.1.1 Morphological characteristics of the 14-days old crosshatch-streak colony of the isolates on ISP no.2 were observed for mature aerial mycelia and spore chain by using light microscopy and scanning electron microscopy.

3.3.1.2 Cultural characteristics, the isolates were cultivated on ISP no.2 broth at 28-30 °C for 4 days and washed the bacterial cells with sterile water for three times. The bacterial cells were cultivated on nine different agar media, which were ISP no.2, oatmeal agar (ISP no.3), inorganic salt-starch agar (ISP no.4), glycerol asparagine agar (ISP no.5), peptone-yeast extract ion agar (ISP no.6), tyrosine agar (ISP no.7), glucose asparagine agar, Czapek's sucrose agar, and nutrient agar at 28-30 °C for 7-14 days. After that the color of mature aerial mycelium, substrate mycelium, diffusible pigment, and spore were determined by the NBS/IBCC color system (Kelly, 1964)

3.3.1.3 Physiological characteristics were observed including the properties of NaCl tolerance (0-10 % (w/v) NaCl), temperature tolerance (4-50 °C), and pH tolerance (pH at 4-10) on ISP no.2 agar for 7-14 days.

3.3.1.4 Biochemical characteristics, the utilization of carbon sources, starch hydrolysis, gelatin liquefaction, milk peptonization and nitrate reduction were determined on various media (Shirling & Gottlieb, 1966).

3.3.2 Chemotaxonomic characterization, the selected isolates will be cultivated in ISP no.2 broth on rotary shaker 200 rpms at 28-30 °C for 7-14 days. The

cell cultures were collected and washed with sterile water for three times before freeze drying.

3.3.2.1 Isomers of diaminopimelic acid analysis

Dried cells (10 mg) were hydrolyzed with 1 ml of 6N HCl at 100 °C for 18 hours. The hydrolyzed solution was filtered and evaporated. To dissolve the dry residue with 400 µl of distilled water, after that will be analyzed by using cellulose TLC plate with twice the developing solvent system as MeOH : H_2O : 6N HCl : pyridine (80:26:4:10, v/v) based on the method of Staneck and Robert (1974). The DAP isomer spots detected as dark-green spots by spraying with 0.5% ninhydrin solution and heating at 100 $^{\circ}$C for 5 minutes, that were compared with the DAP standard solution.

3.3.2.2 Menaquinone analysis

Dried cells (100-500 mg) were extracted with 20 ml of $CHCl_3$: MeOH (2:1, v/v) overnight. The supernatant of suspension were filtered and evaporated. The dried extract was dissolved with small volume of acetone and developed on silica gel TLC plate by using 100 % benzene as the solvent system. The band of menaquinone was detected with UV light at 254 nm, after that the band was scraped off and extracted with acetone. The suspension was filtrated through 0.45 µm membrane filter and dried up with N_2 gas. The menaquinone was analyzed by HPLC (Collins et al., 1977). The menaquinone analysis was compared with the reference strain. The standard solution was the mixture solution of menaquinones extracted from the reference strain and ubiquinone.

3.3.2.3 Whole-cell sugar analysis

Dried cells (50 mg) were hydrolyzed with 1 ml of 1N H_2SO_4 at 100 °C for 2 hours and cooled to room temperature before adjusting to pH 5.2-5.5 with $Ba(OH)_2$. The solution was centrifuged at 3,600 rpms for 10 minutes and collected the supernatant. The supernatant was evaporated. The dry residue was dissolved with 300 µl of distilled water and developed on cellulose TLC plate by using *n*-butanol : water : pyridine : toluene (10:6:6:1, v/v) as the solvent system (Staneck &

Roberts, 1974). The whole-cell sugar was detected by spraying with acid aniline phthalate and heated at 100 $^{\circ}$C for 4 minutes as yellowish-brown spots for the hexose sugars, maroon spots for the pentose sugars. The standard solution was the mixture sugar solution of galactose, arabinose, xylose, rhamnose, mannose, glucose, and ribose.

3.3.2.4 Cellular fatty acids analysis

Dried cells (40 mg) were saponified by well-suspending in 0.1 ml of the reagent 1 (NaOH 15 g, MeOH 50 ml, and milli-Q water 50 ml) and heating at 100 °C for 30 minutes. After cooling to room temperature, the solution was methylated with 2 ml of the reagent 2 (6N HCl 65 ml, MeOH 55 ml) and heated at 80 °C for 10 min. The solution was extracted with 1.25 ml of the reagent 3 (*n*-hexane 50 ml, methyl-tert-butyl ether 50 ml) and transferred the upper layer to another tube. The solution was mixed with 3 ml of the reagent 4 (NaOH 1.2 g, milli-Q water 100 ml) for 5 minutes and transferred the supernatant to a GC vial. The cellular fatty acids were analyzed by using gas chromatography, according to the method of the Sherlock Microbial Identification System (MIDI, version 6.0) (P. Kämpfer & Kroppenstedt, 1996) with the ACTIN1 MIDI database.

3.3.2.5 Polar lipids analysis

Dried cells (150-300 mg) were extracted with 3 ml of MeOH : 0.3% NaCl (100:10) and 3 ml of petroleum ether for 15 minutes. The lower layer was collected to add with 1 ml of petroleum ether for 5 minutes and heated at 100 °C for 5 minutes. After cooling to room temperature, 2.3 ml of $CHCl_3$: MeOH : H_2O (90:100:30, v/v) was added and mixed well for 15 minutes. The lower layer was transferred to another tube and extracted again with 2.3 ml of $CHCl_3$: MeOH : H_2O (50:100:40, v/v). And then, the lower layer was transferred to the previous tube and added with 1.3 ml of each chloroform and water. The mixed solution was dried with N_2 gas (<37 °C) and dissolved with 120 µl of $CHCl_3$: MeOH (2:1, v/v). The extraction of polar lipids were done based on the method of (D.E. Minnikin & Goodfellow, 1980).

The polar lipids were determined by using 2-dimension TLC technique, 10 µl of the mixed solution was applied to the corner of the silica-gel TLC plate (10 x 10 cm) and developed on $CHCl_3$: MeOH : H_2O (65:25:4, v/v) as the first solvent system and $CHCl_3$: CH_3COOH : MeOH : H_2O (40:7.5:6:2, v/v) as the second solvent system. For detection of chromatogram patterns, the TLC plate was sprayed with specific reagents such as Molybdenum blue reagent for detection of lipids containing phosphate ester, Ninhydrin followed by heating at 110 °C for 10 minutes for detection of the polar lipids that contain free amino groups as phosphatidylethanolamine (PE) and its derivatives (lyso-PE, OH-PE and, methyl-PE), Dragendorff's reagent for detection of phosphatidylcholine, Anisaldehyde reagent followed by heating at 110 °C for 10 minutes for detection of glycolipids (green-yellow spot) and other lipid (blue spot), and Dittmer and Lester reagent or phosphomolybdic acid reagent followed by heating 120 at °C for 10 minutes for detection of all lipids in the cells.

3.3.2.6 DNA base composition analysis

. One gram of wet cells were suspended in 2 ml of saline-EDTA (pH 8) with 5-10 mg of lysozyme at 37 $^{\circ}$C for 30 minutes. The solution was added with 8 ml of Tris-NaCl (0.1M Tris and 0.1M NaCl, pH 9) and 0.05-0.1 ml of 10% SDS, and then heated at 55-60 $^{\circ}$C for 10 minutes. The extraction of proteins were carried out by adding 5 ml of phenol : chloroform (1:1) and centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a small beaker and then added with 5-10 ml of 95% ethanol to precipitate the DNA. The DNA was spooled with a glass rod and dry in room temperature. After drying, the DNA was dissolved in 3 ml of 0.1 SSC (0.1 M NaCl and 0.015 M Na-Citrate, pH7) and eliminated the RNA by incubating in 0.3 ml of RNase A solution at 37 $^{\circ}$C for 20 minutes. The DNA solution was added with 0.5 ml of 10 x SSC and extracted with phenol : chloroform (1:1) again. The DNA was precipitated from the supernatant, spooled with a glass rod, dry and dissolved in 0.1 SSC. The DNA solution was measured for the purity by using spectrophotometer at OD_{260}/OD_{280} ($1.8 < OD_{260}/OD_{280} < 2.0$). The DNA solution (10 µg/ml) was heated at 100 $^{\circ}$C for 10 minutes. The denatured DNA was hydrolyzed with 10 µl of nuclease P1

solution at 50 °C for 1 hour and incubated in 10 µl of alkaline phosphatase solution at 37 °C for 1 hour. The DNA G+C content was determined by using HPLC (J. Tamaoka, 1994).

3.3.3 Genotypic characterization

3.3.3.1 16S rRNA gene sequencing and phylogenetic analysis

The chromosomal DNA was prepared according to the method of (Saito & Miura, 1963). The all isolates were cultivated in ISP2 no.2 medium at 28-30 °C for 4-6 days. The cells were collected and washed twice with sterile water. The cells were lysed by using aluminium oxide with a micromixer for 90 seconds. The lyzed cells were added with 300 ml of phenol : chloroform (1:1, v/v) and mixed well for 5 minutes. The solution was centrifuged at 14,000 rpm for 15 minutes, the upper layer was transferred to the other tube and extracted with phenol : chloroform (1:1, v/v). The upper layer was collected and precipitated the DNA. 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) (Yamada et al., 2000). The PCR products were detected by gel electrophoresis and compared the particle size with 1 kb DNA marker. After that the PCR products were analyzed the nucleotide sequence at the Macrogen, Korea. The paired primers were used for sequencing as 20F/1492R, 518F/800R and 357F/920R. Sequence homology was performed on the standard BLAST sequence similarity searching program from website http://eztaxon-e.ezbiocloud.net/ (Chun et al., 2007). The multiple alignments of the sequences were carried out with the BioEdit program. The neighbor-joining tree (Saitou & Nei, 1987) was constructed by using the MEGA 5.0 program (Tamura et al., 2011). The topology was evaluated in a bootstrap analysis (Felsenstein, 1985) based on 1,000 replication.

3.3.3.2 DNA-DNA hybridization

The purification of genomic DNA (chromosomal DNA), 1 g of wet cells were suspended and inoculated in 2 ml of saline-EDTA (pH 8) with 5-10 mg of lysozyme at 37 °C for 30 minutes. The solution was added with 8 ml of Tris-NaCl (0.1M Tris and 0.1M NaCl, pH 9) and 0.05-0.1 ml of 10% SDS and then heated at 55-

60 °C for 10 minutes. The extraction of proteins were carried out by adding 5 ml of phenol : chloroform (1:1) and centrifuged at 10,000 rpms for 10 minutes. The supernatant was transferred to a small beaker and added with 5-10 ml of 95% ethanol to precipitate the DNA. The DNA was spooled with a glass rod and dry in room temperature. After drying, the DNA was dissolved in 3 ml of 0.1 SSC (0.1 M NaCl and 0.015 M Na-Citrate, pH7) and eliminated the RNA by incubating in 0.3 ml of RNase A solution at 37 °C for 20 minutes. The DNA solution was added with 0.5 ml of 10 x SSC and extracted with phenol : chloroform (1:1) again. The DNA was precipitated from the supernatant, spooled with a glass rod, dry and dissolved in 0.1 SSC. The genomic DNA solution was measured for the purity by using spectrophotometer at OD_{260}/OD_{280} ($1.8 < OD_{260}/OD_{280} < 2.0$) (J. Tamaoka, 1994).

The immobilization of single-strand DNA on the 96-well plate (microplate), 0.2 ml of genomic DNA solution (0.1 mg/ml) by boiling for 10 minutes after that immediately cooled in ice. The denature single-strand DNA solution was added with 1 ml of 2xPBS, 0.6 ml of distilled water and 0.2 ml of $MgCl_2$ and then mixed well. The single-strand DNA solution (0.1 ml) was dispensed to each well of the microplate and fixed on the wells at 37 °C for 2 hours. The fixed-well was removed solution and washed with 0.2 ml of PBS. The microplate was dried up at 60 °C for 2 hours and preserved in the desiccators until testing.

The preparation of DNA probe, 10 µl of DNA solution (1mg/ml) was added with 15 µl of photobiotin solution (1mg/ml) and radiated with a sunlamp for 30 minutes. The DNA probe was labeled with photobiotin before adding with 0.2 ml of 0.1 M Tris-HCl buffer (pH 9.0) and 0.4 ml of 2-butanol. The mixed solution was centrifuged at 12,000 rpms for 20 seconds and discarded the upper layer. The lower layer was added with 0.4 ml of 2-butanol. The mixed solution was centrifuged at 12,000 rpm for 20 minutes and discarded the upper layer. The lower layer was transferred to the new microtube and boiled for 15 minutes after that immediately cooled in ice and sonicated for 3 minutes before dissolved with 10 ml of the hybridization solution.

The 0.1 ml of the biotinylated DNA probe solution was dispended to each well of the microplate and incubated at optimal temperature for 15 hours. The solution was discarded and the microplate was washed three times with 0.2 ml of 0.2xSSC. The microplate was added with 0.2 ml of the solution 1 and incubated at room temperature for 10 minutes after that the solution 1 was discarded. The 0.1 ml of the solution 2 was added to each well of the microplate after that incubated at 37 $^{\circ}$C for 30 minutes and the solution 2 was discarded. The microplate was analyzed using calorimetric method by 100 µl of peroxidase-streptavidin solution and the solution was discarded after incubated at 37 $^{\circ}$C for 30 minutes. The microplate was washed twice with 300-400 µl of PBS and incubated in 100 µl of tetramethylbenzidine- H_2O_2 solution at 37 $^{\circ}$C for 5 minutes. The enzyme reaction was stopped with 100 µl of H_2SO_4 after that the absorbance at 450 nm was measured using the microplate reader (Microplate Reader Wallac 1420, PerkinElmer^{TM}). The results were calculated for the value of percentage of DNA homology (Ezaki et al., 1989).

3.4 Screening for antimicrobial activity

The isolates were inoculated into 10 ml of ISP no.2 broth (seed medium) and incubated at 28 $^{\circ}$C for 4-5 days on a shaker at 200 rpms. The 0.1 ml of inocula were incubated in 10 ml of the production medium (ISP no.2) at 28-30 $^{\circ}$C for 14 days on a shaker at 200 rpms (Songsumanaus, 2010). The culture broth was centrifuged to discard the cells. The supernatant was screened for antimicrobial activity by using paper disc diffusion method. The culture broth was extracted with ethyl acetate for 15 minutes and collected the upper layer after that it was evaporated. The crude extracts were dissolved with methanol and screened for antimicrobial activity by using paper disc diffusion method.

The tested bacteria, including *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 will be cultivated on trypticase soy agar (TSA) at 37 $^{\circ}$C for 24 hours, before antimicrobial testing. The tested yeast, *Candida albicans* ATCC 10231 will be cultivated on Sabouraud dextrose agar (SDA) at 30 $^{\circ}$C

for 48 hours. All tested microorganisms will be suspended in 0.85% normal saline solution and standardized the turbidity with McFarland solution No.0.5, approximately 1.5 x10^8 CFU of cell suspension. The tested plates will be swabbed with tested microorganisms, TSA for cultivation of the bacteria and SDA for cultivation of the yeast. The dried discs will be placed on the surface of tested plates and incubated at 37^{o}C 24 hours for the bacteria and 30 ^{o}C, 48 hours for the yeast. The 30 µl of chloramphenicol (1 mg/ml) or 50 µl of ciprofloxacin (0.1 mg/ml) will be used as a position control of antimicrobial activity testing for bacteria. The 20 µl of cycloheximide (2.5 mg/ml) will be used to the position control of antimicrobial activity testing for yeast. Methanol will be used to the negative control of antimicrobial activity testing (Vargas, Sousa, Smania, & Smania, 2007). The inhibition zones will be measured by using vernier calipers.

3.5 Chemical profile analysis of the secondary metabolites

3.5.1 Fermentation of the selected isolates

The isolates with high activity were selected to inoculate in the seed medium (ISP no.2) at 28-30 ^{o}C for 5 days on a shaker at 200 rpms. Approximately 3 ml of the inoculum will be incubated in 350 ml of the production medium (ISP no.2) at 28-30 ^{o}C for 7 days on the shaker at 200 rpms.

3.5.2 Extraction of the secondary metabolites

After the fermentation, the cells were discarded by centrifugation at 4,500 rpms for 10 minutes. The supernatant were collected and extracted with ethyl acetate three times. The ethyl acetate layer was evaporated to dryness.

3.5.3 Chemical profile analysis of the secondary metabolites

The crude extract was dissolved in MeOH and analyzed the chemical profile by using HPLC. The chemical profile was compared with the in-house BIOTEC's compound database. The isolates containing unknown secondary metabolites, will be selected for further study.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Plant sample collection

In this study, the medicinal plant samples containing leaves, stem and roots, were collected from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand, as shown in Table 4.1.

Table 4.1 Plant sample, collected date and isolated date

Plant sample		Collected date	Isolated date
Genus or scientific name	Family name		
Catharanthus roseus	*Apocynaceae*	14/01/2013	14/01/2013
Stemona sp.	*Stemonaceae*	14/01/2013	14/01/2013
Ophiorrhiza sp.	*Rubiaceae*	24/02/2013	27/02/2013
Pseuderanthemum graciliflorum	*Acanthaceae*	27/02/2013	27/02/2013
Phyllanthus amarus	*Euphorbiaceae*	28/03/2014	28/03/2014
Phyllanthus pulcher	*Euphorbiaceae*	28/03/2014	28/03/2014
Vernonia cinerea	*Asteraceae*	10/04/2014	10/04/2014

4.2 Isolation of endophytic actinomycetes

In this study, forty-seven actinomycetes were isolated from roots, stems and leaves of eight healthy medicinal plant samples within 7-21 days after incubation at 30^{o}C by using three different agar media (humic acid-vitamin agar, starch-casein agar and 2.5% water agar) supplemented with cycloheximide (50 µg/ml) and nalidixic acid (25 µg/ml), as shown in Table 4.2. Eleven isolates were collected from root and leaves of *Catharanthus roseus* as CR1-01 to CR1-11. Ten isolates were collected from stem of *Stemona* sp. as ST1-01 to ST1-10. Two isolates were collected from stem and leaves of *Pseuderanthemum graciliflorum* as PG1-01 to PG1-02. Two isolates were collected from stem and leaves of *Ophiorrhiza* sp. as OH1-01 to OH1-02. Eleven

isolates were collected from stem, roots and leaves of *Phyllanthus amarus* as PA1-01 to PA1-11. Six isolates were collected from stem and roots of *Phyllanthus pulcher* as PP1-01 to PP1-06. Five isolates were collected from stem and leaves of *Vernonia cinerea* as VC1-01 to VC1-05. All isolates were cultivated on ISP no. 2 slants (triplicates) and preserved as lyophilized cells in 10% skim milk.

Table 4.2 Host plant, agar media and isolate number of actinomycete isolates

Host plant	Agar medium	Isolate number		
		Root	Stem	Leaf
Catharanthus roseus	HV	CR1-05	-	CR1-01, CR1-03
	SC	CR1-06, CR1-07, CR1-08, CR1-09, CR1-10	-	CR1-02, CR1-04, CR1-11
Stemona sp.	HV	-	ST1-02, ST1-03, ST1-04, ST1-05	-
	SC	-	ST1-01, ST1-07	-
	WA	-	ST1-06, ST1-08, ST1-09, ST1-10	-
Ophiorrhiza sp.	HV	-	-	OH1-01
	WA	-	-	OH1-02
Pseuderanthemum graciliflorum	HV	-	PG1-01	PG1-02
Phyllanthus amarus	HV	-	PA1-07	-
	SC	PA1-01, PA1-03, PA1-05, PA1-06	PA1-09, PA1-11	PA1-10
	WA	-	-	PA1-02, PA1-04, PA1-08
Phyllanthus pulcher	HV	PP1-02	PP1-06	-
	SC	PP1-01, PP1-04, PP1-05	PP1-03	-
Vernonia cinerea	SC	-	VC1-01, VC1-02,	VC1-03, VC1-04 V1-05

HV; humic acid-vitamin agar, SC; starch casein agar, WA; 2.5% water agar

CR; *Catharanthus roseus*, ST; *Stemona* sp., PG; *Pseuderanthemum graciliflorum*, OH; *Ophiorrhiza* sp., PA; *Phyllanthus* sp., PP; *Phyllanthus pulcher*, and VC; *Vernonia cinerea*

4.3 Identification of endophytic actinomycetes

The different thirty-seven isolates were selected from forty-seven isolates based on morphological and cultural characteristics by using light microscopy (500X), color of colony and soluble pigment that observed on ISP no. 2 agar at 30C for 14 days. On the basis of the same colony morphology on ISP no.2, isolate PA1-03 was selected as a representative of the isolates PA1-01, PA1-03, PA1-05 and PA1-06. The isolate PA1-02 was selected as a representative of isolates PA1-02, PA1-04, PA1-08 and PA1-10. The isolate PA1-11 was selected as a representative of isolates PA1-09 and PA1-11. The isolates PP1-01 and PP1-04 were selected as the representatives of isolates PP1-01, PP1-02, PP1-04 and PP1-05. The isolate PP1-03 was selected as a representative of isolates PP1-03 and PP1-16. The isolate VC1-04 was selected as a representative isolate of VC1-04 and VC1-05. They were identified as *Streptomyces* (13 isolates), *Amycolatopsis* (1 isolate), *Nocardia* (2 isolates), *Micromonospora* (3 isolates), *Microbispora* (15 isolates), and *Nonomuraea* (3 isolates) by using 16S rRNA gene sequencing, as shown in Table 4.3 and Figure 4.1.

Table 4.3 Identification of isolates based on 16S rRNA gene sequences

Host plant	Isolate number			Identification
	Root	Stem	Leaf	
Catharanthus roseus	CR1-05, CR1-06, CR1-07, CR1-08, CR1-09, CR1-10	-	CR1-01, CR1-02, CR1-03, CR1-04, CR1-11	*Microbispora*
Stemona sp.	-	ST1-01, ST1-02, ST1-03, ST1-04, ST1-05, ST1-07, ST1-09, ST1-10	-	*Streptomyces*
	-	ST1-06	-	*Nocardia*
	-	ST1-08	-	*Amycolatopsis*
Ophiorrhiza sp.	-	-	OH1-01, OH1-02	*Microbispora*
Pseuderanthemum sp.	-	PG1-01	PG1-02	*Microbispora*
Phyllanthus amarus	PA1-03 (PA1-01, PA1-05, PA1-06)	-	-	*Nocardia*
	-	PA1-07	-	*Streptomyces*
	-	PA1-11 (PA1-09)	PA1-02 (PA1-04, PA1-08, PA1-10)	*Nonomuraea*
Phyllanthus pulcher	PP1-01, PP1-04 (PP1-02, PP1-05)	PP1-03 (PP1-06)	-	*Micromonospora*
Vernonia cinerea	-	VC1-01, VC1-02	VC1-03, VC1-04 (VC1-05)	*Streptomyces*

The isolates in parentheses were not selected to analyze 16S rRNA gene sequence because their morphological and cultural characteristics on ISP no.2 were similar to the representative isolates.

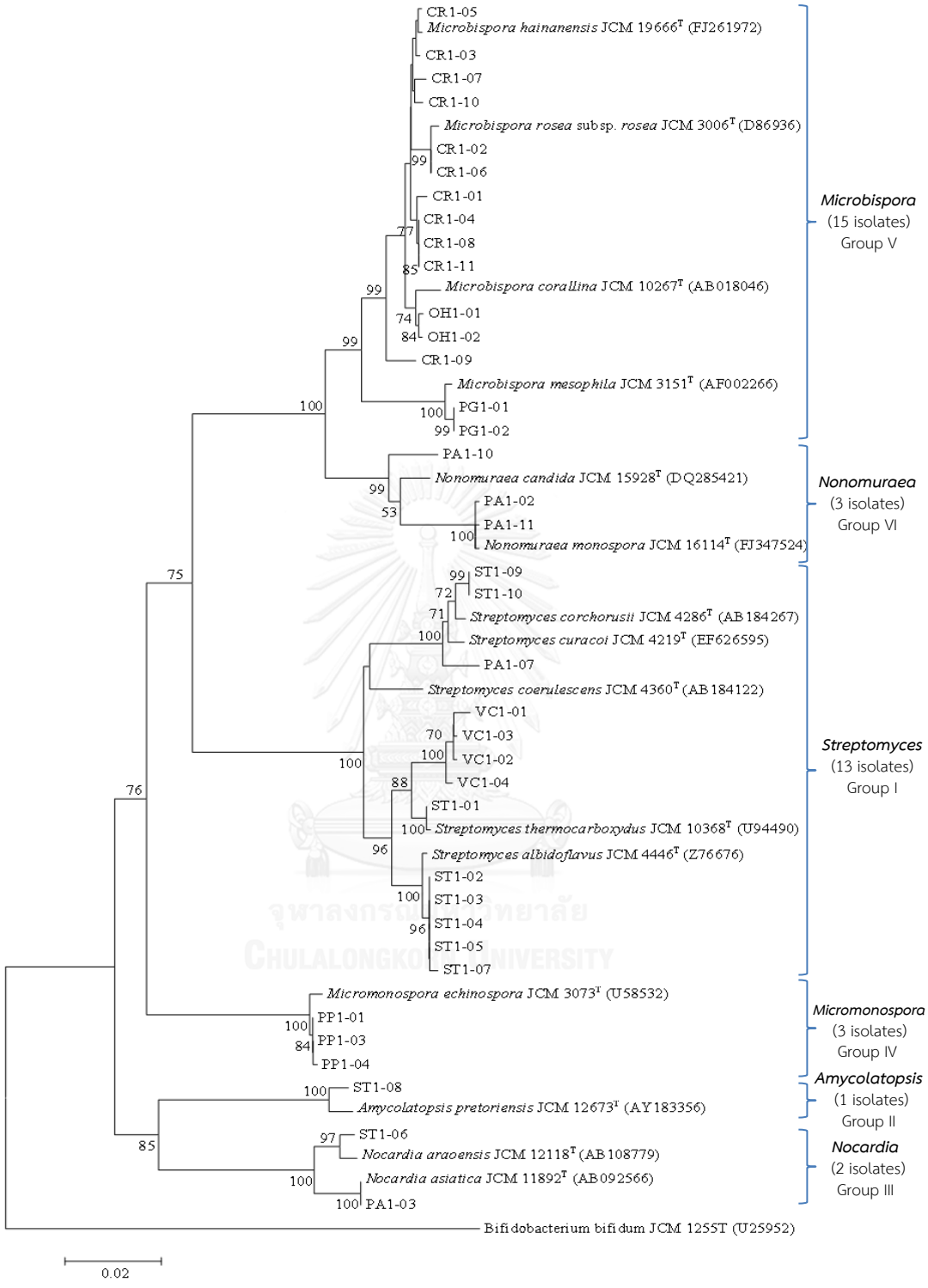


Figure 4.1 Neighbor-joining tree based on the partial 16S rRNA gene sequences of the representative isolates

4.3.1 Characteristics of *Streptomyces* (Group I)

The isolates ST1-01, ST1-02, ST1-03, ST1-04, ST1-05, ST1-07, ST1-09, ST1-10, VC1-01, VC1-02, VC1-03, VC1-04 and PA1-07 were assigned as *Streptomyces* based on their phenotypic, genotypic and chemotaxonomic characteristics (P. Kämpfer, 2012). The cultural characteristics based on colors of aerial mycelium, substrate mycelium and soluble pigment on ISP no. 2 agar of the isolates were shown in Table 4.4. Most of these isolates grew on 4-5 % NaCl, at pH 6-10 and 20-45 °C. Peptonization of milk, gelatin liquefaction and starch hydrolysis were positive and variation characteristics were shown in Table 4.5. The representative isolate, PA1-07 formed rugose spore chains on aerial mycelium cultivated on ISP2 agar for 14 days (Figure 4.2). This isolate contained *LL*-diaminopimelic acid in cell wall peptidoglycan. The whole cell hydrolysates offered ribose, mannose, galactose and glucose, corresponded to pattern B (M. P. Lechevalier & Lechevalier, 1970). Predominant menaquinones were MK-9(H_6) and MK-9(H_8). The major cellular fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$, and anteiso-$C_{17:0}$, corresponded to pattern II (Kroppenstedt, 1985) as in Table 4.6. DNA G+C content was 71 mol%. It grew at 20-40 °C, pH 6-10 and on 5 % (w/v) maximum of NaCl. Gelatin liquefaction, starch hydrolysis and skim milk peptonization were positive. Nitrate reduction and milk coagulation were negative. It used L-arabinose, D-galactose, D-glucose, glycerol, *myo*-inositol, lactose, D-mannitol, D-raffinose, L-rhamnose and D-sorbitol as sole carbon sources while carbon utilization of other isolates in the genus *Streptomyces* was in Table 4.5.

On the basis of 16S rRNA gene sequence analysis indicated that ST1-01 was closely related to *S. thermocarboxydus* JCM 10368^T (99.85% similarity) that was identified as *S. thermocarboxydus* JCM 10368^T. ST1-02, ST1-03, ST1-04, ST1-05 and ST1-07 were closely related to *S. albidoflavus* JCM 4446^T (99.62-99.78 % similarity) that were identified as *S. albidoflavus* JCM 4446^T. ST1-09 and ST1-10 were closely related to *S. corchorusii* JCM 4467^T (99.35-99.55 % similarity) that were identified as *S. corchorusii* JCM 4467^T. VC1-01, VC1-02, VC1-03 and VC1-04 were closely related to *S. coerulescens* JCM 4360^T (99.04-99.48% similarity) that were identified as *S. coerulescens* JCM 4360^T. PA1-07 was closely related to *Streptomyces curacoi* JCM

4219^{T} (98.75% similarity). Neighbor-joining tree exhibited the relative between some isolates and some members of the genus *Streptomyces* as shown in Figure 4.3. Some isolates are possibly identified as new species in the genus *Streptomyces* however they should be confirmed by compared characterization and DNA-DNA hybridization with closely type strains (Stackebrandt & Schumann, 2006).

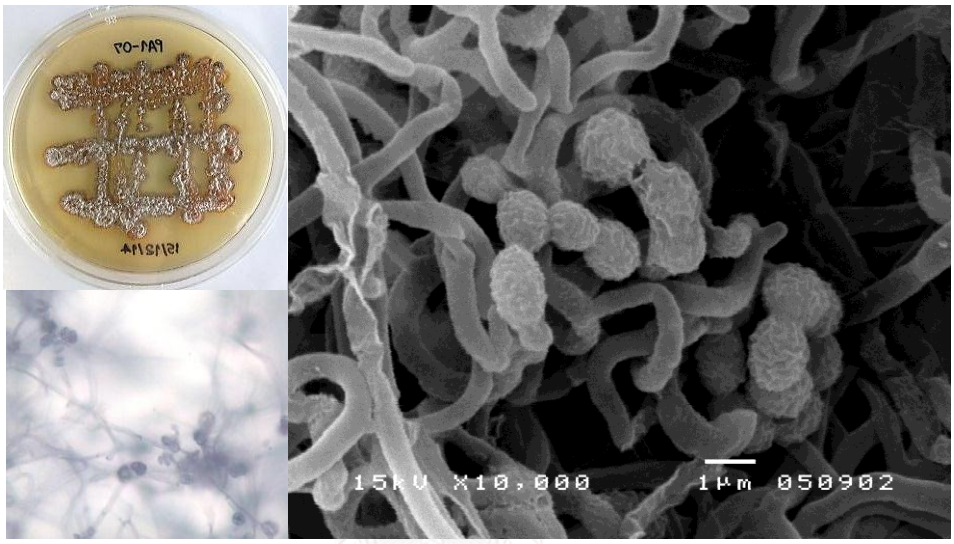


Figure 4.2 Photomicrograph (500x) and scanning electron micrograph of *Streptomyces* sp. PA1-07 on ISP no. 2 agar at 30 °C for 14 days.

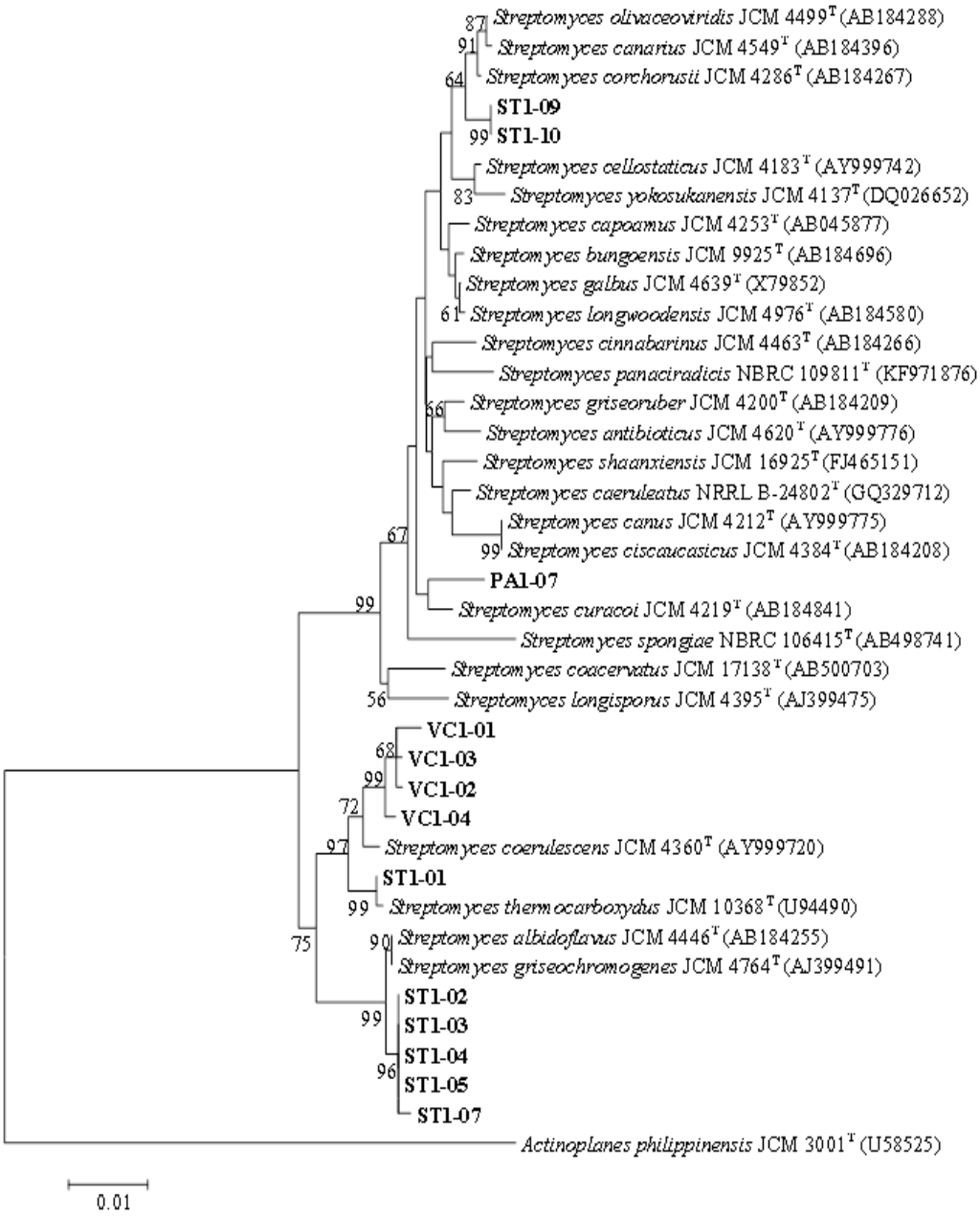


Figure 4.3 Neighbor-joining tree based on partial 16S rRNA gene sequences, showing the *Streptomyces* isolates and related type strains.

Table 4.4 Cultural characteristic of the *Streptomyces* isolates

Isolate No	Color of upper surface	Color of reverse surface	Soluble pigment
ST1-01	White	Strong yellow brown (74)	Moderate yellow (87)
ST1-02	Greenish white (153)	Moderate reddish brown (43)	Strong reddish brown (40)
ST1-03	Greenish white (153)	Moderate reddish brown (43)	Strong reddish brown (40)
ST1-04	Greenish white (153)	Moderate reddish brown (43)	Strong reddish brown (40)
ST1-05	Greenish white (153)	Moderate reddish brown (43)	Strong reddish brown (40)
ST1-07	Pale yellowish green (121)	Moderate reddish brown (43)	Strong yellowish brown (54)
ST1-09	Pale yellowish pink (31)	Strong orange (50)	-
ST1-10	Pale yellowish pink (31)	Strong orange (50)	-
PA1-07	Yellowish white (92)	Pale yellowish pink (31)	-
VC1-01	Yellowish white (92)	Dark yellow (89)	Moderate yellow(87)
VC1-02	Yellowish white (92)	Dark yellow (89)	Moderate yellow(87)
VC1-03	Yellowish white (92)	Dark yellow (89)	Moderate yellow(87)
VC1-04	Yellowish white (92)	Dark yellow (89)	Moderate yellow(87)
VC1-05	Yellowish white (92)	Dark yellow (89)	Moderate yellow(87)

Table 4.5 Physiological and biochemical characteristics of *Streptomyces* isolates

characteristics	ST1-01	ST1-02	ST1-03	ST1-04	ST1-05	ST1-07	ST1-09	ST1-10	PA1-07	VC1-01	VC1-02	VC1-03	VC1-04	VC1-05
% NaCl max	5	4	4	5	4	5	4	4	5	5	5	5	5	5
pH	6-9	6-9	6-9	6-9	6-9	6-9	6-10	6-10	6-10	6-9	6-9	6-9	6-9	6-9
Temperature (°C)	20-45	20-45	20-45	20-45	20-45	20-45	20-45	20-45	20-40	20-45	20-45	20-45	20-45	20-45
Peptonization of milk	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coagulation of milk	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Utilization of:														
L-Arabinose	+	w	w	w	w	-	-	w	w	-	w	-	-	w
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	w	+	w	w	+	+	+	+	+	+	+	+	+
Glycerol	-	w	w	w	w	-	-	-	w	-	-	-	-	-
myo-Inositol	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	w	w	w	w	+	+	+	+	+	+	+	+	+
D-Mannitol	-	w	w	w	w	-	+	+	+	+	+	+	+	+
D-Raffinose	-	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Rhamnose	-	w	w	+	+	-	+	+	+	+	+	+	+	+
D-Sorbitol	w	+	+	+	+	+	+	w	+	+	w	+	+	w

+, positive; w, weakly positive; -, negative

Table 4.6 Cellular fatty acids of representative *Streptomyces* isolates and *S. curacoi* JCM 4219^T

Fatty acid[a]	ST1-01	ST1-02	VC1-01	VC1-04	PA1-07	JCM4219^T
Straight-chain fatty acids						
$C_{14:0}$	2.2	1.6	2.4	1.0	2.6	5.0
$C_{15:0}$	1.3	0.6	4.3	1.2	2.5	3.1
$C_{16:0}$	17.2	10.9	19.0	10.7	14.3	10.9
$C_{17:0}$	0.5	0.3	1.5	0.6	2.2	0.4
$C_{17:0}$ cyclo	2.6	3.7	1.6	2.4	2.8	0.4
$C_{18:0}$	1.1	0.7	0.6	0.9	5.4	-
Unsaturated fatty acids						
$C_{18:1}$ ω7c	2.1	0.4	2.2	0.9	5.5	2.1
$C_{18:1}$ ω9c	1.9	0.6	1.4	0.7	2.5	1.9
Branched fatty acids						
iso-$C_{14:0}$	5.8	4.2	6.9	5.5	3.4	5.8
iso-$C_{15:0}$	6.5	7.0	8.0	6.0	5.9	6.5
anteiso-$C_{15:0}$	13.0	22.6	18.6	17.1	9.0	13.0
iso-$C_{16:0}$	19.4	16.9	15.5	26.9	13.2	19.4
iso-$C_{16:1}$ H	2.0	2.0	0.3	2.9	1.5	2.0
iso-$C_{17:0}$	1.7	3.0	2.3	2.8	4.4	1.7
anteiso-$C_{17:0}$	4.7	12.6	5.1	9.8	7.3	4.7
iso-$C_{17:1}$ ω9c	1.6	1.6	0.8	1.8	1.9	1.6
anteiso-$C_{17:1}$ ω9c	1.4	3.0	0.5	2.7	3.6	1.4
Summed in feature	4.5	2.4	2.8	1.9	7.7	15.8

[a]Values are percentages of total cellular fatty acids. -, not detected.

4.3.2 Characteristics of *Amycolatopsis* (Group II)

The isolate ST1-08 was assigned in the genus *Amycolatopsis* based on its phenotypic, genotypic and chemotaxonomic characteristics (Yuan, Tan, & Goodfellow, 2012). It formed smooth and squarish to ellipsoidal spore-like chain on aerial mycelium when cultured on ISP no. 2 agar for 14 days (Figure 4.4). The colors of aerial mycelium, substrate mycelium and soluble pigment on various agar media

were shown in Table 4.7. The isolate ST1-08 grew on ISP no. 2 media with 1 to 2 % NaCl, pH 6 to 8 and 20 to 45°C. Peptonization of milk, gelatin liquefaction and starch hydrolysis were positive but milk coagulation and nitrate reduction were negative (Table 4.8). It utilized L-arabinose, D-galactose, D-glucose, glycerol, *myo*-inositol, D-mannitol, L-rhamnose as sole carbon sources but not use Lactose, L-raffinose and D-sorbitol. Acid was produced from adonitol, L-arabinose, galactose, *myo*-inositol, maltose, D-mannitol, D-melibiose, α-methyl-D-glucoside, L-raffinose, L-rhamnose, salicin, sucrose, trehalose and xyrose (Table 4.8). It contained *meso*-diaminopimelic acid in cell wall peptidoglycan. The whole cell hydrolysates offered rhamnose, ribose, mannose, glucose, galactose and arabinose corresponded to pattern A (M. P. Lechevalier & Lechevalier, 1970). The predominant menaquinone was MK-9(H_4). The phospholipid profile composed of hydroxylated phosphotidylethanolamine (OH-PE), phisphatidylinositol (PI), diphosphotidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphotidylglycerol (PG) that corresponded to pattern IV (M. P. Lechevalier et al., 1977), shown in Figure 4.5. The major cellular fatty acids were iso-$C_{16:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$, $C_{17:1}$, $C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{15:0}$, $C_{15:0}$, $C_{17:0}$, iso-$C_{14:0}$, $C_{15:1}$ and 10-methyl $C_{17:0}$ that corresponded to pattern III (Kroppenstedt, 1985), as in Table 4.9. DNA G+C content was 71 mol%.

On the basis of 16S rRNA gene sequence analysis and phylogenetic tree investigation indicated that ST1-08 was closely related to *Amycolatopsis pretoriensis* JCM 12673^T (98.99 % similarity) as in Figure 4.6. The levels of DNA-DNA relatedness value among ST1-08^T, *A. pretoriensis* JCM 12673^T (17.4±5.2 to 57.6±6.4) and *A. lexingtonensis* JCM 12672^T (19.9±2.9 to 42.2±1.8) were lower than 70%, the cutoff level for assigning strains to the same species as in Table 4.10 (Wayne *et al*., 1987). This result can be indicated that strain ST1-08^T represents a genomic distinct from some related *Amycolatopsis* species. Therefore, the differences of physiological, biochemical characteristics and DNA-DNA relatedness support that ST1-08^T is a novel species of the genus *Amycolatopsis* and named *Amycolatopsis stemonae* (Type strain ST1-08^T=JCM 30050^T =PCU 339^T =TISTR 2278^T).

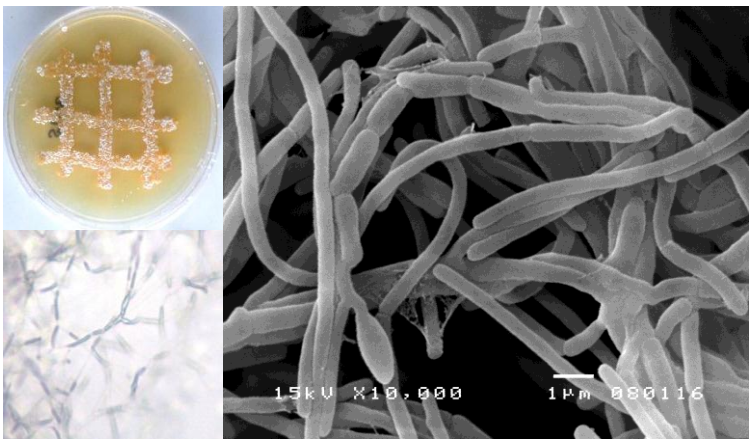


Figure 4.4 Photomicrograph (500x) and scanning electron micrograph of *Amycolatopsis* sp. ST1-08^{T} on ISP no. 2 agar at 30 °C for 14 days.

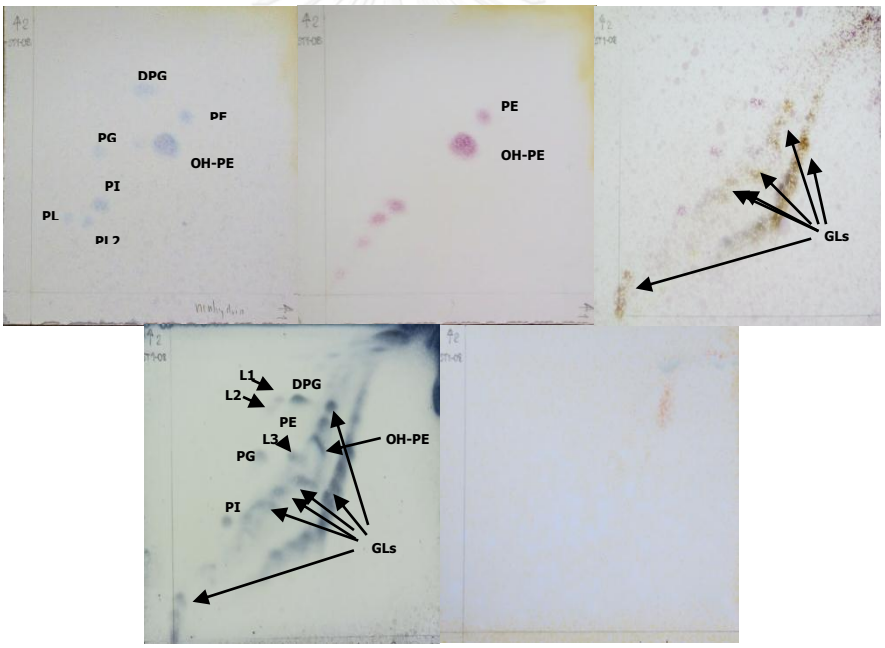


Figure 4.5 Polar lipid profile of isolate ST1-08^{T} after two dimension TLC and detected with Dittmer & Lester (A), ninhydrin (B), anisaldehyde (C), phosphomolybdic acid (D) and dragendorff (E) as spraying reagents.

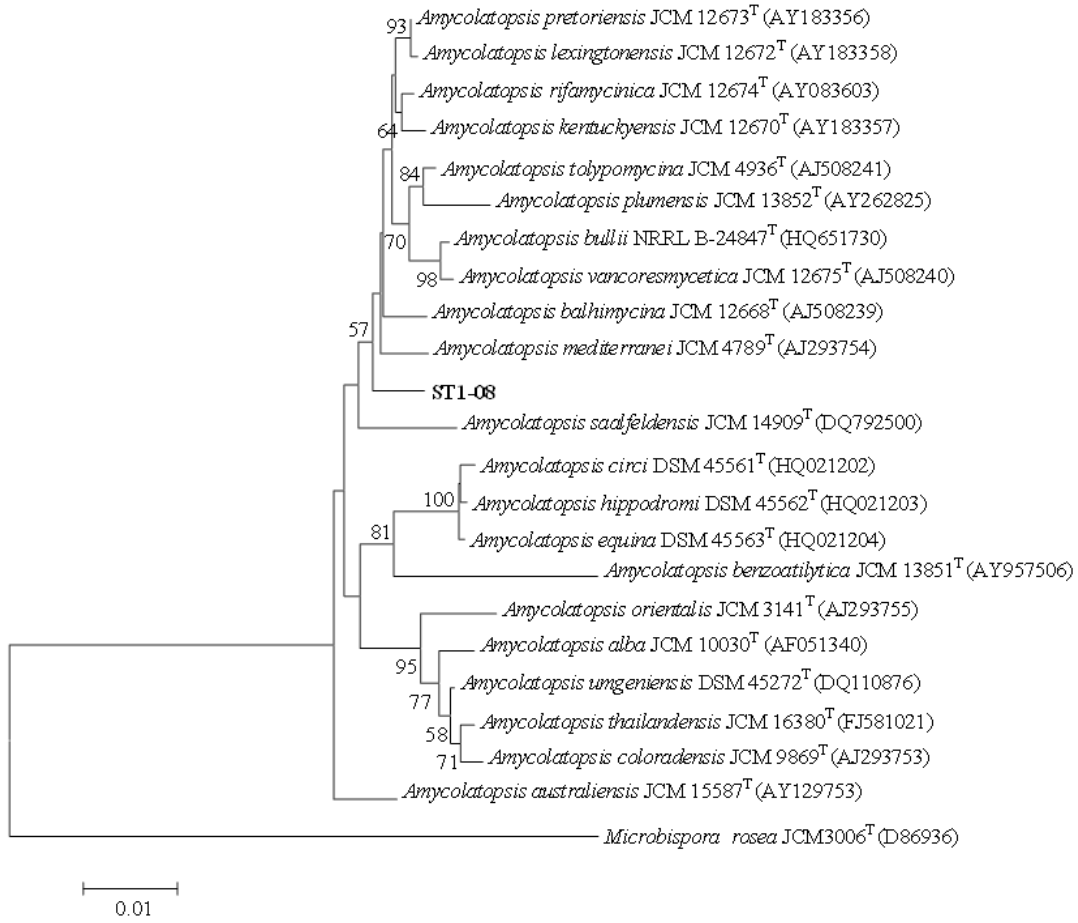


Figure 4.6 Neighbor-joining tree based on partial 16S rRNA gene sequences, showing *Amycolatopsis* isolate and related type strains.

Table 4.7 Cultural characteristics of the ST1-08^{T} and closely related species in the genus *Amycolatopsis* by the NBS/IBCC color System

Media	ST1-08	*A. pretoriensis* JCM 12673^{T}	*A. lexingtonensis* JCM 12672^{T}
Growth on ISP no.2	Good	Good	Good
Color of upper surface	White	Pale yellow	White
Color of reverse surface	Strong reddish orange	Pale yellow	Deep reddish brown
Soluble pigment	-	-	Deep orange
Growth on ISP no.3	Moderate	Moderate	Good
Color of upper surface	White	White	White
Color of reverse surface	Pale yellow	Pale greenish yellow	Moderate orange
Soluble pigment	-	-	Light yellow pink
Growth on ISP no.4	Moderate	Good	Good
Color of upper surface	White	White	White
Color of reverse surface	Pale greenish yellow	Pale yellow	Deep orange yellow
Soluble pigment	-	-	Vivid orange yellow
Growth on ISP no.5	Moderate	Good	Good
Color of upper surface	White	White	Yellowish white
Color of reverse surface	Strong reddish orange	Pale greenish yellow	Deep reddish brown
Soluble pigment	-	-	Deep orange
Growth on ISP no.6	Poor	Poor	Moderate
Color of upper surface	White	Strong reddish orange	Yellowish white
Color of reverse surface	Brilliant orange yellow	Light orange yellow	Strong yellow
Soluble pigment	-	-	Pale yellow
Growth on ISP no.7	Good	Good	Good
Color of upper surface	White	White	white
Color of reverse surface	Light orange yellow	Pale yellow89	Dark reddish brown
Soluble pigment	-	-	Strong brown
Growth on GluA	Moderate	Good	Good
Color of upper surface	White	Yellowish white	White
Color of reverse surface	Yellowish white	Yellowish white	Brownish orange
Soluble pigment	-	-	Light orange
Growth on Czk	Moderate	Moderate	Poor
Color of upper surface	White	White	White
Color of reverse surface	Pale greenish yellow	Yellowish white	Pale yellowish pink
Soluble pigment	-	-	Pale pink
Growth on NA	Moderate	Good	Good
Color of upper surface	White	Pale greenish yellow	White
Color of reverse surface	Pale yellow	Pale greenish yellow	Deep orange
Soluble pigment	-	-	Dark yellowish pink

Table 4.8 Physiological and biochemical characteristics of *Amycolatopsis* isolates

Characteristics	ST1-08^T	*A. pretoriensis* JCM 12673^T	*A. lexingtonensis* JCM 12672^T
% NaCl max	4	1	2
pH	6-11	6-11	6-11
Temperature (°C)	15-40	20-45	20-45
Starch hydrolysis	+	-	-
Peptonization of milk	+	-	-
Nitrate reduction	-	+	+
Gelatin liquefaction	+	-	-
Carbon utilization			
L-Arabinose	+	w	+
D-Galactose	+	+	+
D-Glucose	+	w	+
Glycerol	+	+	+
myo-Inositol	+	+	+
Lactose	-	w	+
D-Mannitol	+	-	+
D-Raffinose	-	w	-
L-Rhamnose	+	w	+
D-Sorbitol	-	+	-
Acid production from			
Adonitol	+	-	-
L-Arabinose	+	w	+
D-Cellobiose	-	w	+
D-Galactose	w	w	+
Lactose	-	+	+
Maltose	+	w	+
D-Mannitol	+	w	+
D-Melibiose	+	-	+
α-Methyl-D-glucoside	+	w	+
D-Sorbitol	-	+	+
Sucrose	+	w	+
D-Trehalose	+	w	+
D-Xylose	+	-	+

+, positive; -, negative; w, weakly positive

Table 4.9 Cellular fatty acids of ST1-08^T and related *Amycolatopsis* species

Fatty acid[a]	ST1-08^T	*A. lexingtonensis* JCM 12672^T	*A. pretoriensis* JCM 12673^T
Straight-chain fatty acids			
$C_{14:0}$	1.2	1.0	0.4
$C_{15:0}$	2.7	2.2	1.2
$C_{16:0}$	11.7	10.1	4.3
$C_{17:0}$	4.0	2.5	1.7
Unsaturated fatty acids			
$C_{17:1}$ ω6c	3.1	5.5	3.6
$C_{17:1}$ ω8c	2.6	2.7	1.9
Branched fatty acids			
iso-$C_{14:0}$	2.5	3.2	3.3
iso-$C_{15:0}$	12.8	17.8	18.8
iso-$C_{16:0}$	26.0	24.6	34.9
iso-$C_{17:0}$	3.8	3.5	5.0
anteiso-$C_{15:0}$	3.5	3.8	4.5
anteiso-$C_{17:0}$	8.5	5.8	10.4
10Me-$C_{16:0}$	1.0	-	0.9
Summed in feature	5.1	8.5	5.0

[a]Values are percentages of total cellular fatty acids. -, not detected

Table 4.10 DNA-DNA relatedness of ST1-08^T and related *Amycolatopsis* species

Strain	DNA-DNA relatedness (%) with labeled strains*		
	ST1-08^T	*A. pretoriensis* JCM 12673^T	*A. lexingtonensis* JCM 12672^T
ST1-08^T	100.0±0.2	57.6±6.4	42.2±1.8
A. pretoriensis JCM 12673^T	17.4±5.2	100.0±13.5	48.6±4.6
A. lexingtonensis JCM 12672^T	19.9±2.9	71.31±12.4	100.0±28.5

* Values obtained from three independent determinations

4.3.3 Characteristics of *Nocardia* (Group III)

The isolates ST1-06 and PA1-03 were indicated in the genus *Nocardia* based on their phenotypic, genotypic and chemotaxonomic characteristics (Stackebrandt *et al.,* 1997). ST1-06, a representative isolate formed moderate orange yellow substrate mycelia and fragmented into rod-shaped and non-motile spores on pale orange yellow aerial mycelium when cultured on ISP no. 2 agar for 14 days (Figure 4.7). The color colony of PA1-03 was yellowish white aerial mycelium and pale yellow substrate mycelium. The soluble pigment was not produced. The cultural characteristics of ST1-06 on various media were shown in Table 4.11. ST1-06 grew on ISP no. 2 media with 1 to 5% NaCl, pH 5 to 8 and at 20 to 40°C. Coagulation of milk was weakly positive. Peptonization of milk, gelatin liquefaction, nitrate reduction and starch hydrolysis were negative. They utilized different carbon sources exhibited the isolate ST1-06 utilized L-arabinose, D-galatose, D-glucose, glycerol, *myo*-inositol, D-mannitol, L-rhamnose and D-sorbitol as sole carbon sources but not lactose and L-raffinose (Table 4.12). The isolate ST1-06 was selected to study chemotaxonomic characteristics that represent all isolates in this group of the genus *Nocardia*. It contained *meso*-diaminopimelic acid in cell wall peptidoglycan. It contained ribose, mannose, arabinose, galactose and glucose as the reducing sugars of whole cell hydrolysates which corresponded to whole cell sugar pattern A (Lechevalier and Lechevalier, 1970). The predominants of isoprenoid quinone were 2,3-epoxy MK-8 (H_4-ωcycl) and MK-8 (H_4-ωcycl). The phospholipid composition composed of diphosphotidylglycerol (DPG), phosphatidylethanolamine (PE), phisphatidylinositol (PI), phosphotidylinositolmannosides (PIMs) and phosphotidylglycerol (PG) which corresponded to phospholipid pattern II (Lechevalier *et al.,* 1977), shown in Figure 4.8. The predominant fatty acids were $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ω9c and 10-methyl $C_{18:0}$ corresponded to fatty acid pattern Ib (Kroppenstedt, 1985), as in Table 4.13. The DNA G+C content was 71 mol%.

On the basis of 16S rRNA gene sequence analysis and phylogenetic tree investigation, PA1-03 was closely related to *N. asiatica* JCM 11892^T (100% similarity) that was identified as *N. asiatica* JCM 11892^T. ST1-06 was closely related to *Nocardia*

araoensis JCM 12118^{T} (99.03% similarity). The neighbor-joining tree showed relative between isolate ST1-06, PA1-03 and related members of genus *Nocardia* (Figure 4.9). The isolate ST1-06 was possibly identified as new species in the genus *Nocardia* however it should be confirmed by DNA-DNA hybridization (Stackebrandt and Evers, 2006).

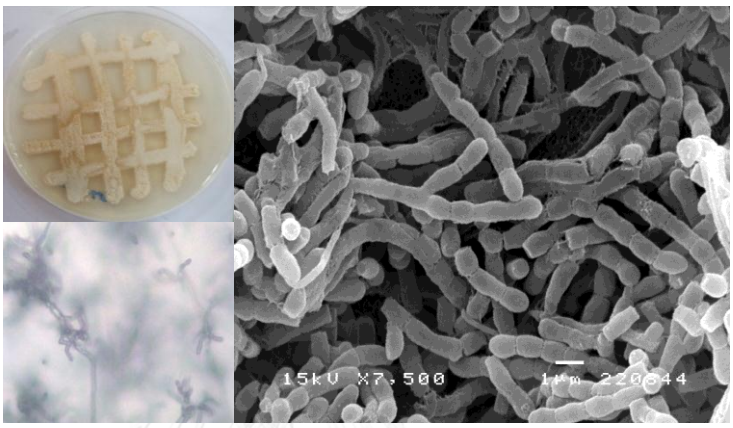


Figure 4.7 Photomicrograph (500x) and scanning electron micrograph of *Nocardia* sp. ST1-06 on agar at 30 °C for 14 days.

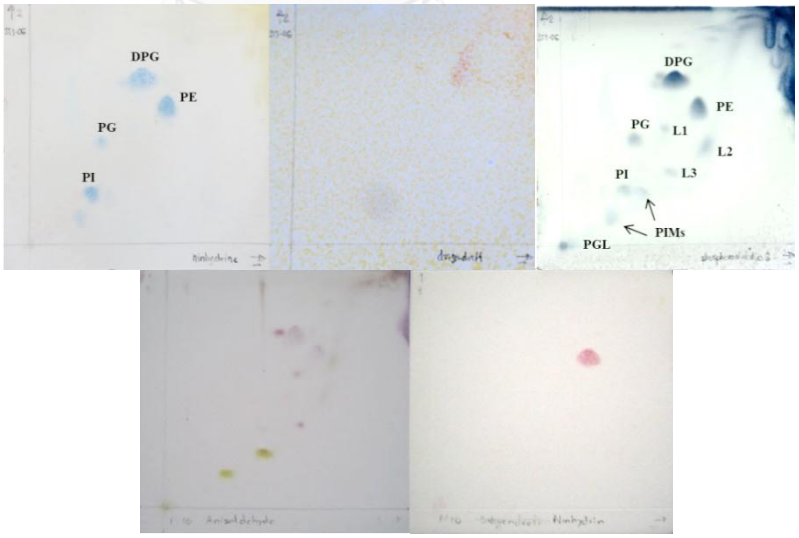


Figure 4.8 Polar lipid profile of isolate ST1-06 after two dimension TLC and detected with Dittmer & Lester (A), ninhydrin (B), anisaldehyde (C), phosphomolybdic acid (D) and dragendorff (E) as spraying reagents.

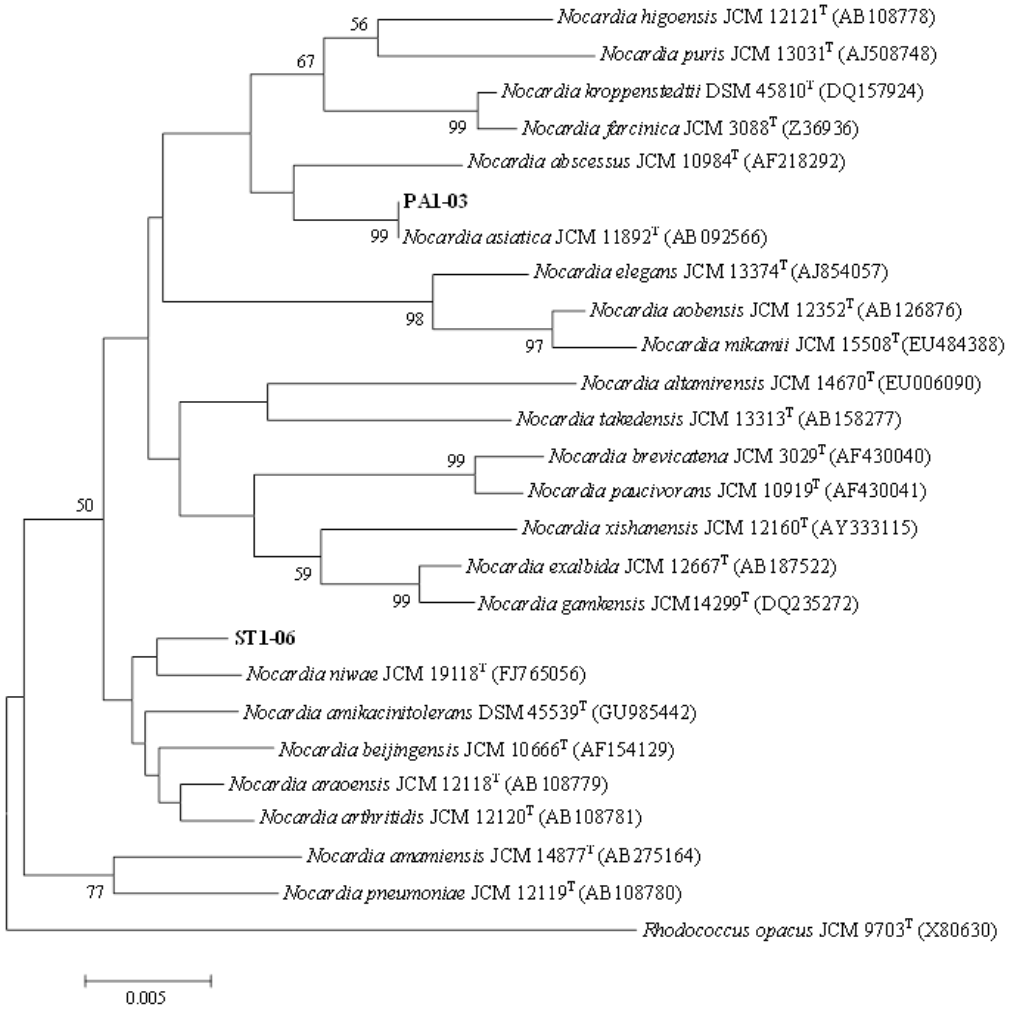


Figure 4.9 Neighbor-joining tree based on partial 16S rRNA gene sequences, showing the *Nocardia* isolates and related type strains.

Table 4.11 Cultural characteristics of the representative isolates and related *Nocardia* species by the NBS/IBCC color System

Media	ST1-06	JCM 10666^T	JCM 12118^T	JCM 12120^T	JCM 19118^T
Growth on **ISP no.2**	Good	Good	Good	Good	Good
Color of upper surface	Pale orange yellow	Pale yellowish pink	Pale yellowish pink	Pale orange yellow	Pale yellowish pink
Color of reverse surface	Moderate orange yello	Strong orange yellow	Strong orange yellow	Strong orange yellow	Deep orange yellow
Soluble pigment	-	-	-	-	-
Growth on **ISP no.3**	Moderate	Moderate	Good	Moderate	Moderate
Color of upper surface	White	White	White	White	White
Color of reverse surface	Pale orange yellow	Pale orange yellow	Pale orange yellow	Pale orange yellow	Pale orange yellow
Soluble pigment	-	-	-	-	-
Growth on **ISP no.4**	Moderate	Moderate	Moderate	Poor	Moderate
Color of upper surface	White	White	Pale orange yellow	Pale yellowish pink	Pale orange yellow
Color of reverse surface	Pale orange yellow	Pale orange yellow	Pale yellowish pink	Pale orange yellow	Pale yellowish pink
Soluble pigment	-	-	-	-	-
Growth on **ISP no.5**	Good	Good	Moderate	Good	Good
Color of upper surface	Pinkish white	Pale yellowish pink	Pinkish white	Pale orange yellow	White
Color of reverse surface	Deep brown	Moderate brown	Deep reddish brown	Strong orange yellow yellow	Strong orange yellow
Soluble pigment	Light yellowish pink	Moderate pink	Light grayish red	-	-
Growth on **ISP no.6**	Poor	Good	Poor	Good	Good
Color of upper surface	Pinkish white	Pale orange yellow	Pinkish white	Light yellowish pink	Pale yellowish pink
Color of reverse surface	Light orange yellow	Moderate orange yellow	Strong orange yellow	Strong orange yellow	Strong orange yellow
Soluble pigment	-	-	-	-	-
Growth on **ISP no.7**	Good	Good	Good	Good	Good
Color of upper surface	White	Pale orange yellow	Pinkish white	White	Moderate orange
Color of reverse surface	Moderate brown	Moderate orange yellow	Moderate orange yellow	Strong orange yellow	Moderate orange yellow yellow
Soluble pigment	Light yellowish brown	-	Light brown	-	Light orange
Growth on **GluA**	Good	Good	Moderate	Good	Good
Color of upper surface	Light orange	Pinkish white	Pale yellowish pink	Pale orange yellow	Strong orange
Color of reverse surface	Deep brown	Strong brown	Strong brown	Strong orange yellow	Moderate orange
Soluble pigment	Dark orange yellow	-	Light yellowish brown	-	-
Growth on **Czk**	Moderate	Moderate	Moderate	Moderate	Moderate
Color of upper surface	Pinkish white	Pinkish white	Pinkish white	Pinkish white	Pinkish white
Color of reverse surface	Pinkish white	Pinkish white	Pinkish white	Pinkish white	Pinkish white
Soluble pigment	-	-	-	-	-
Growth on **NA**	Good	Good	Moderate	Good	Good
Color of upper surface	Pale orange yellow	Pale orange yellow	Pale orange yellow	Pale yellowish pink	Pale yellowish pink
Color of reverse surface	Pale yellowish pink	Strong reddish orange	Strong reddish orange	Pale orange yellow	Pale orange yellow
Soluble pigment	-	-	-	-	-

Table 4.12 Physiological and biochemical characteristics of isolates and closest related *Nocardia* species

Characteristics	PA1-01	PA1-03	PA1-05	PA1-06	ST1-06	JCM10666^T	JCM12118^T	JCM12120^T	JCM19118^T
% NaCl max	5	4	5	4	5	5	5	5	5
pH	6-11	6-10	5-11	6-11	4-11	5-11	5-11	5-11	5-11
Temperature (°C)	20-40	20-40	20-40	20-40	15-40	20-40	15-45	15-40	15-45
Peptonization of milk	-	+	+	+	+	-	-	-	-
Coagulation of milk	-	-	-	-	-	-	-	-	+
Gelatin liquefaction	-	-	-	-	+	-	-	-	-
Nitrate reduction	+	+	-	+	-	+	-	+	+
Starch hydrolysis	-	-	+	-	+	-	-	-	-
Utilization of:									
L-Arabinose	-	-	-	-	+	-	-	-	-
D-Galactose	-	w	w	w	w	-	-	-	-
D-Glucose	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+
myo-Inositol	-	-	-	-	+	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-
D-Mannitol	+	-	-	-	+	-	-	-	-
D-Raffinose	+	-	-	-	-	-	-	-	-
L-Rhamnose	+	+	+	+	+	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	w	-	-

+, positive; -, negative; w, weakly positive

N. beijingensis JCM 10666^T, *N. araoensis* JCM 12118^T, *N. arthritidis* JCM 12120^T and *N. niwae* JCM 19118^T

Table 4.13 Cellular fatty acids of ST1-06 and related *Nocardia* species

Fatty acid[a]	ST1-06	JCM10666^T	JCM12118^T	JCM12120^T	JCM19118^T
Straight-chain fatty acids					
$C_{14:0}$	3.3	2.6	2.5	2.0	3.0
$C_{16:0}$	53.9	43.8	32.2	40.3	43.0
$C_{16:0}$ N alc	-	-	2.0	-	-
$C_{17:0}$	-	2.2	3.0	-	-
$C_{18:0}$	7.9	17.0	11.1	24.5	17.4
$C_{19:0}$	-	-	1.4	-	-
$C_{20:0}$	-	-	1.2	-	-
Unsaturated fatty acids					
$C_{18:1}$ **ω**9c	12.8	6.4	5.6	4.8	11.2
$C_{18:3}$ **ω**9c (6,9,12)	-	-	1.4	-	-
Branched fatty acids					
anteiso-$C_{16:0}$	-	-	1.0	-	-
anteiso-$C_{17:0}$	-	-	2.4	-	-
iso-$C_{18:0}$	-	-	2.3	-	-
10Me-$C_{18:0}$TBSA	11.2	15.3	11.4	16.1	13.2
Summed in feature	7.6	6.8	9.8	7.4	5.5

[a]Values are percentages of total cellular fatty acids. -, not detected

N. beijingensis (JCM 10666^T), *N. araoensis* (JCM 12118^T), *N. arthritidis* (JCM 12120^T) and *N. niwae* (JCM 19118^T)

4.3.4 Characteristics of *Micromonospora* (Group IV)

The isolates PP1-01, PP1-03 and PP1-04 were assigned in the genus *Micromonospora* based on their phenotypic, genotypic and chemotaxonomic characteristics (Genilloud, 2012). The representative isolate, PP1-03 formed single and non-motile spores on substrate mycelium and lack aerial mycelium when cultured on ISP no. 2 agar for 14 days (Figure 4.10). The colors of colony of PP1-01, PP1-03 and PP1-04 were strong orange yellow, and turned to olive black to black after sporulation. The soluble pigment was not produced. They grew on ISP no. 2 media with 1 to 5% NaCl, pH 5 to 8 and at 20 to 40 °C. Coagulation of milk was weakly positive. Peptonization of milk, gelatin liquefaction, nitrate reduction and starch hydrolysis were negative. They utilized different carbon sources as in Table 4.14. Isolate PP1-03 contained *meso*-diaminopimelic acid in cell wall peptidoglycan. It contained ribose, mannose, arabinose, galactose, xylose and glucose as whole cell sugars which corresponded to whole cell sugar pattern D (M. P. Lechevalier & Lechevalier, 1970). The major menaquinones were MK-9(H_4), MK-9(H_6), MK-9(H_8), MK-10(H_4), MK-10(H_6) and MK-10(H_8). The predominant of fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{17:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, $C_{17:1\omega 8c}$ and 10-methyl $C_{17:0}$ corresponding to fatty acid pattern III (Kroppenstedt, 1985). The DNA G+C contents was 73 mol%.

On the basis of 16S rRNA gene sequence analysis and phylogenetic tree investigation indicated that PP1-01, PP1-03 and PP1-04 were closely related to *Micromonospora echinospora* JCM 3073^T (99.54-99.71 % similarity). Neighbor-joining tree based on partial 16S rRNA gene sequences, showing relative between PP1-01, PP1-03, PP1-04 and some members of genus *Micromonospora* was shown in Figure 4.11.

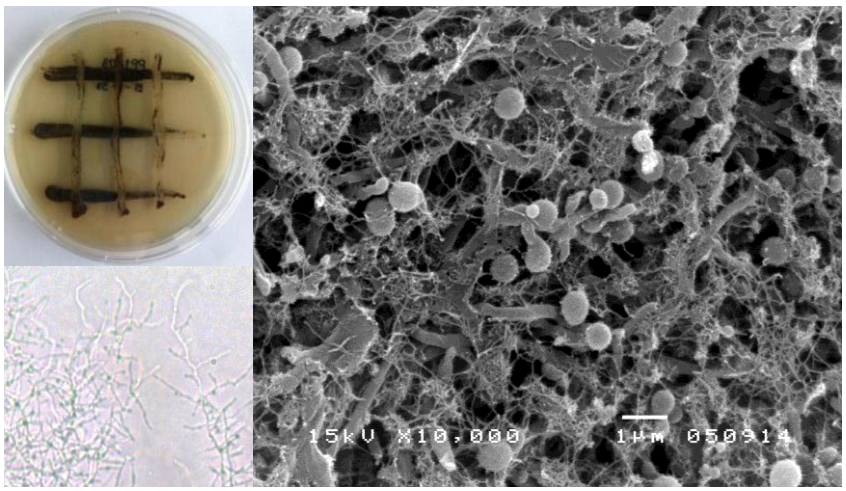


Figure 4.10 Photomicrograph (500x) and scanning electron micrograph of *Micromonospora* sp. PP1-03 on ISP no. 2 agar at 30 °C for 14 days.

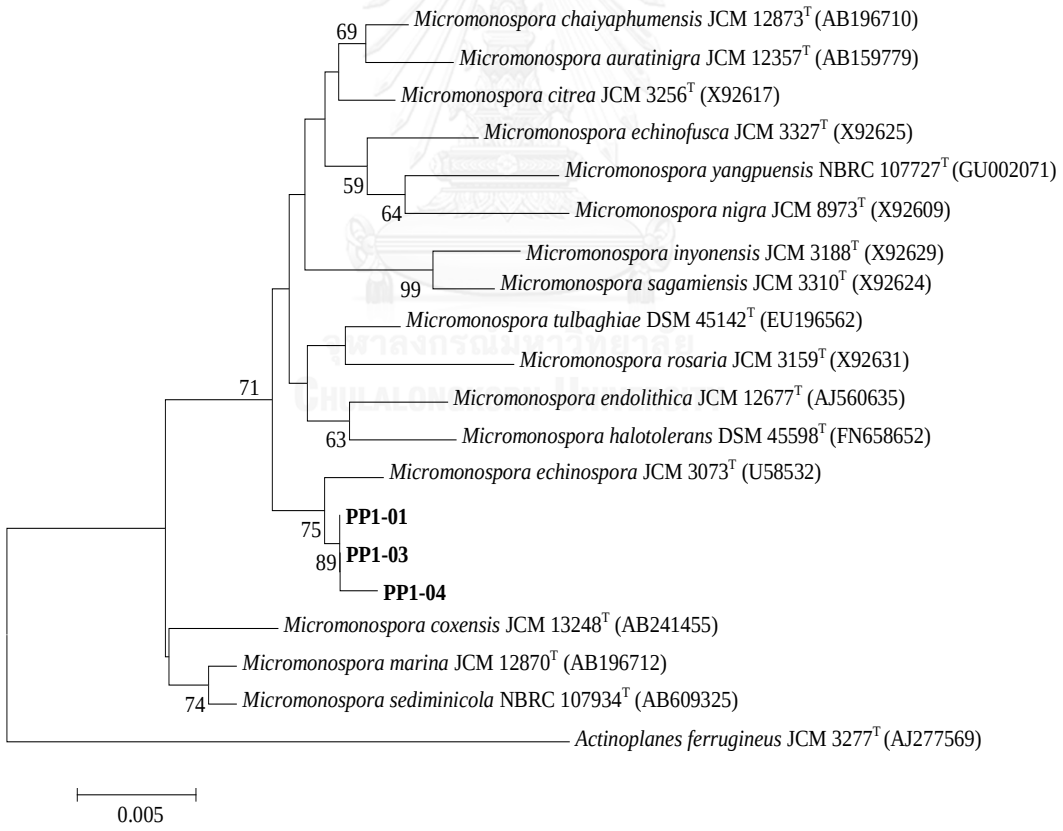


Figure 4.11 Neighbor-joining tree based on partial 16S rRNA gene sequences, showing *Micromonospora* isolates and related type strains.

Table 4.14 Physiological and biochemical characteristics of *Micromonospora* isolates

characteristics	PP1-01	PP1-02	PP1-03	PP1-04	PP1-05	PP1-06
% NaCl max	2	2	1-2	2	2	2
pH	6-11	6-11	6-11	6-11	6-11	6-11
Temperature (°C)	20-45	20-45	20-45	20-45	20-45	20-45
Peptonization of milk	+	+	+	+	+	+
Coagulation of milk	-	-	-	-	-	-
Gelatin liquefaction	w	w	w	w	w	w
Nitrate reduction	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-
Utilization of:						
L-Arabinose	-	w	w	w	-	w
D-Galactose	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
Glycerol	w	-	w	-	w	w
myo-Inositol	-	-	-	-	-	-
Lactose	w	-	w	-	w	w
D-Mannitol	w	w	w	w	w	w
D-Raffinose	+	+	+	+	+	+
L-Rhamnose	w	-	w	-	w	w
D-Sorbitol	+	+	+	+	+	+

+, positive; w, weakly positive; -, negative

4.3.5 Characteristics of *Microbispora* (Group V)

The fifteen isolates, CR1-01, CR1-02, CR1-03, CR1-04, CR1-05, CR1-06, CR1-07, CR1-08, CR1-09, CR1-10, CR1-11, OH1-01, OH1-02, PG1-01 and PG1-02 were assigned in the genus *Microbispora* based on phenotypic, genotypic and chemotaxonomic characteristics (Franco, 2012). The representative isolate, CR1-06 formed smooth and non-motile oval paired spores on aerial mycelium and substrate mycelium when cultivated on ISP no. 2 agar for 14 days (Figure 4.12). The color of aerial mycelium, substrate mycelium and soluble pigment on ISP no. 2 was shown in Table 4.15. All isolates grew on ISP no. 2 media with 2 to 3 % NaCl, pH 6 to 8 and at 20 to 45°C. Peptonization of milk, gelatin liquefaction, starch hydrolysis and nitrate reduction were shown in Table 4.16. The isolate CR1-01, CR1-03, CR1-04, CR1-07, CR1-08, CR1-09, CR1-10, CR1-11 and OH1-01 were selected to study chemotaxonomic characteristics that represent all isolates in this group of the genus *Microbispora*. They contained *meso*-diaminopimelic acid in cell wall peptidoglycan. The whole cell hydrolysates offered ribose, madurose, galactose and glucose, corresponded to pattern B (M. P. Lechevalier & Lechevalier, 1970). Predominant menaquinones were MK-9(H_2), MK-9(H_4) and MK-9(H_6). The phospolipid profile of representative isolate, CR1-09 composed of diphosphotidylglycerol (DPG), hydroxylated phosphotidylethanolamine (OH-PE), phosphotidylinositolmannosides (PIMs), phosphotidylglycerol (PG), phosphatidylethanolamine (PE), glucosamine-containing phospholipid (GluNu), phisphatidylinositol (PI) and NPPG that corresponded to pattern IV (Lechevalier *et al.,* 1977), shown in Figure 4.13. The major cellular fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{15:0}$, $C_{16:0}$, iso-$C_{16:0}$, $C_{17:0}$ and 10-methyl $C_{17:0}$ corresponded to pattern III (Kroppenstedt, 1985), as in Table 4.17. The DNA G+C contents were 72-73 mol%. They utilized different carbon sources as shown in Table 4.16.

On the basis of 16S rRNA gene sequence analysis and phylogenetic tree investigation indicated that CR1-01, CR1-02, CR1-04, CR1-06, CR1-07, CR1-08 and CR1-11 were closely related to *Microbispora rosea* subsp. *rosea* JCM 3006^T (98.83–99.71% similarity), while CR1-03, CR1-05, CR1-09 and CR1-10 were closely related to *M.*

hainanensis JCM 19666^{T} (98.98-99.93 % similarity). OH1-01 was closely related to *M. corallina* JCM 10267^{T} (98.96 % similarity), while OH1-02 was closely related to *M. hainanensis* JCM 19666^{T} (99.24% similarity). PG1-01 and PG1-02 were closely related to *M. mesophila* JCM 3151^{T} (99.63 and 99.70% similarity, respectively). The neighbor-joining tree showed relative between some isolates and related members of genus *Microbispora* (Figure 4.14). Some isolates were possibly identified as new species in the genus *Microbispora*, however they should be confirmed by DNA-DNA hybridization (Stackebrandt & Schumann, 2006).

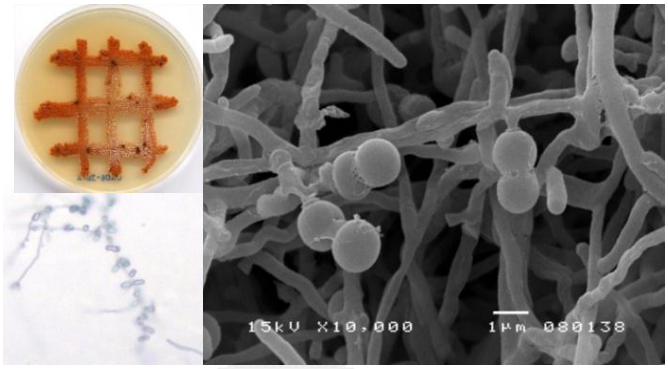


Figure 4.12 Photomicrograph (500x) and scanning electron micrograph of *Microbispora* sp. CR1-06 on ISP no. 2 agar at 30 °C for 14 days.

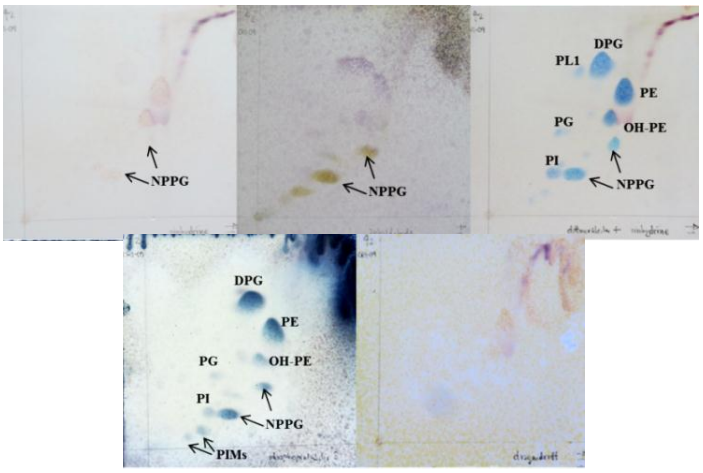


Figure 4.13 Polar lipid profiles of CR1-09

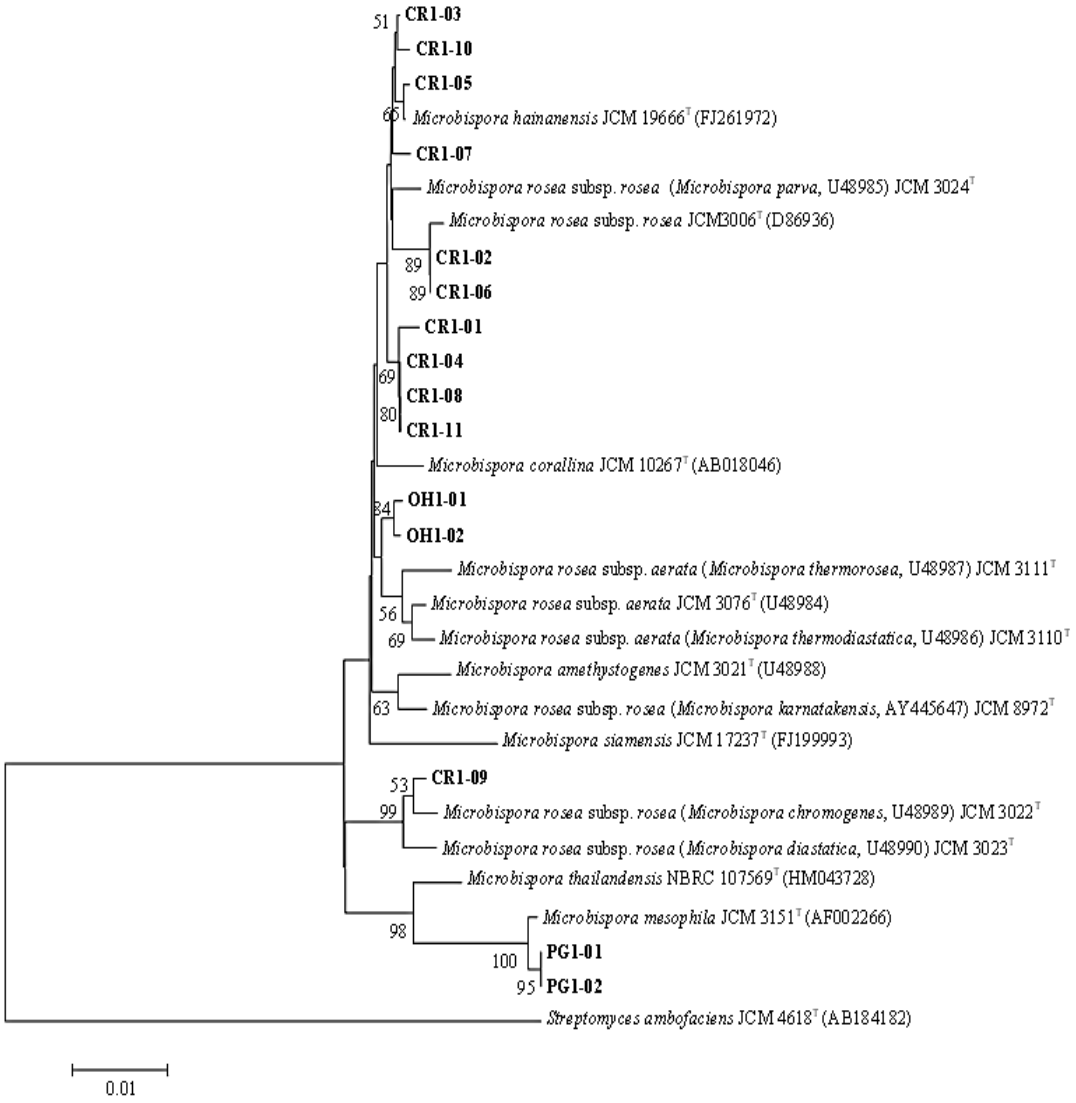


Figure 4.14 Neighbor-joining tree based on partial 16S rRNA gene sequences, showing *Microbispora* isolates and related type strains.

Table 4.15 Cultural characteristics of the representative isolates and related *Microbispora* species

Media	CR1-01	CR1-03	CR1-04	CR1-07	CR1-08	CR1-09
Growth on **ISP no.2**	Good	Moderate	Good	Good	Good	Good
Color of upper surface	Pinkish white9	Very dark red17	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9
Color of reverse surface	Strong yellow brown74	Dark grayish red20	Strong brown55	Strong brown55	Strong yellow brown74	Blackish red21
Soluble pigment	-	-	-	-	-	Very dark purple225
Growth on **ISP no.3**	Good	Good	Good	Good	Good	Moderate
Color of upper surface	Pale yellowish pink31	Pinkish white9	Pale yellowish pink31	Pale yellowish pink31	Pale yellowish pink31	Light pink4
Color of reverse surface	Light orange52	Strong yellowish pink26	Light orange52	Light orange52	Light orange52	Strong brown55
Soluble pigment	-	-	-	-	-	Moderate purple223
Growth on **ISP no.4**	Moderate	Poor	Moderate	Moderate	Moderate	Poor
Color of upper surface	Pinkish white9	Light olive brown94	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9
Color of reverse surface	Pale orange yellow73	Moderate orange yellow71	Pale orange yellow73	Pale orange yellow73	Pale orange yellow73	Vivid yellowish pink25
Soluble pigment	-	-	-	-	-	-
Growth on **ISP no.5**	Moderate	Moderate	Moderate	Moderate	Moderate	Poor
Color of upper surface	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9
Color of reverse surface	Brownish orange54	Dark orange yellow72	Deep orange yellow69	Deep orange yellow69	Brownish orange54	Pale yellowish pink31
Soluble pigment	-					Pale purple227
Growth on **ISP no.6**	Poor	Poor	Poor	Poor	Poor	Poor
Color of upper surface	Pinkish white9	Dark brown59	Pinkish white9	Pinkish white9	Pinkish white9	Dark grayish brown62
Color of reverse surface	Moderate orange yellow71	Brownish gray64	Moderate orange53	Moderate orange53	Moderate orange yellow71	Dark orange yellow72
Soluble pigment	-	-	-	-	-	-
Growth on **ISP no.7**	Good	Moderate	Good	Moderate	Good	Moderate
Color of upper surface	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9
Color of reverse surface	Moderate orange yellow71	Grayish yellow brown	Moderate orange53	Moderate orange53	Moderate orange yellow71	Moderate yellow brown77
Soluble pigment	-	-	-	-	-	-
Growth on **GluA**	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Color of upper surface	Pale pink7	Pinkish white9	Pinkish white9	Pinkish white9	Pale pink7	Pinkish white9
Color of reverse surface	Dark orange yellow72	Light yellowish brown76	Light olive brown95	Dark orange yellow72	Dark orange yellow72	Yellowish white92
Soluble pigment	-	-	-	-	-	-
Growth on **Czk**	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Color of upper surface	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9
Color of reverse surface	Pale orange yellow73	Yellowish white92	Pale orange yellow73	Pale orange yellow73	Pale orange yellow73	Pinkish gray10
Soluble pigment	-	-	-	-	-	-
Growth on **NA**	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Color of upper surface	Pale orange yellow73	Pinkish white9	Pale orange yellow73	Pale orange yellow73	Pale orange yellow73	Pinkish white9
Color of reverse surface	Moderate orange53	Deep yellowish brown75	Moderate orange53	Moderate orange53	Moderate orange yellow71	Moderate orange53
Soluble pigment	-	-	-	-	-	-

Table 4.15 Cultural characteristics of the representative isolates and related *Microbispora* species (continued)

Media	CR1-10	CR1-11	OH1-01	JCM 3006^T	JCM 3023^T	JCM 17237^T	JCM 19666^T
Growth on **ISP no.2**	Moderate	Good	Moderate	Moderate	Good	Good	Moderate
Color of upper surface	Very dark red17	Pinkish white9	Vivid greenish yellow	Pinkish white9	Pale yellowish pink31	Pinkish white9	Very dark red17
Color of reverse surface	Dark grayish red20	Strong yellow brown74	Grayish yellow90	Strong brown55	Vivid orange48	Deep orange yellow69	Dark grayish red20
Soluble pigment	-	-	-	-	-	-	-
Growth on **ISP no.3**	Good	Good	Moderate	Good	Good	Good	Good
Color of upper surface	Pinkish white9	Pale yellowish pink31	Pale greenish yellow	Pale yellowish pink31	Pale yellowish pink31	Pale pink7	Pinkish white9
Color of reverse surface	Strong yellowish pink26	Light orange52	Light yellow86	Light orange52	Pale orange yellow73	Light orange yellow70	Vivid yellowish pink25
Soluble pigment	-	-	-	-	-	Light greenish yellow84	-
Growth on **ISP no.4**	Poor	Moderate	Good	Moderate	Good	Good	Poor
Color of upper surface	Light olive brown94	Pinkish white9	Pale greenish yellow	Pinkish white9	Light yellowish pink28	Pinkish white9	Moderate orange yellow7
Color of reverse surface	Moderate orange yellow7	Pale orange yellow73	Pale yellow89	Pale orange yellow73	Vivid yellowish pink25	Dark reddish gray23	Moderate orange
Soluble pigment	-	-	-	-	-	-	-
Growth on **ISP no.5**	Moderate	Moderate	Poor	Good	Good	Good	Moderate
Color of upper surface	Pinkish white9	Pinkish white9	Pale yellow89	Pinkish white9	Pinkish white9	Pinkish white9	Pinkish white9
Color of reverse surface	Dark orange yellow72	Brownish orange54	Brilliant yellow83	Brownish orange54	Dark grayish red20	Pale orange yellow73	Dark orange yellow72
Soluble pigment	-	-	-	-	Brownish pink33	-	-
Growth on **ISP no.6**	Poor	Poor	Poor	Moderate	Good	Moderate	Poor
Color of upper surface	Dark brown59	Pinkish white9	Pale yellow89	Pinkish white9	Pinkish white9	Pale orange yellow73	Dark brown59
Color of reverse surface	Brownish gray64	Moderate orange yellow	Pale yellow89	Moderate orange yellow7	Moderate yellow87	Strong yellow84	Brownish gray64
Soluble pigment	-	-	-	-	-	-	-
Growth on **ISP no.7**	Moderate	Moderate	Moderate	Good	Good	Good	Moderate
Color of upper surface	Pinkish white9	Pinkish white9	Yellowish white92	Pinkish white9	Pale pink7	Pinkish white9	Pinkish white9
Color of reverse surface	Grayish yellow brown	Moderate orange yellow7	Brilliant yellow83	Moderate orange yellow7	Dark yellowish brown78	Light orange52	Grayish yellow brown
Soluble pigment	-	-	-	-	Light yellowish brown76	-	-
Growth on **GluA**	Moderate	Moderate	Good	Moderate	Good	Good	Moderate
Color of upper surface	Pinkish white9	Pinkish white9	Yellowish white92	Pale pink7	Pale pink7	Pale pink7	Pinkish white9
Color of reverse surface	Light yellowish brown76	Dark orange yellow72	Light greenish yellow	Dark orange yellow72	Dark grayish red20	Yellowish white92	Light yellowish brown76
Soluble pigment	-	-	-	-	Light yellowish pink28	-	-
Growth on **Czk**	Moderate	Moderate	Good	Moderate	Moderate	Good	Moderate
Color of upper surface	Pinkish white9	Pinkish white9	Yellowish white92	Pinkish white9	Light orange 52	Pinkish white9	Pinkish white9
Color of reverse surface	Yellowish white92	Pale orange yellow73	Pale greenish yellow	Pale orange yellow73	Pale yellow89	Pale greenish yellow	Yellowish white92
Soluble pigment	-	-	-	-	-	-	-
Growth on **NA**	Moderate	Moderate	Good	Moderate	Good	Good	Moderate
Color of upper surface	Pinkish white9	Pale orange yellow73	Pale greenish yellow	Pale orange yellow73	Pinkish white9	Pinkish white9	Pinkish white9
Color of reverse surface	Deep yellowish brown75	Moderate orange yellow7	Light greenish yellow	Moderate orange53	Moderate orange yellow7	Moderate yellow87	Deep yellowish brown75
Soluble pigment	-	-	-	-	-	-	Pale yellow89

Table 4.16 Physiological and biochemical characteristics of *Microbispora* isolates

Characteristics	CR1-01	CR1-04	CR1-07	CR1-08	CR1-09	CR1-11	OH1-01	JCM3006^T	JCM3022^T	JCM3023^T	JCM10267^T	JCM17237^T	JCM19666^T
% NaCl max	2	3	3	3	3	3	2	2	1	2	-	2	2
pH	6-11	6-10	6-10	6-10	6-10	6-10	6-10	6-11	6-11	6-11	6-9	5-11	6-10
Temperature (°C)	20-45	20-45	20-40	20-45	20-45	20-45	20-45	20-45	20-45	20-45	20-37	20-45	20-45
Peptonization of milk	-	-	-	-	+	-	-	-	-	+	+	+	+
Coagulation of milk	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	-	-	+	+	+	-	+	+	-	+	-	+
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	+	-
Utilization of:													
L-Arabinose	+	-	-	-	-	-	w	+	-	w	+	w	w
D-Galactose	-	-	-	-	+	-	-	-	w	w	w	w	-
D-Glucose	+	+	+	+	+	+	+	+	w	+	+	+	w
Glycerol	-	-	+	-	-	-	+	-	-	w	+	w	w
myo-Inositol	-	+	w	+	w	w	+	-	+	+	+	+	+
Lactose	-	+	+	-	-	-	-	-	-	w	+	w	w
D-Mannitol	-	+	+	-	w	-	+	-	-	w	w	w	w
D-Raffinose	-	+	+	+	+	+	+	-	+	+	w	+	+
L-Rhamnose	-	+	+	w	-	-	+	-	-	w	w	w	+
D-Sorbitol	w	+	+	w	+	+	w	w	+	+	w	w	+

+, positive; -, negative; w, weakly positive

Microbispora rosea subsp. *rosea* JCM 3006^T, *M. chromogenes* (*M. rosea* subsp. *rosea*) JCM 3022^T, *M. diastatica* (*M. rosea* subsp. *rosea*) JCM 3023^T, *M. corallina* JCM 10267^T, *M. siamensis* JCM 17237^T and *M. hainanensis* JCM 19666^T

Table 4.17 Cellular fatty acids of *Microbispora* isolates and related type strain

Fatty acid[a]	CR1-01	CR1-04	CR1-07	CR1-08	CR1-09	CR1-11	OH1-01	JCM4219^T	JCM4219^T	JCM4219^T	JCM4219^T	JCM4219^T	JCM4219^T
Straight-chain fatty acids													
$C_{14:0}$	-	1.8	1.6	1.0	1.6	1.6	-	1.4	1.5	-	2.4	1.4	2.1
$C_{15:0}$	9.8	7.6	9.8	6.4	8.7	6.3	4.2	6.7	5.6	-	-	-	-
$C_{16:0}$	7.3	12.2	9.5	5.8	9.9	11.4	9.7	13.8	6.7	5.8	18.5	11.6	22.3
$C_{17:0}$	7.1	8.5	6.3	3.8	5.3	8.1	14.3	4.8	2.2	8.4	5.6	9.6	4.1
$C_{18:0}$	-	1.2	-	1.2	-	1.2	2.9	1.2	-	-	1.9	1.6	3.2
Unsaturated fatty acids													
$C_{17:1}$ ω6c	-	-	-	6.2	-	3.8	2.8	-	-	7.1	4.5	4.8	1.0
$C_{17:1}$ ω8c	2.2	4.0	2.5	5.6	2.0	3.7	2.5	4.6	4.7	3.0	2.0	3.6	3.2
Branched fatty acids													
iso-$C_{14:0}$	2.0	1.4	1.9	2.6	2.5	1.4	-	1.7	5.2	1.8	1.5	1.3	1.3
iso-$C_{15:0}$	4.0	7.3	4.0	8.7	5.8	5.6	5.5	2.0	6.0	8.1	7.0	9.2	5.2
anteiso-$C_{15:0}$	3.3	2.2	3.6	2.6	3.6	1.8	1.1	2.2	6.2	3.6	3.9	3.4	5.9
iso-$C_{16:0}$	40.5	29.5	34.7	28.2	34.0	29.2	23.8	36.4	32.8	33.5	22.6	21.6	20.2
iso-$C_{17:0}$	1.7	2.1	1.5	2.0	2.0	1.7	4.2	1.0	1.2	3.4	2.3	3.9	1.6
anteiso-$C_{17:0}$	4.0	2.5	3.3	2.6	4.1	2.0	4.2	2.8	3.4	3.0	3.0	4.1	3.9
10Me-$C_{16:0}$	3.5	4.9	4.2	4.5	4.8	5.1	4.7	5.4	5.7	-	-	-	-
10Me-$C_{17:0}$	8.7	6.6	9.3	9.4	8.0	6.6	10.3	7.9	6.2	6.6	3.0	8.0	4.0
Summed feature	1.7	3.8	2.1	3.5	2.2	2.8	2.3	4.1	5.0	6.1	3.6	3.0	4.9

[a]Values are percentages of total cellular fatty acids. -, not detected.

Microbispora rosea subsp. *rosea* JCM 3006^T, *M. chromogenes* (*M. rosea* subsp. *rosea*) JCM 3022^T, *M. diastatica* (*M. rosea* subsp. *rosea*) JCM 3023^T, *M. corallina* JCM 10267^T, *M. siamensis* JCM 17237^T and *M. hainanensis* JCM 19666^T

4.3.6 Characteristics of *Nonomuraea* (Group VI)

The isolate PA1-02, PA1-10 and PA1-11 were assigned in the genus *Nonomuraea* based on phenotypic, genotypic and chemotaxonomic characteristics (P. Kämpfer, 2012). The representative isolate, PA1-10 formed rugose spore chain on extensively branch substrate mycelium and aerial mycelium when cultivated on ISP no. 2 agar for 14 days, as shown in Figure 4.15. The cultural characteristics on ISP no.2 of PA1-02 and PA1-11 showed while aerial mycelium on deep red substrate mycelium while PA1-10 showed pinkish white aerial mycelium on pale orange yellow substrate mycelium. They grew on 1 to 5 % NaCl, on pH 6 to 9 and at 20 to 37 °C. Peptonization of milk, gelatin liquefaction, starch hydrolysis and nitrate reduction were shown as in Table 4.18. The isolate PA1-10 utilized L-arabinose, D-glucose, glycerol, *myo*-inositol, Lactose, D-mannitol, L-raffinose, L-rhamnose and D-sorbitol as sole carbon sources but not use D-galactose while the others utilized different carbon sources, shown in Table 4.18. The isolate PA1-10 contained *meso*-diaminopimelic acid in cell wall peptidoglycan. It contained ribose, mannose, madurose, galactose and glucose as the reducing sugars of whole cell hydrolysates which corresponded to pattern B (M. P. Lechevalier & Lechevalier, 1970). Predominant menaquinones were MK-9(H_2), MK-9(H_4) and MK-9(H_6). The major cellular fatty acids were iso-$C_{15:0}$, $C_{15:0}$, $C_{17:1\omega6c}$, $C_{17:1\omega8c}$, iso-$C_{16:0}$, $C_{16:0}$, 10-methyl $C_{16:0}$ anteiso-$C_{17:0}$ and 10-methyl $C_{17:0}$ that corresponded to pattern III (Kroppenstedt, 1985). DNA G+C content was 72 mol%.

On the basis of 16S rRNA gene sequence analysis and phylogenetic tree investigation indicated that PA1-10 was closely related to *Nonomuraea candida* JCM 15928^T (98.31% similarity), while PA1-02 and PA1-11 were closely related to *N. monospora* JCM 16114^T (99.63% and 99.70% similarity, respectively). The neighbor-joining tree showed relative between isolate PA1-02, PA1-10, PA1-11 and other members of genus *Nonomuraea* (Figure 4.16). The isolate PA1-10 was possibly identified as new species in the genus *Nonomuraea* however it should be confirmed by DNA-DNA hybridization (Stackebrandt & Schumann, 2006).

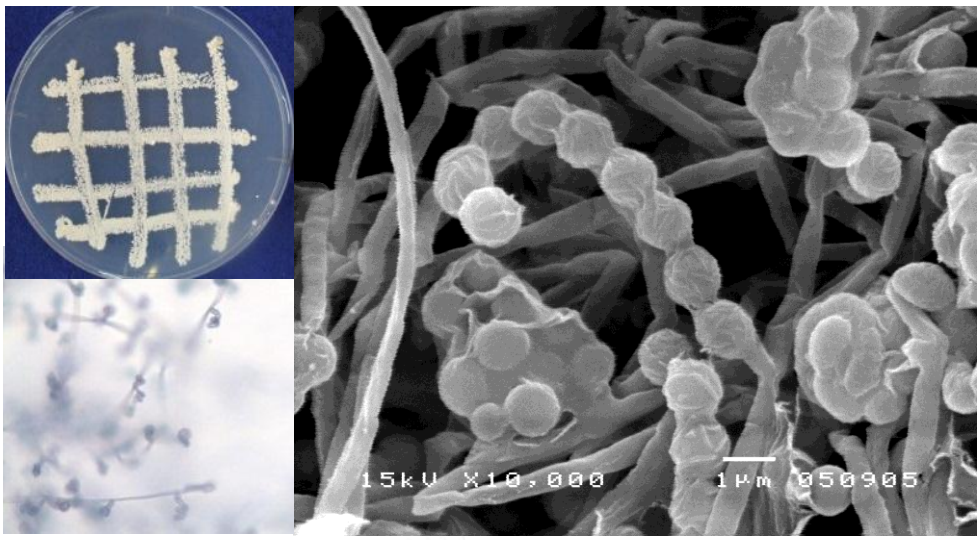


Figure 4.15 Photomicrograph (500x) and scanning electron micrograph of *Nonomuraea* sp. PA1-10 on ISP no. 2 agar at 30 °C for 14 days.

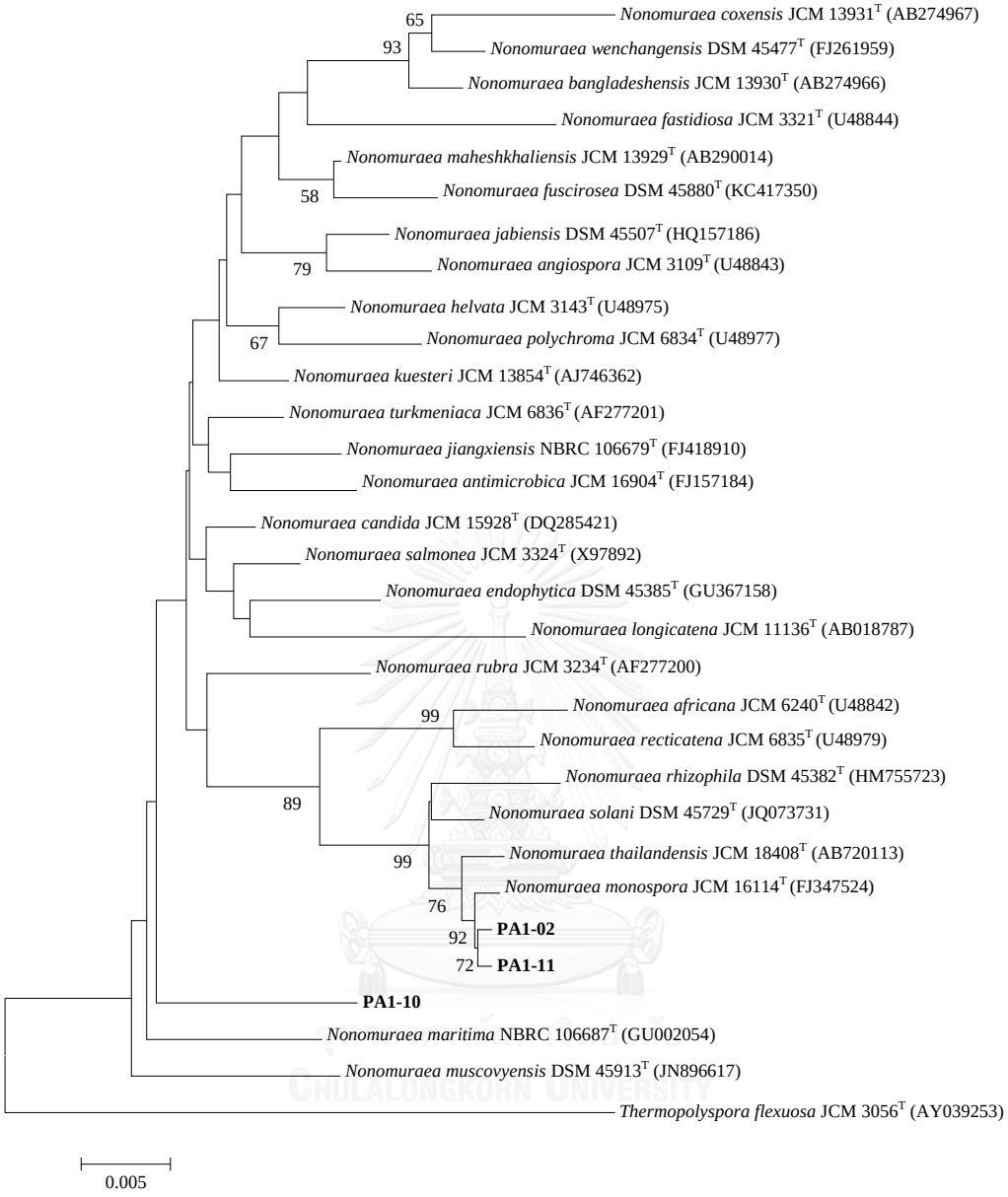


Figure 4.16 Neighbor-joining tree based on partial 16S rRNA gene sequences, showing *Nonomuraea* isolates and related type strains.

Table 4.18 Physiological and biochemical characteristics of *Nonomuraea* isolates

characteristics	PA1-02	PA1-04	PA1-08	PA1-09	PA1-10	PA1-11
% NaCl max	2	2	2	2	5	2
pH	6-9	6-9	6-9	6-9	6-9	6-9
Temperature (°C)	20-37	20-37	20-37	20-37	20-37	20-37
Peptonization of milk	+	+	+	+	+	+
Coagulation of milk	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	-	+
Nitrate reduction	-	-	-	-	+	-
Starch hydrolysis	+	+	+	+	-	+
Utilization of:						
L-Arabinose	+	+	+	+	+	+
D-Galactose	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+
myo-Inositol	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
D-Mannitol	+	+	+	+	w	+
D-Raffinose	-	-	-	-	w	-
L-Rhamnose	+	+	+	+	w	+
D-Sorbitol	w	w	w	w	w	w

+, positive; w, weakly positive; -, negative

4.4 Screening for antimicrobial activity

The fermented ISP no.2 broth of forty-seven endophytic actinomycete strains was screened for antimicrobial activity by using disc diffusion method (Bauer-Kirby test). The result was shown in Table 4.19. The isolate CR1-01 and CR1-08 showed inhibitory effect against *S. aureus* ATCC 25923 (10.7-11.2 mm) and *K. rhizophila* ATCC 9341 (10.4-10.8 mm) while CR1-05 showed inhibitory effect against *K. rhizophila* ATCC 9341 (10.0 mm). The isolate ST1-02 and ST1-05 showed inhibitory effect against *C. albican* ATCC 10231 (10.2-11.1 mm). The antimicrobial activity of ethyl acetate extract (10 mg/ml in MeOH) of isolate CR1-01 and CR1-08 showed inhibitory effect against *S. aureus* ATCC 25923 (21.0-25.3 mm) and *K. rhizophila* ATCC 9341 (11.4-16.0 mm) while CR1-05 showed inhibitory effect against *K. rhizophila* ATCC 9341 (10.5 mm). The isolate ST1-02 and ST1-05 showed inhibitory effect against *C. albican* ATCC 10231 (11.4-11.8 mm), respectively as shown in Table 4.20 and Figure 4.17. The inhibition zone size from the isolates CR1-01, CR1-05 and CR1-08 were smaller than the inhibition zone size from ciprofloxacin (5 µg) but were closed to the inhibition zone size from chloramphenicol (30 µg) against *S. aureus* ATCC 25923. The antimicrobial activity of crude extract showed higher activity than activity of ISP no.2 fermented broth that the concentration of crude extract was probably higher than fermented broth. In this study, 5 µg ciprofloxacin was used as the positive control for antibacterial activity screening because it was a broad-spectrum antibiotic. In this study, only ISP no.2 medium was used as production medium, suggesting in other production media as A16, A11M or the medium comprised glucose (0.4%), yeast extract (0.4%), malt extract (1.0%) and $CaCO_3$ (0.1%) for comparison of the differentiated productive condition of each isolates should be included in the further studies.

Table 4. 19 Screening for antimicrobial activity of the fermented ISP no.2 broth of all isolates based on disc diffusion method

Isolate No.	Inhibition zone size of antimicrobial activity by disc diffusion method (mm)					
	S. aureus ATCC 25923	*B. Subtilis* ATCC 6633	*K. rhizophila* ATCC 9341	*E. coli* ATCC25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CR1-01	10.7	0	10.4	0	0	-
CR1-05	0	0	10.0	0	0	0
CR1-08	11.2	0	10.8	0	0	0
ST1-02	0	0	0	0	0	10.2
ST1-05	0	0	0	0	0	11.1
Ciprofloxacin (5 µg)	34.5	28.2	27.6	36.1	31.4	ND
Cycloheximide (50 µg)	ND	ND	ND	ND	ND	34.7

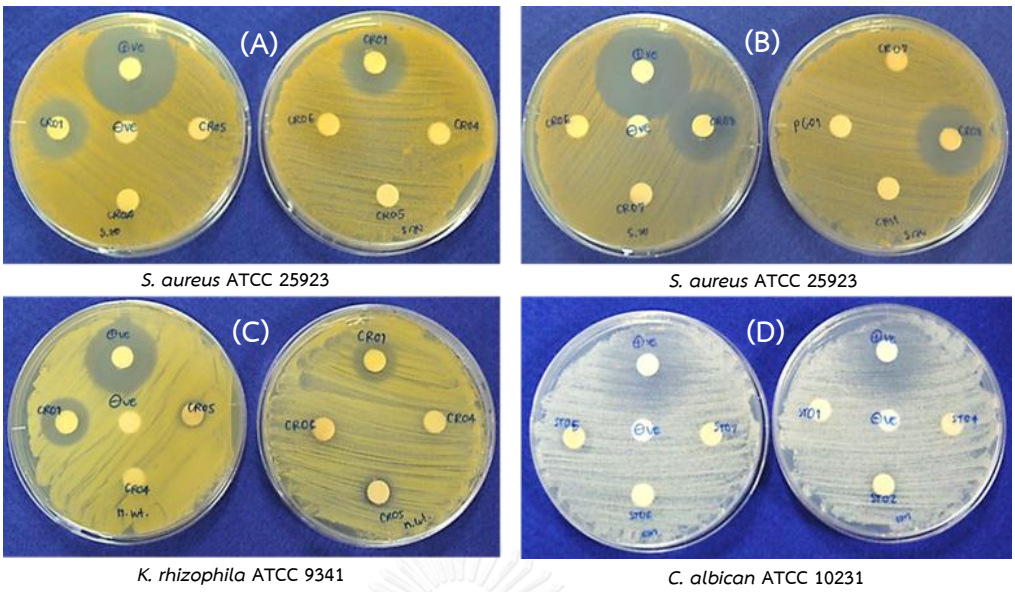
CR1-02 to CR1-04, CR1-06, CR1-07, CR1-09 to CR1-11, ST1-01, ST1-03, ST1-04, ST1-06 to ST1-10, OH1-01, OH1-02, PG1-01, PG1-02, PA1-01 to PA1-11, PP1-01 to PP1-06, VC1-01 to VC1-05 did not show inhibit tested microorganism.

ND; not determined

Table 4. 20 Screening for antimicrobial activity of EtOAc extract of all isolates based on disc diffusion method

Isolate No.	Inhibition zone size of antimicrobial activity by disc diffusion method (mm)					
	S. aureus ATCC 25923	*B. Subtilis* ATCC 6633	*K. rhizophila* ATCC 9341	*E. coli* ATCC25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CR1-01	21.0	0	11.4	0	0	0
CR1-08	25.3	0	16.0	0	0	0
CR1-05	0	0	10.5	0	0	0
ST1-02	0	0	0	0	0	11.4
ST1-05	0	0	0	0	0	11.8
The others	0	0	0	0	0	0
Ciprofloxacin (5 µg)	36.2	29.4	28.1	38.7	33.9	ND
Chloramphenicol (30 µg)	26.4	ND	34.5	ND	ND	ND
Cycloheximide (50 µg)	ND	ND	ND	ND	ND	34.2

ND; not determined



S. aureus ATCC 25923 *S. aureus* ATCC 25923

K. rhizophila ATCC 9341 *C. albican* ATCC 10231

Figure 4. 17 Antimicrobial activity of EtOAc extract of CR1-01 (A), CR1-08 (B), CR1-01 and CR1-05 (C), ST1-02 (D); positive control (top disc on left plate) and negative control (central disc on left plate).

4.5 Chemical profile of secondary metabolites

The forty-seven isolates were cultivated on ISP no. 2 and screened for antimicrobial activity. Five isolates, CR1-01, CR1-05, CR1-08 belonging to *Microbispora* sp. and ST1-02, ST1-05 belonging *to Streptomyces* sp., were selected to analyze the chemical constituents by using high performance liquid chromatography (HPLC) equipped with UV-VIS spectrophotometry due to giving positive biological activity of isolate. For the EtOAc extract of isolate CR1-01 showed three unknown peaks (in red circle) at RT 7.74, 8.25 and 8.78 min while CR1-08 showed three unknown peaks (in red circle) at RT 7.72, 8.26 and 8.78 min (Figures 4.18-4.20). Scanning spectrum profiles (190-600 nm) of the unknown peaks at RT 7.74, 8.25 and 8.78 minutes from *Microbispora* sp. isolate CR1-01 (Figure 4.21 (A)) were similar with spectrum profiles (190-600 nm) of the unknown peaks at RT 7.72, 8.26 and 8.78 min from *Microbispora* sp. isolate CR1-08 (Figure 4.21 (B)). In this study, the chemical profiles of the isolates CR1-01 and CR1-08 showed that their three unknown peaks were at similar retention

times. Therefore, these crude extracts deserved purification and identification of chemical constituents.

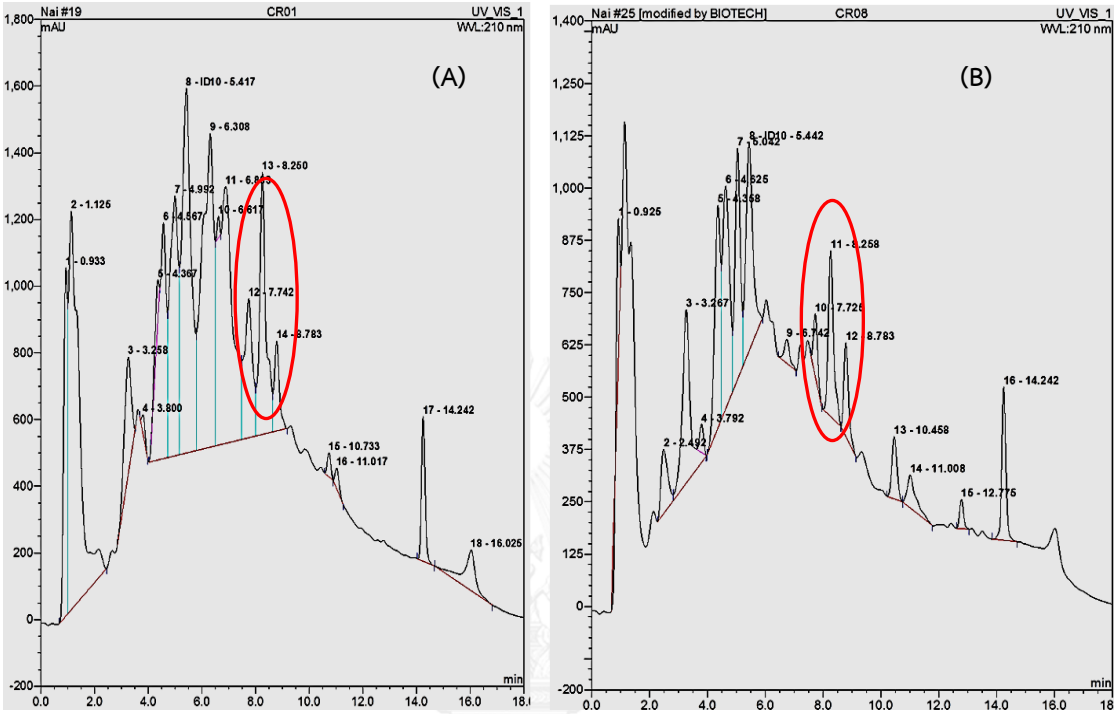


Figure 4. 18 Chemical profiles (λ=210 nm) of the EtOAc crude extract from *Microbispora* sp. A) All peaks of CR1-01 and B) All peaks of CR1-08

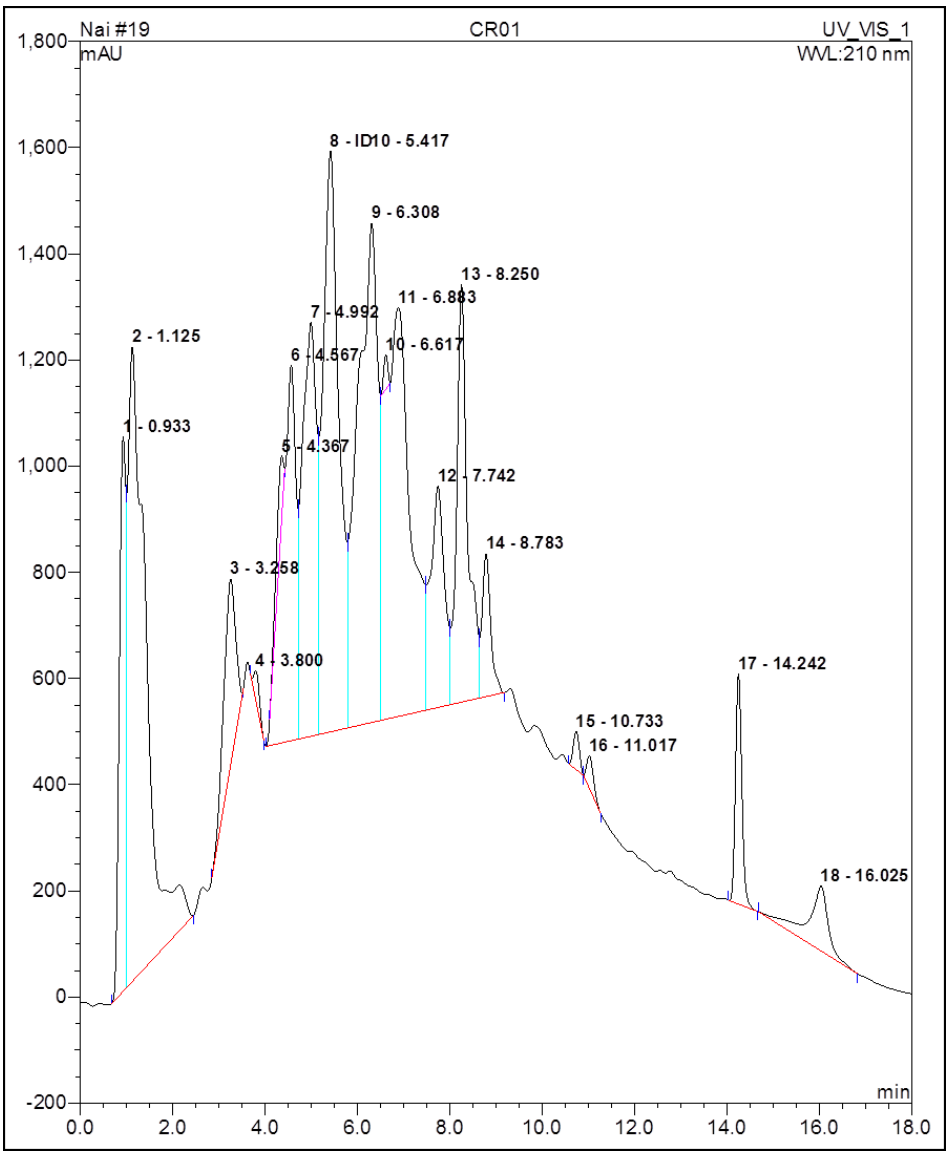


Figure 4. 19 Chemical profiles by using HPLC/UV-VIS (at λ = 210 nm) of the EtOAc crude extract from *Microbispora* sp. isolate CR1-01

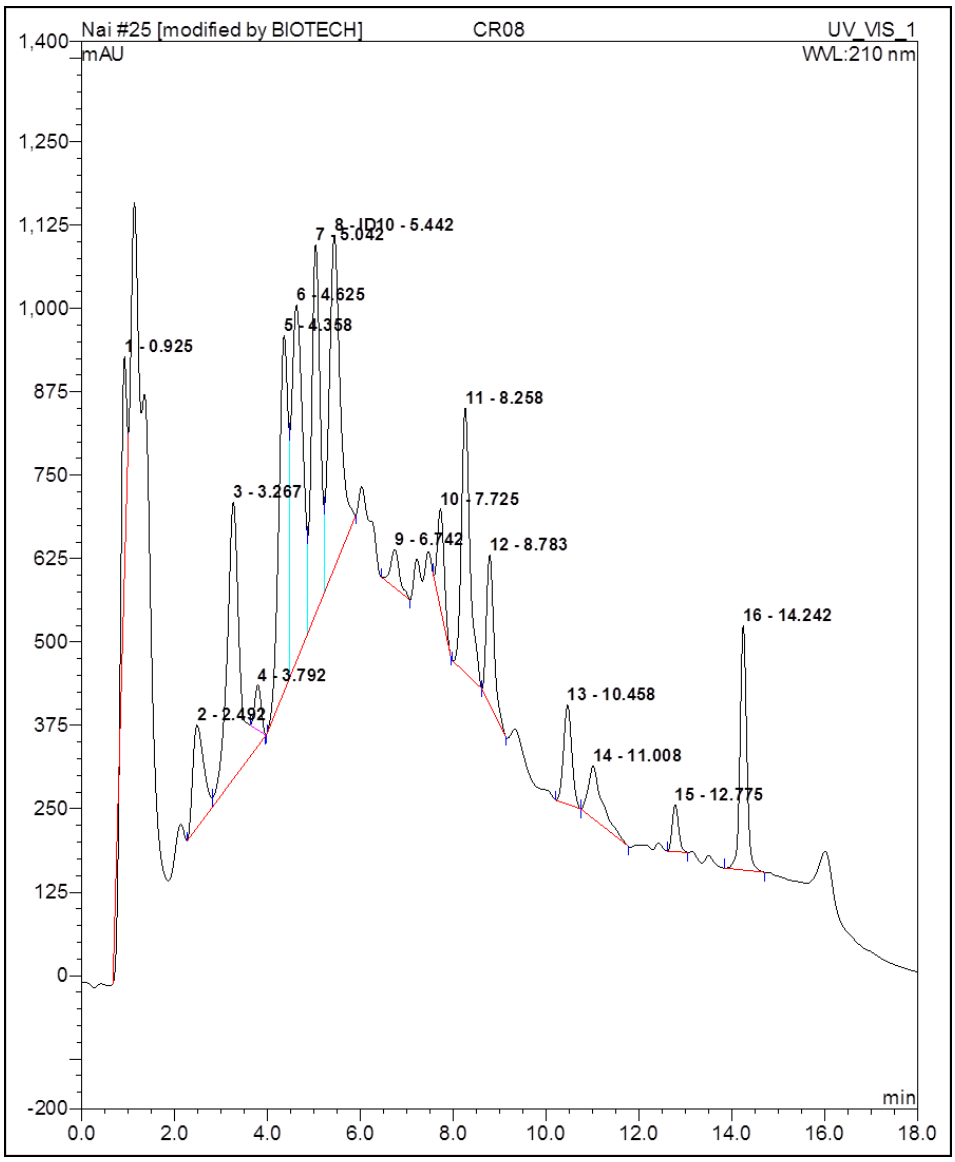


Figure 4. 20 Chemical profiles by using HPLC/UV-VIS (at λ = 210 nm) of the EtOAc crude extract from *Microbispora* sp. isolate CR1-08

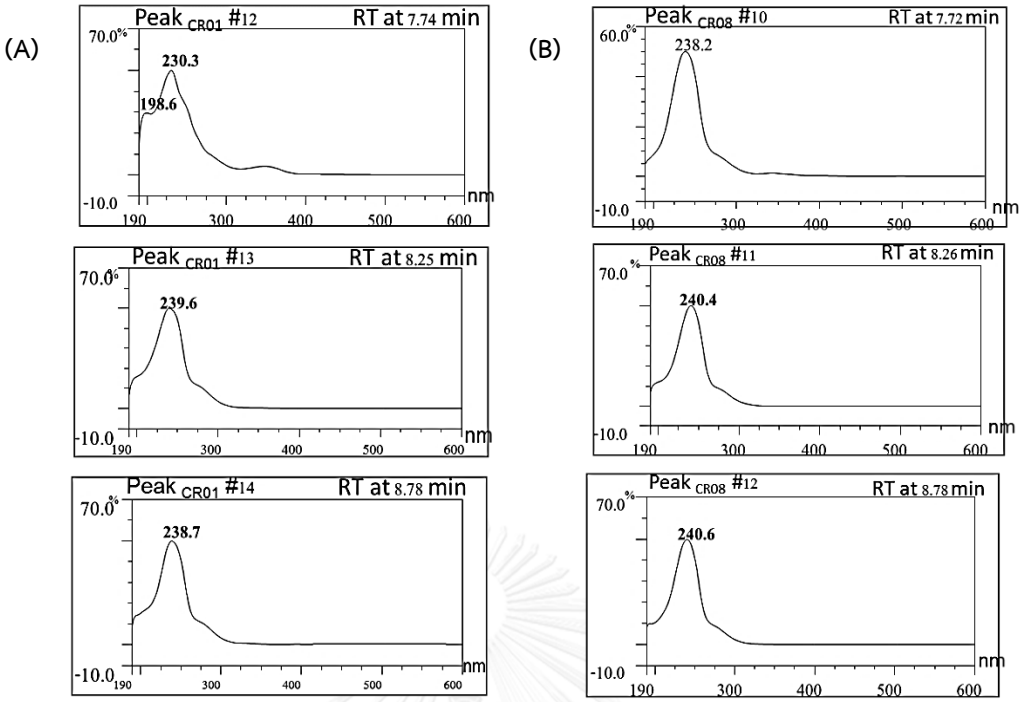


Figure 4. 21 Spectrum profiles (190-600 nm) of the interested peaks from *Microbispora* sp. A) Three peaks of Isolate CR1-01 at RT 7.74, 8.25 and 8.78 min and B) Three peaks of Isolate CR1-08 at RT 7.72, 8.26 and 8.78 min.

CHAPTER V

CONCLUSION

This study, forty-seven actinomycetes distributed in seven medicinal plants in Thailand. Thirty-seven representative isolates were selected for 16S rRNA gene sequence analysis and were identified as *Streptomyces* (13 isolates from *Stemona* sp. and *Phyllanthus amarus*) and *Amycolatopsis* (1 isolate from *Stemona* sp.), *Nocardia* (2 isolates from *Stemona* sp.and *Phyllanthus amarus*), *Micromonospora* (3 isolates from *Phyllanthus pulcher*), *Microbispora* (15 isolates from *Catharanthus roseus*, *Ophiorrhiza* sp. and *Pseuderanthemum graciliflorum*) and *Nonomuraea* (3 isolates from *Phyllanthus amarus*).

Streptomyces (Group I, 13 isolates), ST1-01 from *Stemona* sp. was identified as *Streptomyces thermocarboxydus* JCM 10368^{T} (99.85% similarity), while ST1-02, ST1-03, ST1-04, ST1-05 and ST1-07 were identified as *S. albidoflavus* JCM 4446^{T} (99.78–99.91% similarity). ST1-09 and ST1-10 from *Stemona* sp. were identified as *S. corchorusii* JCM 4286^{T} (99.35-99.55% similarity), while VC1-01 to VC1-04 from *Vernonia cinerea* were identified as *S. coerulescens* JCM 4360^{T} (99.04-99.41% similarity). Strain PA1-07 from *Phyllanthus amarus*was was closely related to *S. curacoi* JCM 4219^{T} (98.75% similarity), possibly identified as a new species of group I. The representative isolate PA1-07 contained *LL*-diaminopimelic acid in cell wall peptidoglycan and ribose, mannose, madurose, galactose and glucose as whole cell sugars. Predominant menaquinones were MK-9(H_6) and MK-9(H_8). The major cellular fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$, and anteiso-$C_{17:0}$. The DNA G+C content was 71 mol%.

Amycolatopsis (Group II, 1 isolate), ST1-08 was closely related to *Amycolatopsis pretoriensis* JCM 12673^{T} (99.17% similarity), while the level of DNA-DNA relatedness with its closest related species was less than 70%. ST1-08 contained *meso*-diaminopimelic acid in cell wall peptidoglycan andrhamnose, ribose, mannose, glucose, galactose and arabinose as whole cell sugars. Predominant menaquinone was MK-9(H_4). Phospolipid profile was hydroxylated phosphotidylethanolamine (OH-

PE), diphosphotidylglycerol (DPG), phisphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphotidylglycerol (PG). Major cellular fatty acids were iso-$C_{16:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$, $C_{17:1}$, $C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{15:0}$, $C_{15:0}$, $C_{17:0}$,iso-$C_{14:0}$, $C_{15:1}$ and 10-methyl $C_{17:0}$. The DNA G+C content was 71 mol%. The chemotaxonomic, phenotypic and genotypic characteristics revealed that isolate ST1-08 which isolated from *Stemona* sp. was a novel species and named *Amycolatopsis stemonae* (type strain ST1-08^T=JCM 30050^T=PCU 339^T =TISTR 2278^T) is proposed.

Nocardia (Group III, 2 isolates), PA1-03 from *Phyllanthus amarus* was identified as *N. asiatica* JCM 11892^T (100% similarity), while ST1-06 from *Stemona* sp. was closely related to *Nocardia araoensis* JCM 12118^T (99.03% similarity) that was possibly identified as a new species of group III. The isolate ST1-06 contained *meso*-diaminopimelic acid in cell wall peptidoglycanand ribose, mannose, arabinose, galactose and glucose as whole cell hydrolysates. Predominants isoprenoid quinone were 2,3-epoxy MK-8 (H_4-ωcycl) and MK-8 (H_4-ωcycl). Phospholipid wasphosphotidylinositolmannosides (PIMs), phisphatidylinositol (PI), phosphatidyl-ethanolamine (PE), diphosphotidylglycerol (DPG) and phosphotidylglycerol (PG). Predominant fatty acids were $C_{16:0}$, $C_{18:0}$, $C_{18:1\omega9c}$ and 10-methyl $C_{18:0}$.The DNA G+C content was 71 mol%

Micromonospora (Group IV, 3 isolates), PP1-01, PP1-03 and PP1-04 from *Phyllanthus pulcher*were identified as *Micromonospora echinospora*JCM 3073^T (99.54-99.71% similarity). The representative isolate PP1-03 contained *meso*-diaminopimelic acid in cell wall peptidoglycan and ribose, mannose, arabinose, galactose, xylose and glucose as whole cell sugars. Major menaquinones were MK-9(H_4), MK-9(H_6), MK-9(H_8), MK-10(H_4), MK-10(H_6) and MK-10(H_8). Phospholipid profile composed of diphosphotidylglycerol (DPG), phosphotidylglycerol (PG), phosphatidylethanolamine (PE), phosphotidylinositolmannosides (PIMs) and phisphatidylinositol (PI) but not phosphotigylcholine (PC). Predominant of fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{17:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, $C_{17:1\omega8c}$ and 10-methyl $C_{17:0}$. The DNA G+C contents was 73 mol%.

Microbispora (Group V, 15 isolates), CR1-01, CR1-02, CR1-04, CR1-06, CR1-07, CR1-08 and CR1-11 from *Catharanthus roseus* were closely related to *Microbispora rosea* subsp. *rosea* JCM 3006^T (98.83–99.71% similarity), while CR1-03, CR1-05, CR1-09 and CR1-10 were closely related to *M. hainanensis* JCM 19666^T (98.98-99.93% similarity). OH1-01 from *Ophiorrhiza* sp.was closely related to *M. corallina* JCM 10267^T (98.96% similarity), while OH1-02 was identified as *M. hainanensis* JCM 19666^T (99.24% similarity). PG1-01 and PG1-02 from *Pseuderanthemum graciliflorum* were identified as *M. mesophila* JCM 3151^T (99.63 and 99.70% similarity, respectively).

Isolates CR1-01, CR1-04, CR1-07, CR1-08, CR1-09, CR1-11 and OH1-01 contained *meso*-diaminopimelic acid in cell wall peptidoglycan. Whole cell hydrolysates offered ribose, madurose, galactose and glucose. Predominant menaquinones were MK-9(H_2), MK-9(H_4) and MK-9(H_6). Phospholipid profile was composed ofdiphosphotidylglycerol (DPG), hydroxylated phosphotidyl-ethanolamine (OH-PE), phosphotidylglycerol (PG), phosphotidylinositolmannosides (PIMs), phosphatidylethanolamine (PE), glucosamine-containingphospholipid (GluNu) and phisphatidylinositol (PI). Major cellular fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{15:0}$, $C_{16:0}$, iso-$C_{16:0}$, $C_{17:0}$ and 10-methyl $C_{17:0}$. The DNA G+C contents were 72-73 mol%. The isolates CR1-01, CR1-04, CR1-07, CR1-08, CR1-09 and OH1-01 were possibly identified as new species of group V based on the phenotypic, chemotaxonomic and genotypic characteristics.

Nonomuraea (Group VI, 3 isolates), PA1-10 from *Phyllanthus amarus*was closely related to *Nonomuraea candida* JCM 15928^T (98.31% similarity), while PA1-02 and PA1-11 were identified as *N. monospora* JCM 16114^T (99.63% and 99.70% similarity, respectively). Isolate PA1-10 contained *meso*-diaminopimelic acid in cell wall peptidoglycan and ribose, mannose, madurose, galactose and glucose as whole cell hydrolysates. Predominant menaquinones were MK-9(H_2), MK-9(H_4) and MK-9(H_6). The major cellular fatty acids were iso-$C_{15:0}$, $C_{15:0}$, $C_{17:1\omega6c}$, $C_{17:1\omega8c}$, iso-$C_{16:0}$, $C_{16:0}$, 10-methyl $C_{16:0}$ anteiso-$C_{17:0}$ and 10-methyl $C_{17:0}$. The DNA G+C content was 72 mol%. The isolate PA1-10 was possibly identified as a new species of group VI by 16S rRNA gene sequence analysis. All the new species of isolates in this study are required

further studies on DNA-DNA hybridization and other polyphasic taxonomic characteristics.

The determination of antimicrobial activity tests of the crude extracts revealed that 5 isolates belonging to *Microbispora* sp. and *Streptomyces* sp. produced the bioactive secondary metabolites. CR1-01 and CR1-08 showed the antimicrobial activity against Gram-positive microorganisms (*S. aureus* ATCC 25923 and *K. rhizophila* ATCC 9341) while CR1-05 exhibited antimicrobial activity against *K. rhizophila* ATCC 9341. ST1-02 and ST1-05 were actived against *C. albicans* ATCC 10231. The EtOAc extracts of the isolates CR1-01 and CR1-08 were obtained from the fermented ISP no.2 broth by using ethyl acetate. These crude extract showed three interesting peaks that exhibited antimicrobial activity against to *S. aureus* ATCC 25923 and *K. rhizophila* ATCC 9341. These peaks will be further purified and identified these chemical components.

As the results of this research, the diverse actinomycetes are distributed in all parts of plant sample such as root, stem or leaf, that are sources of novel actinomycetes and secondary bioactive metabolites. However there are still many strains to be discovered for new taxon and new secondary metabolites.

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APPENDICES

APPENDIX A
CULTURE MEDIA

Almost of media were sterilized in autoclave at 121 °C, 15 pounds pressure for 15 min, except skim milk and media for carbon utilization test that were sterilized in autoclave at 110 °C, 110 pounds pressure for 10 min. All media were prepared in 1 Litre of distilled water.

1. Humic acid vitamin agar (HV)

Humic acid (dissolved in 1 ml of 0.2 N NaOH)	1	g
Na_2HPO_4	0.5	g
KCl	1.71	g
$MgSO_4 \cdot 7H_2O$	0.05	g
$FeSO_4 \cdot 7H_2O$	0.01	g
$CaCO_3$	0.02	g
Vitamin B solution	10	mL
Agar	18	g

Vitamin B solution

Thiamine-HCl	0.05	g
Riboflavin	0.05	g
Nicothinate (Niacin)	0.05	g
Pyridoxine-HCl	0.05	g
Inositol	0.05	g
Ca-pantothenate	0.05	g
p-Aminobenzoate	0.05	g
d-Biotin	0.025	g

To add vitamin B solution, cycloheximide (50 mg/l) and nalidixic acid (25 mg/l) that was filter-sterilized.

2. Starch-casein nitrate agar (SCN)

Soluble starch	10	g
Sodium caseinate	1	g
KNO_3	2	g
KH_2PO	0.5	g
$MgSO_4$	0.5	g
Agar	15	g

To add cycloheximide (50 mg/l) and nalidixic acid (25 mg/l) that was filter-sterilized.

3. 2.5% Water agar

Agar	25	g

To add cycloheximide (50 mg/l) and nalidixic acid (25 mg/l) that was filter-sterilized.

4. Yeast extract-malt extract agar (ISP no.2), pH 7.2 $\pm$ 0.2

Yeast extract	4	g
Malt extract	1	g
Glucose (dextrose)	4	g
Agar	15	g

5. Oatmeal agar (ISP no.3), pH 7.2 $\pm$ 0.2

Oatmeal	20	g
Agar	15	g

6. Inorganic salts-starch agar (ISP no.4), pH 7.2 ± 0.2

Soluble starch	1	g
K_2HPO_4	1	g
$MgSO_4 \cdot 7H_2O$	1	g
NaCl	1	g
$(NH_4)_2SO_4$	2	g
$CaCO_3$	2	g
Trace salts solution A	1	mL
Agar	18	g

7. Glycerol-asparagine agar (ISP no.5), pH 7.2 ± 0.2

Glycerol	10	g
L-Asparagine	1	g
K_2HPO_4	1	g
Trace salts solution A	1	mL
Agar	15	g

8. Peptone-yeast extract iron agar (ISP no.6), pH 7.2 ± 0.2

Peptone Iron agar, dehydrated	3.6	g
Yeast extract	0.1	g
Agar	15	g

9. Tyrosine agar (ISP no.7), pH 7.2 ± 0.2

Glycerol	15	g
L-Tyrosine	0.5	g
L-Asparagine	10	g
K_2HPO_4	0.5	g

$MgSO_4 \cdot 7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4 \cdot 7H_2O$	0.1	g
Trace salts solution A	1	mL
Agar	18	g

Trace salts solution A

$FeSO_4 \cdot 7H_2O$	0.1	g
$MnCl_2 \cdot 4H_2O$	0.1	g
$ZnSO_4 \cdot 7H_2O$	0.1	g
Distilled water	100	mL

10. Glucose asparagine agar (GluA), pH 7.2 $\pm$ 0.2

Glucose (dextrose)	10	g
Asparagine	0.5	g
K_2HPO_4	0.5	g
Agar	15	g

11. Czapek's sucrose agar (Czk), pH 7.2 $\pm$ 0.2

Sucrose	30	g
K_2HPO_4	1	g
$MgSO_4$	0.5	g
KCl	0.5	g
$FeSO_4$	0.01	g
Agar	18	g

12. Nutrient agar (NA), pH 7.2 ± 0.2

Meat extract	10	g
Peptone	10	g
NaCl	1	g
Agar	18	g

13. Carbon utilization medium (ISP no.9), pH 7.2 ± 0.2

Carbohydrate	10	g
$(NH_4)_2SO_4$	2.64	g
$K_2HPO_4 \cdot 3H_2O$	5.65	g
KH_2PO_4 anhydrous	2.38	g
$MgSO_4 \cdot 7H_2O$	1	g
Trace salts solution B	1	mL
Agar	15	g

Trace salts solution B (Pridham and Gottlieb trace salts)

$CuSO_4 \cdot 5H_2O$	0.64	g
$FeSO_4 \cdot 7H_2O$	0.11	g
$MnCl_2 \cdot 4H_2O$	0.79	g
$ZnSO_4 \cdot 7H_2O$	0.15	g
Distilled water	100	mL

14. Bouillon gelatin broth, pH 7.2 -7.8

Peptone	1	g
Meat extract	0.5	g
NaCl	0.5	g
Gelatin	15	g

Distilled water	100	mL

15. Peptone KNO_3 broth, pH 7.2 -7.8

Peptone	1	g
KNO_3	0.1	g
NaCl	0.5	g
Distilled water	100	mL

16. 10% Skim milk

Skim milk	100	g

To sterilize at 110 °C for 10 min

17. Mueller-Hinton agar (Difco)

Beef infusion form	30	g
Casamino acid, technical	17.5	g
Starch	1.5	g
Agar	17	g

18. Sabouraud's dextrose agar (Difco)

Neopeptone	10	g
Glucose (dextrose)	40	g
Agar	15	g

APPENDIX B

REAGENTS AND BUFFER

1. Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	mL

N,N-dimethyl-1-naphthylamine solution

N,N-dimethyl-1-naphthylamine	0.5	g
5 N Acetic acid	100	mL

Both of them were dissolved by gentle heating in fume hood.

2. 2 N H_2SO_4

Conc. H_2SO_4	2	mL
Distilled water	34	mL

To add conc. H_2SO_4 into the distilled water for cell hydrolysis process in the whole-cell sugar analysis.

3. 6 N HCl

Conc. HCl	60	mL
Distilled water	60	mL

To add conc. HCl into the distilled water for cell hydrolysis process in the diaminopimelic acid analysis.

4. Dittmer & Lester reagent

Solution A

MoO_3	4.011	g
25 N H_2SO_4	100	mL

To dissolve MoO_3 in hot acid.

Solution B

Molybdenum powder	0.178	g
Solution A	50	mL

To add molybdenum powder in solution A and boil for 15 minutes. After cooling, remove the precipitate by decantation. And then, mix solution A (50 mL), solution B (50 mL) and water (100 mL) before use.

5. Anisaldehyde reagent

EtOH	90	mL
H_2SO_4	5	mL
p-Anisaldehyde	5	mL
Acetic acid	1	mL

6. Ninhydrin solution

Ninhydrin	0.3	g
n-Butanol	100	mL
Glacial acetic acid	3	mL

7. Dragendroff's reagent

Solution A

Basic bismuth nitrate	1.7	g
Acetic acid	20	mL
Distilled water	80	mL

Solution B

KI	40	g
Distilled water	100	mL

To mix solution A (10 ml), solution B (10 ml) and acetic acid (10 ml), before use.

8. Reagents for fatty acid analysis

Reagent 1 (Saponification reagent)

NaOH	15	g
MeOH	50	mL
Ultrapure water	50	mL

Reagent 2 (Methylation reagent), pH below 1.5

6N HCl	65	mL
MeOH	55	mL

Reagent 3 (Extraction solvent)

n-Hexane	50	mL
Methyl-tert-butyl ether	50	mL

Reagent 4 (Base wash reagent)

NaOH	1.2	g
Ultrapure water	100	mL

Reagent 5 (Saturated sodium chloride)

Sodium hydroxide saturated in ultrapure water.

9. Phenol : Chloroform (1 : 1 v/v)

Crystalline phenol (melted)	50	mL
Chloroform	50	mL

To melted crystalline phenol in water bath at 65 °C and mix with chloroform. The solution was stored in a light tight bottle.

10. 0.2 M Tris-HCl buffer, pH 8.5

Tris	24.22	g
Distilled water	700	mL

To adjust pH 8.5 with conc. HCl and adjust volume to 1 Litre with distilled water.

11. DNA extraction buffer (Grind method)

0.2 M Tris-HCl buffer, pH 8.5	900	mL
NaCl	14.61	g
EDTA·2Na	9.31	g
SDS	5	g

To adjust volume to 1 Litre with 0.2 M Tris-HCl buffer. The solution was sterilized by autoclaving at 121°C, 15 lb/in^2 for 15 minutes.

12. 3 M NaCl

NaCl	17.55	g
Distilled water	100	mL

The solution was autoclaved at 121°C, 15 lb/in^2 for 15 minutes.

13. RNase A solution

RNase A	20	mg
0.15 M NaCl	10	mL

To dissolve RNase A in 0.15 M NaCl and heat at 95°C for 5-10 minutes. To keep this solution in –20°C.

14. 0.1 M Tris-HCl buffer, pH 7.5

Tris	1.2	g
Distilled water	70	mL

To adjust pH 7.5 with conc. HCl and adjust volume to 100 mL with distilled water. The solution was sterilized by autoclaving at 121°C, 15 lb/in^2 for 15 minutes.

15. RNase T_1 solution

RNase T_1	80	μL
0.1 M Tris-HCl, pH 7.5	10	mL

To mix RNase T_1 in 0.1 M Tris-HCl (pH 7.5) and heat at 95° C for 5 minutes. To keep this solution in –20° C.

16. 100xDenhardt solution

Bovine serum albumin	2	mL
Polyvinylpyrrolidone	2	mL
Ficoll 400	2	mL
Distilled water	700	mL

17. 20xSSC solution

NaCl	175.3	g
Citrate·3Na·2H_2O	88.2	g
Distilled water	700	mL

To adjust pH to 7.0 with 10 M NaOH and adjust volume to 1 Litre with distilled water. The solution was autoclaved at 121°C, 15 lb/in^2 for 15 minutes.

18. 10XPBS

Na_2HPO_4	5.68	g
KH_2PO_4	1.02	g
NaCl	40.07	g
KCl	1	g
Distilled water	1	L

To adjust pH 7.0 with 1N NaOH or 1N HCl. The solution was autoclaved at 121°C, 15lb/in^2 for 15 minutes.

19. 10 mg/mL Salmon sperm DNA

Salmon sperm DNA	10	mg
10 mM TE buffer, pH7.6	1	mL

To boil for 10 minutes. After immediately cooling in ice, sonicate for 3 mins.

20. Proteinase K

Proteinase K	4	mg
50 mM Tris-HCl, pH 7.5	1	mL

To use freshly prepared solution.

21. 40 mM CH_3COONa

CH_3COONa	3.2812	g
Distilled water	1	L

22. 12 mM $ZnSO_4$

$ZnSO_4$ (anhydrous)	1.9376	g
Distilled water	1	L

23. Nuclease P1 solution

Nuclease P1	0.1	mg
40 mM CH_3COONa	500	µL
12 mM $ZnSO_4$, pH 5.3	500	µL

24. Alkaline phosphatase solution

Alkaline phosphatase	2.4	units
0.1 M Tris-HCl, pH 8.1	1	mL

25. TE buffer

10 mM Tris HCl, pH 8.0	10	mL
1 m M Na_2-EDTA, pH 8.0	10	mL
Distilled water	980	mL

26. 10 mg/mL TMB in 10% DMSO

3,3',5,5'-Tetramethylbenzidine (TMB)	0.001	g
DMSO	10	µL
Distilled water	90	µL

27. 0.3% H_2O_2

3% H_2O_2	10	µL
Distilled water	90	µL

28. 0.1 M Citric acid in 10% DMSO

Citric acid	0.05	g
DMSO	250	µL
Distilled water	2.25	mL

29. 0.2 M Na_2HPO_4 in 10% DMSO

Na_2HPO_4	0.07	g
DMSO	250	µL
Distilled water	2.25	mL

30. TE buffer + RNase A

TE buffer	960	mL
Rnase A (2 mg/mL)	100	µL

31. Reagent and buffer for DNA-DNA hybridization

Prehybridization solution

100xDenhardt solution	5	mL
10 mg/ml Salmon sperm DNA	1	mL
20xSSC	10	mL
Formamide	50	mL
Distilled water	34	mL

Hybridization solution

Prehybridization solution	100	mL
Dextran-sulfate	5	g

Solution I

Bovine serum albumin (Fraction V)	0.25	g
Titron X-100	50	µL
1X PBS	50	mL

Solution II

Streptavidin-POD	1	µL
Solution I	4	mL

Solution III, pH 6.2

10 mg/mL TMB in 10% DMSO	100	µL
0.3% H_2O_2	100	µL
0.1 M Citric acid in 10% DMSO	2.5	mL
0.2 M Na_2HPO_4 in 10% DMSO	2.5	mL

2M H_2SO_4

Conc. H_2SO_4	22	mL

Distilled water	178	mL

32. Ethidium bromide solution (10 mg/mL)

Ethidium bromide	1	g
Distilled water	100	mL

33. 5XTris-acetate EDTA buffer (5XTAE)

Tris	5.4	g
Boric acid	2.75	g
Na_2-EDTA	0.47	g
Distilled water	100	mL

34. Agarose gel

Agarose	0.8	g
1xTAE buffer	100	mL

35. 0.1 M $MgCl_2$ in 1xPBS (PBSM)

$MgCl_2$ (anhydrous)	0.95211	mg
10xPBS	10	mL
Distilled water	90	mL

APPENDIX C

PRIMERS AND 16S rRNA GENE SEQUENCES

1. Primers

20F	5'-AGTTTGATCCTGGCTC-3'
1530R	5'-AAGGAGGTGATCCAGCC-3'
27F	5'-AGAGTTTGATCMTGGCTCAG-3'
1492R	5'-TACGGYTACCTTGTTACGACTT-3'
518F	5'-CCAGCAGCCGCGGTAATACG-3'
800R	5'-TACCAGGGTATCTAATCCC-3'
920F	5'-AAACTCAAATGAATTGACGG-3'
357R	5'-CTGCTGCCTCCCGTAG-3'

2. 16S rRNA gene sequences

>CR1-01 (1369 nt)

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>CR1-02 (1370 nt)

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>CR1-03 (1393 nt)

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>CR1-04 (1466 nt)

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>CR1-05 (1362 nt)

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>CR1-06 (1363 nt)

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>CR1-07 (1461nt)

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>CR1-08 (1485 nt)

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>CR1-09 (1482 nt)

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>CR1-10 (1397nt)

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>CR1-11 (1467 nt)

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>ST1-01 (1368 nt)

CAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGC
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GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGC
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AGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTG
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>ST1-02 (1340 nt)

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>ST1-03 (1351 nt)

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CAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTC
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>ST1-04 (1346 nt)

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GGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCG
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GGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTT
GTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTA
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AGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACT
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>ST1-05 (1360 nt)

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CGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGG
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>ST1-06 (1453 nt)

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>ST1-07 (1322 nt)
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>ST1-08 (1462 nt) LC011703; JCM 30050^{T}, PCU 339^{T}, TISTR 2278^{T}
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>ST1-09 (1345 nt)

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>ST1-10 (1388 nt)

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>OH1-01 (1496nt)

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>OH1-02 (1491 nt)

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CATGCTACAATGGTCGGTACAGAGGGTTGCGATACCGTGAGGTGGAGCGAATCCCTAAAAGCCGGTCTCA
GTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGCAACACCCGAAGCCC
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>PG1-01 (1365 nt)

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CTCTGGGATAAGCCTGGGAAACCGGGTCTAATACCGGATACGACCATTCTCGGCATGGGATGGTGGTG
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CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGT
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GGTCGGTACAGAGGGCTGCTAACCCGTGAGGGGGAGCGAATCCCTAAAAGCCGGTCTCAGTTCGGATT
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CCAACCCGT

>PG1-02 (1344 nt)

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CTAACCCGTGAGGGGGAGCGAATCCCTAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACC
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>PA1-02 (1347 nt)

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GGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTAGTGGCCTACCAAGGCGACGACGGGTAGC
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TGGGGAATATTGCGCAATGGGCGGAAGCCTGACGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGG
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CCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAATGCTGCGGTGAATACGTTCCCGGGCCTT
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>PA1-03 (1479 nt)

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TCGACCCCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGG
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>PA1-07 (1356 nt)

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GGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGT
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CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTT
CGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTA
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GCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCAGTGCTGCGGTGAATACGTTC
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>PA1-10 (1371 nt)

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GGTGGAAAGTTTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTAGTGGCCTAC
CAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGA
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AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTGG
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GCGAAGAACCTTACCAAGGTTTGACATCACCCGGACAACTGCAGAGATGTGGTTTTCTTCGGACTGGG
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CAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCT
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GGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAATGCTGCG
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GTGGCCCAACCACTTG

>PA1-11 (1366 nt)

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TGGTGGAAAGTTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTAGTGGCCTAC
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GGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGTTGACGTGTACCTGCAGAAG
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TGGCCGGTACAGAGGGTTGCGATACCGTGAGGTGGAGCGAATCCCTAAAAGCCGGTCTCAGTTCGGAT
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CCCAACCCG

>VC1-01 (1356 nt)

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TACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTC
TGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTT
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>VC1-02 (1351 nt)

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GAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAAC
TCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGG
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>VC1-03 (1351 nt)

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TGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGTCAACTCGGAGGAAAGT
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GAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAAC
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>VC1-04 (1362 nt)

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CGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCG
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ACCCC

>PP1-01 (1342nt)

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TGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGG
GTTGTAAACCTCTTTCAGCAGGGACGAAGCGTAAGTGACGGTACCTGCAGAAGAAGCGCCGGCCAACT
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>PP1-03 (1393nt)

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>PP1-04 (1301nt)

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TGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTT
CCCGGGCCTTGTA

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POSTER :

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