PRODUCT DEVELOPMENT FOR FUSARIUM WILT DISEASE CONTROL IN BANANA AND PLANT GROWTH PROMOTING ACTIVITY FROM ACTINOMYCETES

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY
DEPARTMENT OF BIOLOGY SCHOOL OF SCIENCE
KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG
2022

KMITL-2022-SC-D-020-113

Thesis Title	Product Development for Fusarium Wilt Disease Control in Banana and Plant Growth Promoting Activity from Actinomycetes
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Degree	Doctor of Philosophy (Biotechnology)
Department	Biology
Year	2022
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Abstract

Fusarium wilt is one of the most serious diseases of banana plants caused by soil–borne pathogen *Fusarium oxysporum* f.sp. *cubense* (*Foc*). In this study, a total of 173 actinomycetes were isolated from the compost types and soil samples. Among these isolates, 24 isolates showed antagonistic activity against *Foc* with the percentage of inhibition of radial growth values of more than 21% and at least 50% of *Foc* strains. These isolates were identified as *Streptomyces*. Three actinomycete products (ATU–Bio 1: RBST1–4, RCPT3–28, CH9–7, ATU–Bio 2: CH5–8, RCPT1–4, RBST2–54, and ATU–Bio 3: RCPT1–4, RCPT3–40, RBST2–21) were formulated based on antifungal activity, plant growth–promoting traits and their properties. The actinomycete products enhanced the growth of banana in field experiment compared to the control (no supplement) and commercial PGPR–2 ($P<0.05$) and ATU–Bio 1 and ATU–Bio 2 showed plant growth promoters better than ATU–Bio 3. For the protective experiment, the final severity index, for leaf symptom (LSI) and rhizome discoloration (RDI) of actinomycete products were reduced with maximum values of about 63 and 57% of ATU–Bio 1, 50 and 50% of ATU–Bio 2, and 50 and 46% of ATU–Bio 3, respectively, compared to untreated plantlets (no supplement). For the curative experiment, the final severity index, for LSI and RDI of the actinomycete products were reduced with maximum values of about 21 and 25% of ATU–Bio 1, 15 and 9% of ATU–Bio 2, and 11 and 7% of ATU–Bio 3, respectively, compared to untreated plantlets (no supplement). For plant growth promoters under *Foc* infection, the actinomycete products in the protective experiment still enhanced banana growth, while the curative experiment had a poor–efficiency capacity to cure *Foc* infection and plant growth promoters in banana. Chitinase from strains RCPT1–4, CH5–8, and CH9–7 and CMCase from strains RCPT1–4 and CH5–8

were one of an antifungal mechanism of fungal cell wall degradation. Moreover, albocycline from strain CH5–8 and nocardamine from strain CH9–7 were found to have antifungal activity. Three strains, RCPT1–4, CH5–8, and CH9–7, were represented the novel species as *Streptomyces sennicomposti* RCPT1–4^{T}, *Streptomyces musisoli* CH5–8^{T}, and *Streptomyces* sp. CH9-7^{T}, respectively. Hence, the actinomycete products, especially ATU–Bio 1 and ATU–Bio 2, could be useful for the biological control of Fusarium wilt disease and plant growth promoters of banana.

Keywords : Banana, Biocontrol, Bio–product, Fusarium wilt disease, Actinomycetes

Acknowledgements

I would like to express my special appreciation and thanks to my thesis advisors and co–advisor, Associate Professor Dr. Chitti Thawai and Dr. Pramote Sirirote, for their kind recommendation, guidance, mention, and enthusiastic encourangement throughout this research. They have inspired me to become an independent researcher and helped me realize the power of critical reasoning. They also demonstrated what a brilliant and hard–working scientist can accomplish.

I would also like to thank my examination committee, Professor Dr. Somboon Tanasupawat, Associate Professor Dr. Saranya Phunpruch, Assistant Professor Dr. Nattawut Rungjindamai, and Dr. Pattama Pittayakhajonwut for letting my defense be an enjoyable moment, and for your brilliant comments and valuable suggestions toward improving my work.

The following persons are also greatly acknowledged: many specific tasks involved in this study that performed by cooperative with other members of the Department of Biology and Actinobacterial Research Unit, School of Science, KMITL.

I would like to thank the National Center for Genetic Engineering and Biotechnology (BIOTEC) for providing all the research facilities. Special thanks are given to the BIOTEC staff, especially Mr. Chakapong Intaraudom for all supporting and Dr. Chanwit Suriyachadkun for supporting the actinobacterial type strains.

I would like to thank Micro–Biotec Co., Ltd., especially, Mr. Pornarun Suwanplai and Mr. Yutthachai Pun–nga for the preparation of soil for field experiments.

I cannot forget all my old friends who went through hard times together, cheered me on, and celebrated each accomplishment.

I deeply thankful my family. Words cannot express how grateful I am to my deceased father, Mr. Thongsin Duangupama, my mother, Mrs. Suporn Duangupama, my older sister, Miss Chompunut Duangupama, and two beloved nephews, Chatchanun Chaiyong and Niraphat Chaiyong, for their take care and supporting me for everything.

Thitikorn Duangupama

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Abbreviations/Symbols

AAI	=	Average amino acid identity
ACC	=	1–Aminocyclopropane–1–carboxylate
ANIb	=	Average nucleotide identity–blast
ANIm	=	Average nucleotide identity–MUMmer
ANOVA	=	Analysis of variance
APG	=	Acylphosphatidylglycerol
$Ba(OH)_2$	=	Barium hydroxide
BCAs	=	Biocontrol agents
BLAST	=	Basal local alignment search tool
BPB	=	Bromophenol blue
^{13}C–NMR	=	Carbon–13 nuclear magnetic resonance
CAS	=	Chrome Azurol S
CFU	=	Colony forming unit
CH_3CN	=	Acetonitrile
cm	=	Centimeter
CMC	=	Caboxymethy cellulose
CRD	=	Completely randomized design
CTAB	=	Cetyltrimethylammonium bromide
δ	=	Chemical shift
DAP	=	Diaminopimelic acid
dDDH	=	Digital DNA–DNA hybridization
DF–ACC	=	Dworkin and Foster's salt minimal–ACC
DMRT	=	Duncan's new multiple range test
DMSO–d_6	=	Deuterated dimethysulfoxide
DPG	=	Diphosphatidylglycerol
DSI	=	Disease severity index
EDTA	=	Ethylenediaminetetraacetic acid
f.sp.	=	formae specialis
Foc	=	*Fusarium oxysporum* f.sp. *cubense*
g	=	Gram
g/l	=	Gram per liter
GAs	=	Gibberellic acid
GC	=	Gas chromatography
GGDC	=	Genome–to–genome distance calculator
GL	=	Glycolic acid
GluNU	=	Glucosamine containing unknown phospholipids

Abbreviations/Symbols (Continued)

^{1}H–NMR	=	Proton nuclear magnetic resonance
H_2S	=	Hydrogen sulfide
HCl	=	Hydrochloric acid
HCN	=	Hydrocyanic acid
HPLC	=	High performance liquid chromatography
HPTLC	=	High–performance thin-layer chromatography
HRESIMS	=	High–resolution electrospray ionization mass spectrometry
Hz	=	Hertz
IAA	=	Indole acetic acid
iPSBs	=	Inorganic–phosphate–solubilizing bacteria
ISP 2	=	Yeast malt agar
ISP 3	=	Oatmeal agar
ISP 4	=	Inorganic salt–starch agar
ISP 5	=	Glycerol–asparagine agar
ISP 6	=	Peptone–yeast extract iron agar
ISP 7	=	Tyrosine agar
ISP	=	International *Streptomyces* Project
ITS	=	Internal transcribed spacer
J	=	Coupling constant
K	=	Potassium
KSB	=	Potassium solubilizing bacteria
LEA	=	Levels of enzymatic activity
Ls	=	Lipids
LSI	=	Leaf symptom index
$[M+Na]^{+}$	=	Protenated molecular ion
M	=	Molar
MEGA	=	Molecular Evolutionary Genetics Analysis
MHz	=	Megahertz
MK	=	Menaquinone
ML	=	Maximum–likelihood
µl	=	Microliter
ml	=	Milliliter
mm	=	Millimeter
MS	=	Mass spectroscopy
N_2	=	Gaseous nitrogen
NA	=	Nutrient agar

Abbreviations/Symbols (Continued)

Na_2SO_4	=	Sodium sulfate
NaCl	=	Sodium chloride
NAG	=	N–Acetylglucosamine
NAM	=	N–Acetylmuramic acid
NBRIP	=	National Botanical Research Institute's phosphate growth medium
NFb	=	Nitrogen–free bromothymol blue
NH_3	=	Ammonia
NH_4Ac	=	Ammonium acetate
NJ	=	Neighbor–joining
nm	=	Nanometer
NMR	=	Nuclear magnetic resonance
NO_2	=	Nitrite
NO_3	=	Nitrate
NPL	=	Ninhydrin–positive lipid
nt	=	Nucleotide
OH–PE	=	Hydroxyphosphatidylethanolamine
P	=	Phosphate
PC	=	Phosphatidylcholine
PCR	=	Polymerase chain reaction
PDA	=	Potato dextrose agar
PDB	=	Potato dextrose broth
PE	=	Phosphatidyl choline
PEG	=	Polyetheleneglycol
PG	=	Phosphatidyl glycerol
Pg	=	Picogram
PI	=	Phosphatidyl inositol
PIMs	=	Phosphatidyl inositol mannosides
PIRG	=	Percentage of inhibition of radial growth
PLs	=	Polar lipids
PME	=	Phosphatidyl methyl ethanolamine
pmol	=	Picomole
PS	=	Phosphatidylserine
PSE	=	Potassium solubilization efficiency
RDI	=	Rhizome discoloration index
rpm	=	Round per minute
rRNA	=	Ribosomal ribonucleic acid

Abbreviations/Symbols (Continued)

SDS	=	Sodium dodecyl sulphate
SE	=	Phosphate solubilization efficiency
sp.	=	species
SPI	=	Siderophore production index
TAE buffer	=	Tris–Acetate–EDTA buffer
TE buffer	=	Tris–EDTA buffer
TES	=	Tris (hydroxymethyl) methyl–2–amino ethane sulfonic acid
Tetra	=	Tetra–nucleotide signature
TLC	=	Thin–layer chromatography
TR4	=	Tropical race 4
TSI	=	Triple sugar iron
TYGS	=	Type strain genome server
v/v	=	Volume by volume
w/w	=	Weight by weight
ZSSE	=	Zhang's starch soil extract

Chapter 1
Introduction

1.1 Research motivation

Actinomycetes are a group of Gram–positive prokaryotic organisms, filamentous bacteria with high genomic G+C content. These bacteria produce asexual spores on aerial or substrate mycelia. Actinomycetes are widespread environmental organisms and are found in both aquatic and terrestrial habitats such as soil, sediment, plant tissue, animal tissue, freshwater, seawater, etc.

The use of biological control for the management of plant diseases plays a role in modern agriculture practice. Increasing rate of using synthetic chemicals has led to environmental pollution and the development of resistant pathogens. Over 25–30 years, alternative control methods, including the use of microorganisms, were developed (Pakdeevaraporn *et al*., 2005). Actinomycetes, especially *Streptomyces* species are one of the most attractive sources of biological substances or antibiotics (Demain and Fang, 1995 ; Ilic *et al*., 2007). Moreover, actinomycetes played an extensive role in the plant rhizosphere and produced different agroactive compounds such as indole acetic acid, gibberellic acid, cytokinin, siderophore, nutrient solubilization (potassium and phosphate), or asymbiotic nitrogen fixation (Khan, 2005). Antagonistic properties against phytopathogenic microorganisms by producing antimicrobial compounds or active substances such as antifungal compounds (Barriuso *et al*., 2008 ; Majeed *et al*., 2015) are interesting effects of actinomycetes.

Fusarium wilt of banana or Panama disease is the influential disease of banana, menacing 80% of the world's banana (Ingle and Ingle, 2013). This disease is caused by fungi "*Fusarium oxysporum* f.sp. *cubense* (*Foc*)", the natural habitat of soil, (Green, 1981) that infect roots of susceptible and resistant banana cultivars, entry to the water–conducting xylem vessels of banana, and lead to the characteristic symptoms (leaf chlorosis and necrosis). About 100% of infected bananas died from the disease. Control of this pathogen by chemical methods using fungicides is ineffective like biological methods and/or cannot be eliminated from soil using fumigants. In cases of biological control, some researchers have successfully hired antagonistic microorganisms. For example, Sivamani and Gnanamanickam (1988) inoculated *Pseudomonas fluorescens* to control *Foc* in banana, Raguchander *et al*. (1997) used *Trichoderma viride* to prevent this fungus. So far, few workers have studied antagonistic actinomycetes to regulate the cause of disease such as Meredith (1946) applied soil actinomycetes to control banana wilt in field experiments,

Cao *et al*. (2004) isolated endophytic actinomycetes from roots and leaves of banana to study its activities against *Foc.* Getha and Vikineswary (2002) and Getha *et al*. (2005) evaluated *Streptomyces* sp. strain g10 to suppress Fusarium wilt and rhizosphere colonization in pot–grown banana plantlets.

Due to the biodiversity of beneficial actinomycetes species in Thailand, these microorganisms are more attractive in the production of bioactive and/or agroactive compounds. Therefore, the investigation of actinomycetes from a new different ecosystem has been focused. In this study, actinomycetes isolated from the different ecosystems in Thailand have screened the antifungal activities against *Foc* isolated from infected banana and banana planting soil. The study also includes production of plant growth promotion substances, extracellular enzymes, tolerance properties, and measurement of actinobacterial growth. The selected isolates were characterized and identified by a polyphasic taxonomy approach including phenotypic characteristics, chemotaxonomic characteristics, and genotypic characteristics. Isolation, purification, and identification of secondary metabolite were also done. Additionally, the formulation of the actinomycete products from the selected isolates was tested in their activities in field–grown banana plantlets experiment.

1.2 Objectives of the study

1) To isolate and screen actinomycetes having anti–*Fusarium oxysporum* f.sp. *cubense* activities and plant growth–promoting substances from the compost types and soils.

2) To identify and characterize the selected actinomycete isolates.

3) To study the actinomycete properties, formulate the actinomycete products and study their activities based on field experiments.

4) To evaluate the mechanism against *Fusarium oxysporum* f.sp. *cubense*.

1.3 Scopes of the study

1) Isolation of the actinomycetes from the compost types: four samples from aerobic bio–sludge compost, two samples from anaerobic bio–sludge compost, five samples from agricultural waste compost, and two samples from the soil collected from Rayong, Suphanburi, Ratchaburi, Pichit, and Nakhon Sawan provinces.

2) Screening of the antifungal activity against *Fusarium oxysporum* f.sp. *cubense* that was isolated from infected banana and banana planting soil.

3) Screening of the plant growth–promoting ability of the selected isolates to produce indole acetic acid, gibberellic acid, cytokinin, siderophore, solubilization of

inorganic phosphate and potassium, nitrogen fixation properties, and 1–aminocyclopropane–1–carboxylate (ACC) deaminase production.

4) Identification and characterization of the selected actinomycete isolates using a polyphasic taxonomy approach including phenotypic characteristics, chemotaxonomic characteristics, and genotypic characteristics.

5) Study of the selected actinomycetes properties such as extracellular enzyme production, growth measurement, tolerance property, antagonistic activity among the actinomycetes, and toxicity on the banana plant.

6) Formulation of the actinomycete products based on the anti–*Fusarium oxysporum* f.sp. *cubense* activity, plant growth–promoting ability and related properties, and study of the product based on field experiment with plant growth promotion, the protective and curative experiments.

7) Evaluation of the mechanism against *Fusarium oxysporum* f.sp. *cubense* including extracellular enzyme and secondary metabolite production.

1.4 Benefits of the study

1) Exploring the novel actinomycete species from new sources and types of compost and soil.

2) Discovering the actinomycete species that show several activities such as anti–*Fusarium oxysporum* f.sp. *cubense*, plant growth–promoting ability, extracellular enzyme, and secondary metabolite production.

3) Formulating the actinomycete products for banana growth promotion and control Fusarium wilt disease that can be used for agriculture as an alternative choice for the organic farming systems.

Chapter 2
Theory and literature reviews

2.1 Fusarium wilt of banana

The banana of Thailand is called “Kluai” is one of the main fruits cultivated in subtropical and tropical regions and the world’s most important fruit in terms of production volume and trade (FAOSTAT, 2017). Edible banana cultivars are offsprings from two ancestral wide *Musa* species, *Musa acuminate* (AA), and *Musa balbisiana* Colla (BB) (Simmonds, 1992). These diversified into various edible varieties comprising diploids (AA, BB), triploids (AAA, AAB, ABB), and tetraploids (ABBB). From the data of the Horticulture Research Institute of Thailand have seventy varieties of banana. Only four varieties, “Kluai Khai” (*Musa*, AA), “Kluai Tani” (*Musa*, BB), “Kluai Namwa” (*Musa*, ABB), and “Kluai Hom” (*Musa*, AAA) were exported as the economic fruits in Thailand. In 2020, Thailand has accounted area for banana planting of about 653,408 rais (104,545.28 hectares) with approximately yielded 798,938 tons and exported about 2–3% of the total (Department of Agriculture Extension, 2021). Panama disease, which is also known Fusarium wilt, is regarded as one of the most destructive diseases of banana production in the world including Thailand (Moore *et al*., 2001).

2.1.1 Disease background and distribution

Panama disease or Fusarium wilt is a destructive fungal disease of banana plants. The pathogen, possibly originated from Southeast Asia and the disease was first described in Australia in 1876 (Bancroft, 1876) and it first became an epidemic in Panama in 1890. At the beginning of the 20th century, the information about the incidence of the disease rose rapidly, mostly on commercial plantations. Its global distribution has an important anthropogenic component: the infected but symptomless rhizomes or suckers used for planting material were introduced into different areas with conventional plantation material (Ploetz and Pegg, 2000). *Fusarium oxysporum* formae specialis *cubense* (*Foc*) exists as different pathogenic races such as *Foc* races 1, 2, 3, and 4, classified according to their ability to cause disease in a set of different banana cultivars. Table 2.1 and Figure 2.1 showed pathogenic races of *Foc* on varieties of banana and the geographical distribution of its.

Table 2.1 Pathogenic races of *Fusarium oxysporum* f. sp. *cubense* (*Foc*).

Cultivars	Race 1	Race 2	Race 3	Race 4	TR4*
Gros Michel (AAA)	+	-	+	+	+
Pome, Manzano (AAB)	+	-	-	+	+
Pisang awak (ABB)	+	-	-	+	+
Bluggoe (cooking banana) (ABB)	-	+	-	+	-
Heliconia spp. (tropical american banana relatives)	-	-	+	-	-
Cavendish (AAA)	-	-	-	+	+

* TR4 (subtropic) = Cavendish suscepts to *Foc* occur in the subtropics region

Source: Adapted from Pérez–Vicente and Dita (2014)

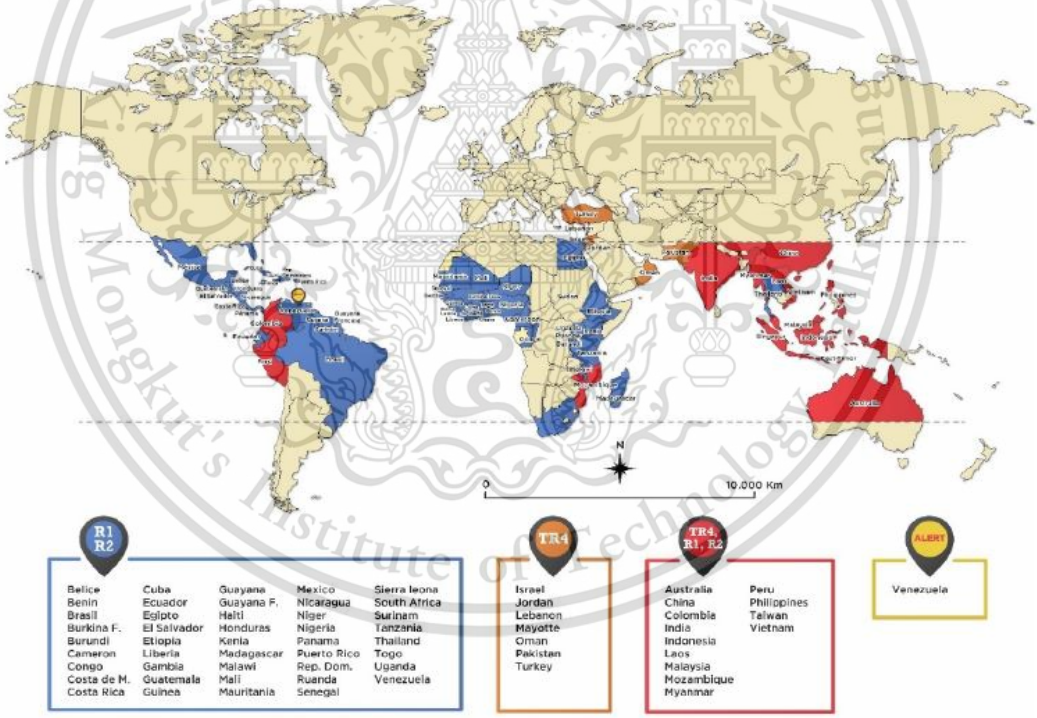


Figure 2.1 Geographical distributions of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) races.

Source: Adapted from EPPO (2014), CABI/EPPO (2015) and PROMUSA (2021)

2.1.2 Causal agent

Fusarium wilt of banana is the fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (E.F.Sm) W.C. Snyder & H.N. Hansen as the causal agent. Its taxonomic position is as follows: Domain: Eukaryota, Kingdom: Fungi, Phylum: Ascomycota, Class: Ascomycetes, Subclass: Sordariomycetidae, and Order: Hypocreales.

Fusarium oxyporum is a complex of anamorphic, filamentous, morphologically undifferentiated fungal species featuring saprophytes. It mostly causes wilting, damping–off, root and organ necrosis, and rots. The formae specialis (f. sp.) is a special form of *F. oxysporum* with a specialization of pathogenicity to plant genera and families that are related to the fungal host. Up to date, more than 150 formae speciales and races in 16 of them have been described (Gordon and Martyn, 1997 ; Baayen *et al*., 2000 ; O'Donnell *et al*., 2009 ; Edel–Hermann and Lecomte; 2019). Moreover, it was separated by using vegetative compatibility groups (VCG) and restriction fragment length polymorphism (RFLP) (Kistler, 1997).

Foc is a fungus without a sexual stage (telemorph). It produces macroconidia, microconidia, and chlamydospores for reproduction and dispersal. Both conidia are produced in orange structures that called sporodochia. The sexual stage has not been found even in isolates carrying gene *Mat* 1 and *Mat* 2 (Fourie *et al*., 2011). On potato dextrose agar (PDA) medium, colonies have variable morphology. Mycelia can be hairy to cottony, spaced or abundant, and variable from salmon, white to pale violet. Black to violet sclerotia can be produced in some isolates. Additionally, it commonly produced pale violet to dark red color pigments in PDA (Pérez–Vicente *et al*., 2003). Some isolates mutate rapidly from pionnotal (with abundant greasy or brilliant conidia aggregates) to flat humid mycelia of white–pale yellowish to peach color on a PDA culture (Ploetz, 1990) (Figure 2.2).

Foc can be isolated from infected banana tissue with vascular vessels or contaminated soil. It was isolated, purified, and preserved on PDA, water agar (WA), carnation leaves agar (CLA) (Burguess *et al*., 1988), Komada modified media (K2) (Sun *et al*., 1978) or Spezieller Nährstoffarmer agar (SNA) (Nirenberg, 1981) by using dilution technique. The morphology of *Foc* on PDA with micro– and macroscopic techniques was observed as shown in Figure 2.2.

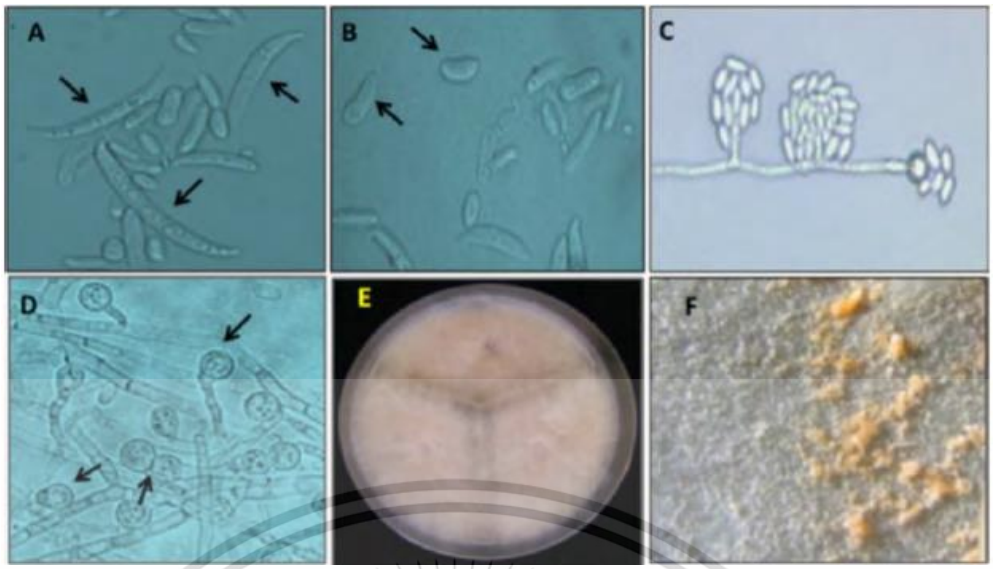


Figure 2.2 Reproductive structure of *Fusarium oxysporum* f.sp. *cubense* (*Foc*).
A. Macroconidia (27–55 x 3.3–5.5 µm, 4–8 cells straight to lightly falcate and foot–shaped basal cells)
B. Microconidia (5–16 x 2.4–3.5 µm, 1 or 2 cells from oval to kidney–like shape)
C. Phialides and microconidia grouped in false heads
D. Chlamydospores (7–11 µm diameter, usually globose developed isolate or in chains)
E. *Fusarium oxysporum* f.sp. *cubense* tropical race 4 in PDA media
F. Orange–colored sporodochia developed in a PDA culture media

Source: Pérez–Vicente and Dita (2014)

For confirmation of the isolate, the molecular technique was used to identify *Foc*. Polymerase chain reaction (PCR)–based identification has a high analytical sensitivity in differentiating *F. oxysporum* strains (Alves–Santos *et al*., 2002 ; Lin *et al*., 2008). The gene generally used in phylogenetic analysis and characterization of *Foc* such as the Intergenic spacer (IGS) and Internal transcribed spacer (ITS) of ribosomal operon, the elongation translation factor–1α (TEF–1α), histone (H3) genes and mitochondrial β–tubulin genes (*tub*–2) (Parte *et al*., 2014). Moreover, Lin *et al*. (2008) developed a molecular marker for the identification of *Foc* race 4. The author achieved the specific detection of *Foc* race 4 by PCR amplification with primers *Foc*–1(5′–CAGGGGATGTATGAGGAGGCT–3′) and *Foc*–2 (5′–GTGACAGCGTCGTCTAGTTCC–3′). The results showed that *Foc* race 4 was detected on DNA samples from 67 both pure isolates and infected plant materials. Other studies on the taxonomic position of *Foc* such as randomly amplified polymorphic DNA (RAPD), RFLP, amplified fragment length polymorphism (AFLP), electrophoretic karyotyping (EK), DNA fingerprint (FP), isoenzyme analysis (IS), plasmid DNA profile

(PL), microsatellite/single sequence repeat (SSR) markers and methylation-sensitive amplification polymorphism (MSAP) were used (Kistler, 1997 ; Fourie *et al*., 2011 ; Luo *et al*., 2016).

Additionally, pathogenicity test from *Foc* may be necessary to confirm an effect of them on the banana plants. Several protocols to infect banana with *Foc* under greenhouse conditions have been reported (Mohamed *et al*., 2000 ; Smith *et al*., 2008 ; Dita *et al*., 2011 ; Ribeiro *et al*., 2011 ; Udompongsuk and Soytong, 2016).

The life cycle of the pathogen, *Foc* infect via the root system entering the xylem vessels, closing up them, and limiting the nutrient and water absorption by the plant. The fungi can grow and multiply saprophytically in neighboring plant tissues as infected plants die, forming chlamydospores that return to the soil (Figure 2.3, 1). The spores in the soil germinate and grow towards the nearby roots of banana plants in response to chemical compounds exuded from the roots (Figure 2.3, 2). Infection takes place on the secondary or tertiary banana roots (Figure 2.3, 3). In susceptible plants, the pathogen is not blocked by the host's defense mechanisms and the infection turns systematic through the vascular system of the pseudo–stem (corm) and stem (Figure 2.3, 4). The water flux in xylem vessels was blocked by the fungus, and the leaves turn yellow and wilt. Distinctive symptoms occur within the pseudo–stem, whose main characteristics are brown, red or yellow ring–shaped lines. Brown stripes or specks appear in the corm (Figure 2.3, 5, and 6). Infected banana plants normally do not produce fruit or their size of them is reduced and their production decrease. The fungus is a soil–borne pathogen, movement of infested soil can disperse soil particles infested by fungal structures (e.g., spores and mycelium). The planting material also plays a significant role in pathogen dissemination (Figure 2.3, 7).

For pathogen dispersal, *Foc* does not spread in soil by active vegetative growth as other soil–borne pathogens. Dispersal mainly takes place by passive movement of pathogen propagules at short and long–distances, from farm to farm or other locations locally or between countries or continents. For long distance dispersal is mainly due to anthropogenic–related factors and wind, whereas dispersal at short distances may be associated with both anthropogenic such as weevil (*Cosmopolites sordidus*) and natural factors such as planting material, drainage water, and runoff, irrigation water, workers, and animal movement, weeds and nematodes (Figure 2.3, A–F) (Dita *et al*., 2018 ; Olivares *et al*., 2021).

Mycotoxin from *Foc* has impacted banana plants, including browning of vascular cells and plant necrosis, inducing cell membrane early super polarization, suppressing H^+ pumping and causing K^+ leaking, suppressing mitochondrial oxygen

absorption and causing malic acid oxidation, conjugating with Cu, Co, Fe, and Zn, forming chelates which make these minerals unavailable to plants and inhibiting the activities of plant defensive enzymes and leads to a reduction in plant cell viability (Li *et al*., 2013). Many researchers studied the effect of mycotoxin on banana plants such as Li *et al*. (2013) indicated that beauvericin and fusaric acid from *Foc* race 1 and 4 in diseased field bananas. Fumonisin B1 from *Foc* race 1 acts as a competitive inhibitor of ceramide synthase and disrupts sphingolipid metabolism in plants (Portal *et al*., 2018). Enniatin A, the non–specific phytotoxins, from *Foc* race 2 produce a complex with metal cations in biological membranes disrupting cation gradients through the banana cell (González *et al*., 2021).

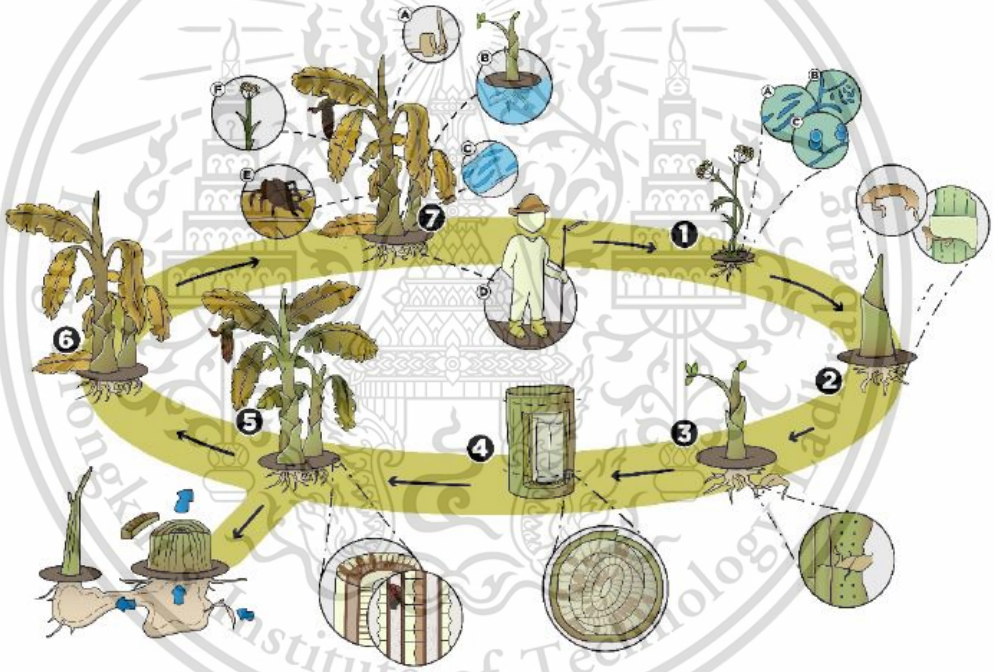


Figure 2.3 Schematic representation of the disease cycle of Fusarium wilt of banana.
(1) *Foc* spores (micro (A) and macroconidia (B) and chlamydospores (C))
(2) Germination of the chlamydospores
(3) Colonization of the roots
(4) Corm infestation
(5) Development of wilt symptoms
(6) Complete wilting of the mother plant
(7) Pathogen dissemination: A. Planting material, B. Drainage water, and runoff, C. Irrigation water, D. Workers, E. The weevils and F. Weeds

Source: Olivares *et al*. (2021)

2.1.3 Disease symptoms and damages

Fusarium wilt of banana is a typical vascular wilt disease. The case of "tolerance" is used to explain cultivars that tolerance infection by *Foc* without developing severe symptoms, while "resistance" is used for cultivars that overcome to some degree the effects of *Foc*. The plant is often able to prevent infection by producing tyloses, gels and gums in xylem lumena, but in resistant cultivars these host products are produced earlier and far more raplidly than in susceptible cultivars. This situation does not occur in tolerant cultivars, due to the obstruction of the pathogen by these host products.

After banana plants were infected, the symptoms do not show sign until advanced infection. The first evident external signs of disease in most varieties are wilting and light yellow coloring of the lower leaves, most prominent around the margins. They finally turn a bright yellow color with dead leaf margins (Figure 2.4, a). As the disease advances, more of the leaves become yellow, wilt die, and often split at their base. A "skirt" of dead leaves often surrounds the pseudostem. In the advancement stages, infected plants may have a spiky appearance due to prominent upright apical leaves in contrast to the skirt of dead lower leaves (Figure 2.4, b).

The first internal symptoms become obvious in the xylem (water–conducting) vessels of the roots and the rhizome. It turns a reddish–brown to marron colour as the fungus grows through the tissue (Figure 2.4, c). Infrequently, the discoloration first appears yellow in plants showing early stages of infection (Figure 2.4, d). In cross–section technique, the discoloration appears in a circular pattern around the center of the rhizome where the infection concentrates due to the arrangement of the vessels (Figure 2.4, e). As symptoms advance into the pseudostem, continuous lines of discolouration are evident when the banana plant is cut longitudinally. The infection may move up to the top of pseudostem. In severe cases, it may even enter the leaf petioles and the peduncle (bunch stalk) of bunched plants. However, the infection has not been expressed to progress into the fruit (Daly and Walduck, 2006).

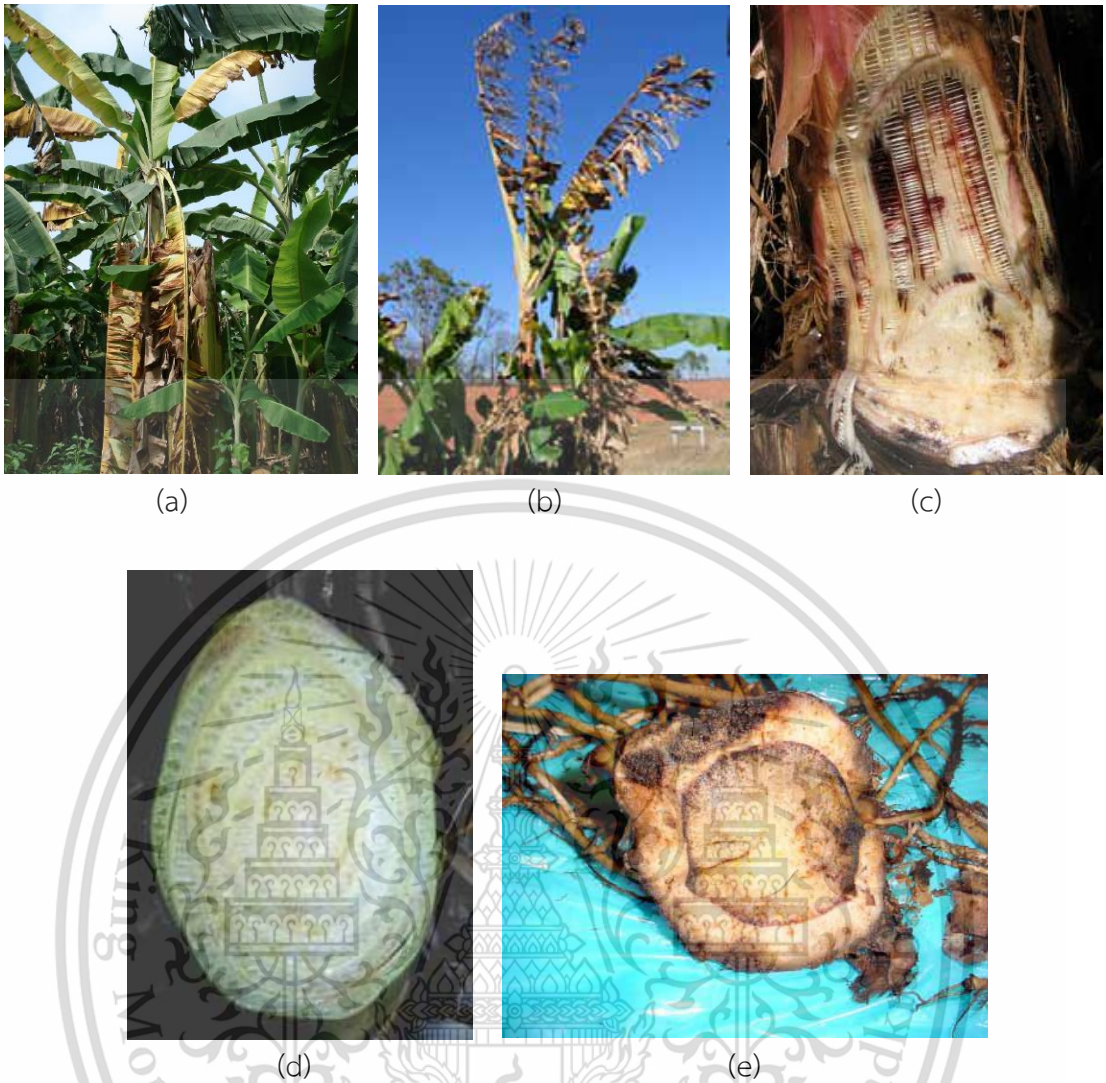


Figure 2.4 *Fusarium oxysporum* f.sp. *cubense* (*Foc*) symptoms in banana plants.
(a) Banana plant showing early symptoms of Fusarium wilt
(b) Banana plant showing advanced symptoms of Fusarium wilt
(c) Reddish–brown to maroon staining of vessels in the pseudo-stem
(d) Yellow staining of xylem vessels in the pseudostem
(e) Reddish–brown to maroon staining of vessels in the rhizome

Source: Daly and Walduck (2006) and Olivares *et al*. (2021)

2.1.4 Disease impact

Fusarium wilt disease has been spread in any countries of the world in commercial banana production (Zheng *et al*., 2018a). Recently, *Foc* caused a serious epidemic throughout the banana–growing regions of the world.

In Asia and Australia region, the disease in Australia has been reported for the first time (Bancroft, 1876), while *Foc* race 4 has been reported in the Northern

Territory since 1997 (Bentley *et al*., 2001 ; Conde and Pitkethley, 2001). In 1967, the pathogen has caused severe damage in Taiwan and 1970 in Philippines (Molina *et al*., 2009). During a survey in 2006 and 2012, the banana plantation in China (Yi *et al*., 2007 ; Farquhar, 2012) was found severely affected by the *Foc*. India found *Foc* race 4 can inflict losses for 7 billion dollars in banana industry (Kulkarni, 2018 ; Thangavelu *et al*., 2019). In other countries, the most records of race 4 have come from Indonesia, Jordan, Lebanon, Pakistan, Laos, Vietnam and Myanmar (Molina *et al*., 2009 ; Zheng *et al*., 2018a).

In Latin America and the Caribbean region, *Foc* race 4 is not present, therefore pathogen exclusion using quarantine procedures is compulsory (Clercx, 2013).

In Africa, *Foc* race 1 has first reported from West Africa in 1924 and then from Tanzania in 1951 (Blomme *et al*., 2013 ; Viljoen *et al*., 2018).

In Carary Island, *Fusarium* wilt was first detected in Tenerife in the 1920s (Blomme *et al*., 2013).

In Thailand, Singburaudom (1999) reported the status of *Fusarium* wilt of banana in Thailand spreading into the banana–growing areas in more than 37 provinces in 1999. In November 2016, “Kluai Hom Thong” banana farms area in Surat Thani province was infected with *Foc*. *Foc* spreaded on “Kluai Namwa” and “Kluai Hin” farm in Songkla province. Seriously case, *Foc* TR 4 was initially detected in confined Cavendish banana farms in Chiang Rai province in 2019. An emergency action plan for the *Foc* TR4 was urgently established and implemented by the Department of Agriculture of Thailand (2019). To avoid the spreading of this disease, the DOA’s Notification on the pest control areas according to the Plant Quarantine Act B.E.2507 and Amended, Section 17 was released as the official measures to control the movement of banana plant parts (fruit is not included) and enforce the eradication program in the infected areas. The surveys on infected banana plants have been continually performed in the pest control areas to ensure that the pest status of *Foc* TR4 would soon be declared absent. Recently, the occurrence of *Foc* TR4 infected in banana plants was eliminated from the farm (Department of Agriculture of Thailand, 2020).

2.1.5 Disease management

Principally, *Foc* is difficult to control for many reasons. Firstly, it is a soil–borne pathogen with a long survival (more than 20 years) in the soil, even in the absence of plant hosts (Buddenhagen, 2009) or within alternate hosts which do not necessarily show disease symptoms (Hennessy *et al*., 2005). Second, it passed through into the plant as a vascular pathogen. Third, it can be spread by banana

vegetative propagation material, soil vectored by workers and machinery, irrigation water, and/or pest. Last, the agricultural practice for banana planting is a monoculture, especially Cavendish varieties in the case of *Foc* TR4, which facilitates the pathogen spread.

There are limited choices for managing Fusarium wilt of banana. Researchers and workers attempt to study many ways such as using pathogen quarantine systems, cultural control, physical control, chemical control, and biological control.

2.1.5.1 Quarantine, exclusion, and personal awareness

In pathogen–free regions, effective quarantine and exclusion are necessary. The pathogen cannot be eradicated from the soil once it is infested and exclusion of the pathogen from noninfested plantations can be very difficult once it moves into a region. For example, Venezuela separated banana farms into three areas, high–risk areas, moderate to high–risk areas, and moderate to low–risk areas, which differ in management (Olivares *et al*., 2021). For Thailand, the quarantine area appeared in the Year 2019 in Chiang Rai province (Department of Agriculture of Thailand, 2019). On personal awareness, each person working on the farm must be aware of the measures that they have to take in preventing the spread of the disease such as using some disinfectants to sterilize the farming tools, equipment, and footwear.

2.1.5.2 Cultural control

The selection of resistance varieties is the choice for banana farming because they have the most effective to manage this disease. Three levels of resistance are complete resistance (qualitative resistance), intermediate resistance (quantitative resistance), and susceptibility (Dita *et al*., 2018). Cavendish (AAA)–*Foc* R1 interaction is a complete resistance variety. Intermediate resistance shows less severe symptoms or damage than susceptible varieties when grown under similar environmental conditions and inoculum pressure. The Prata (AAB)–*Foc* R1 interaction is a variety of this group. Most of the varieties are susceptible including *Gros Michel*–*Foc* R1, Cavendish–*Foc* TR4, and Silk–*Foc* R1 which the pathogen causes serious physiological disturbances and yield losses. In progress, using of new technologies such as molecular techniques, genomic and proteomic studies helps researchers discover the method to control the disease. For example, non–traditional improvement, mutation breeding, somaclonal mutants, or genetic transformation of banana were suggested, but the fruit products from these techniques will be accepted in the marketplace is not clear (Ploetz, 2015).

In susceptible cultivars, mixed plantings in small–scale or subsistence agriculture have been suggested as an alternative. The diverse banana cultivars are planted with different crops, and regulary develop more moderate losses than if they had been planted in monocultures (Stover, 1962). A mixed culture system involving cereals, legumes, and multipurpose trees in banana plantations can also improve the production yield of banana and developed tolerance against the disease (Dowiya *et al*., 2009). Interestingly, crop rotation systems were studied to reduce the occurrence of disease. Different crop rotation systems have a varied effect on the suppression of different diseases via mechanisms such as interrupting the pathogen life cycle, variation in the establishment of antagonistic microbes showed due to variation in plant root exudates, and production of allelochemicals (Christen and Sieling, 1995 ; Winter *et al*., 2014). The example of crop plants such as Chinese leek and paddy could control Fusarium wilt, and it is speculated that the control is due to the release of antifungal compounds from root exudates or leaf leachates such as 1–dimethyl–2–pentenal and dimethyl trisulphide (Zhang *et al*., 2013a). Pattison *et al*. (2014) found that Pinto peanut (*Arachis pintoi*) as ground cover reduced the intensity of Fusarium wilt in Ducasse bananas (Pisang awak, ABB) by 20%. Pineapple or maize suppressed the *Foc* growth (Wang *et al*., 2015a).

2.1.5.3 Physical control

Physical methods of disease control and prevention are based on the physiological tolerance of disease agents to negative conditions such as increased or decreased temperature, lack of moisture, presence of deleterious irradiation, and the removal of pathogen sources or presence of physical barriers to prevent contact between the disease agents and host.

Rice hull burning (hot sterilization of the soil) has been used in Phillipines and Indonesia (Molina *et al*., 2010). The material is mounded on the top of an affected mat and burned, apparently generating sufficient heat to kill the pathogen. Short–term control using solarization with methyl bromide fumigation can be reduced *Foc* for one cycle (Herbert and Mark, 1990) and the symptom developed after solarization for ten months (Hermanto *et al*., 2012). Stover (1962) used flood fallow to rejuvenate *Foc*–infested soil but flooded soil was rapidly recolonized by the pathogen and uneconomic that has a high cost of labor, machinery, and engineering processes. On laboratory scale, Wen *et al*. (2015) reported that *Foc*–infested soil populations rapidly reduced by more than 90% in the first 15 days and then fluctuated till the end of incubation after incorporation with rice or maize straw at rates of 1.5 tons/ha and 3.0 tons/ha was incubated under flooded or water–saturated (100% water holding capacity) conditions at 30 °C for 30 days.

Soil amendments by adding nitrate or ammoniacal nitrogen to the planting soil was used (Elmer, 2012). Nitrate decreases the severity of these diseases, while high levels of ammonium could inhibit penetration of banana roots by *Foc* (Zhang *et al*., 2013b). Fortunato *et al*. (2012) evaluated the effect of silicon (Si) in reducing the symptoms of Fusarium wilt on banana plants. Their results showed that supplying Si to banana plants, especially to a susceptible cultivar against *Foc*, had great potential in reducing the intensity of Fusarium wilt.

2.1.5.4 Chemical control

The use of chemical control has been limited or has questionable efficacy. *In vitro* and greenhouse experiments, root dips with benzimidazole, demethylation inhibitors, phosphonate, and strobilurin fungicides cannot destroy the pathogen (Nel *et al*., 2007). Rhizome injection with 2% carbendazim (Bavistin 50 WP) protected Rasthali banana from *Foc* (Lakshmanan *et al*., 1987) but had no effect in a severe cases in South Africa (Herbert and Mark, 1990). The Cu (II) complexes of 2–(diphenylmethylene) hydrazinecarboxamide derivatives inhibit the growth of *Foc* TR4 (Chan *et al*., 2017). Commercial products were used to control the fungal such as Sporekill (poly dimethyl ammonium chloride), which killed conidia of *Foc* in the 30s, Jik (sodium hypochlorite), and Prazin agri (polymeric biquanidine hydrochloride and quaternary ammonium compound), which killed conidia in 5 min (Nel *et al*., 2007 ; Meldrum *et al*., 2013). These products were more effective disinfectants than copper oxychloride, Farmcleanse (quaternary ammonium compound), Domestos (sodium hypochlorite), which had been used in banana plantations (Meldrum *et al*., 2013). In Thailand, Singburaudom (2009) and the Plant Protection Research and Development Office, Department of Agriculture suggested that injected 2% carbendazim in root areas with 3 ml in months 5, 7, and 9 after planting.

2.1.5.5 Biological control

Biological controls of Fusarium wilt in banana offers a complementary disease management approach (Gang *et al*., 2013 ; Raza *et al*., 2016). However, there has been little field research in which long–term biocontrol efficacy has been evaluated for Fusarium wilt of banana (Ploetz, 2004). The use of biocontrol agents (BCAs) has been proved to be an environmentally friendly disease management strategy in recent years (Kavino *et al*., 2016 ; Deltour *et al*., 2017 ; Fu *et al*., 2017).

Figure 2.5 show schematize show BCAs can act directly or indirectly against *Foc*. On the left, direct antagonism may be due to antibiosis

(antibiotic, lytic enzymes, volatile organic compounds), parasitism, targeting pathogenic potential (detoxification of virulence factors), and competition for nutrients and space (siderophore or biofilm production). On the right, induction of plant local/systemic resistance, plant growth–promoting or changes of soil/plant microbiota in favor of more beneficial microbial taxa are typical mechanisms that indirectly act against the pathogen, or at least contribute to decreasing the infections or the disease (Bubici *et al*., 2019).

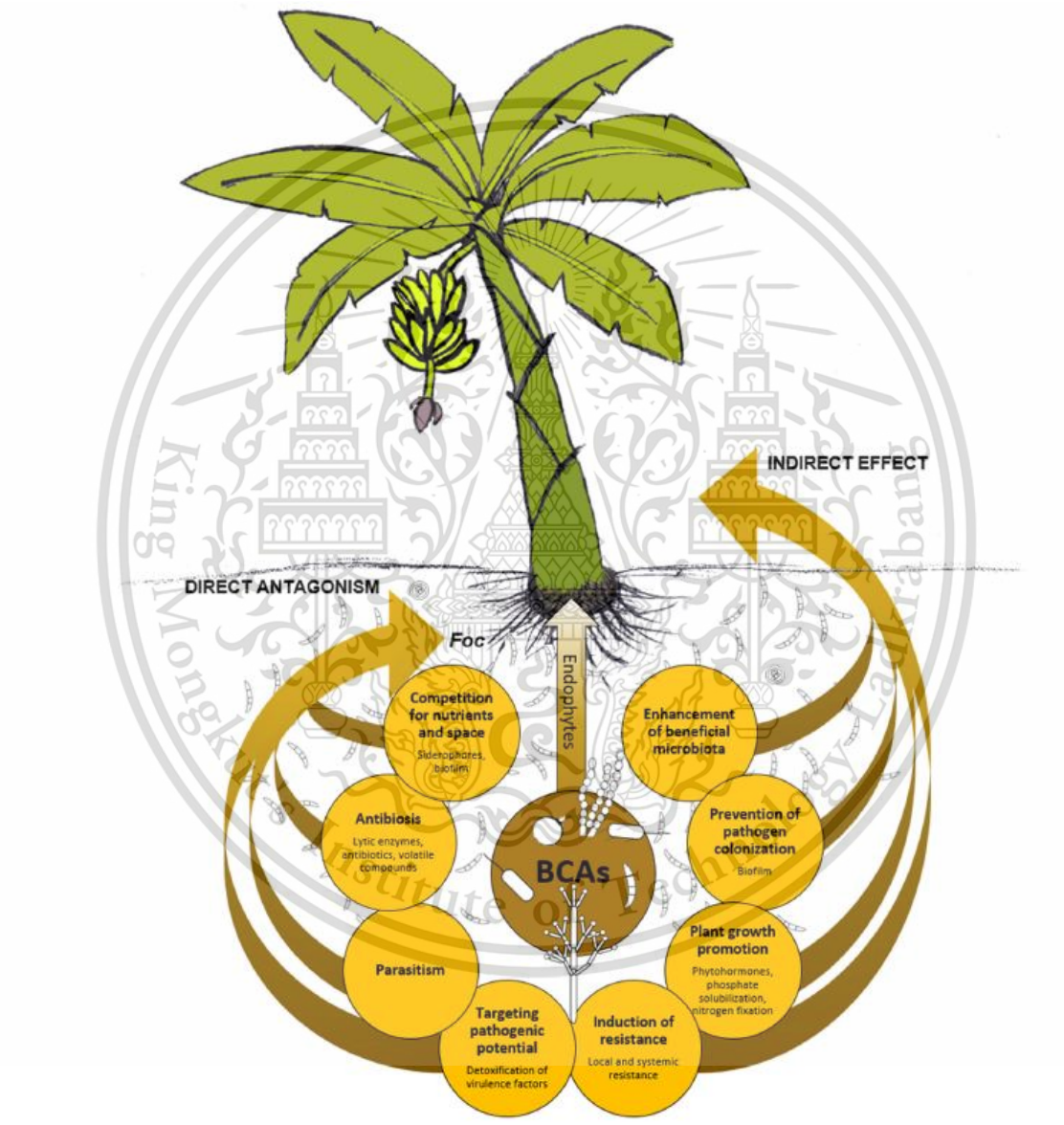


Figure 2.5 Possible modes of action of biological control agents (BCAs). Beneficial microorganisms can show direct antagonism against *Fusarium oxysporum* f.sp. *cubense* (*Foc*) and can affect the plant physiology and/or the microbiota with a consequent, indirect effect against the pathogen.

Source: Bubici *et al*. (2019)

In its natural habitat, it has much diversity of microbes that had been tested by the early 2000s. Most of the published work resulted from laboratory tests or short–term greenhouse studies (Table 2.2). In the field trial test, many researchers used the microorganisms to control Fusarium wilt of banana that can increase the fruit yield such as Thangavelu and Goli (2015a) showed mixed endophytic bacteria, *Pseudomonas putida* C4r4, *Achromobactrum* sp. Gcr1, *Rhizobium* sp. Lpr2 and *Bacillus flexus* Tvpr1, formulation products to control the pathogens that can increase the fruit yield to 214%. Kavino and Manoranjitham (2018) used banana root dipping technique with the suspension of *Pseudomonas fluorescens* Pf1 and *Bacillus subtilis* EPB 10 and EPB 56. The other microorganisms that were tested in the field experiment are shown in Table 2.3.

In actinobacteria genera, Cao *et al*. (2004) found *Streptomyces griseorubiginosus*–like strains can against *Foc* in banana roots. *Streptomyces griseus* produced chitinase and β–1, 3–glucanase to lysis of fungal cell wall and inhibit sporulation (Zacky and Ting, 2013). *Streptomyces griseoruber* sp. nov., *Streptomyces chrysomallus* and *Streptomyces alanosinicus* isolated from rhizosphere soil showed antagonistic activity against *Foc* on plate assay (Ramasamy *et al*., 2017). Duan *et al*. (2020 ; 2021) reported that the crude extract of *Streptomyces* ma. FS–4 at 100 μg/ml significantly reduced the activity of the mitochondrial complex III of *Foc*4 (52.45%) and succinate dehydrogenase (60.20%). Li *et al*. (2021) reported that the crude extract of *Streptomyces* sp. H4 inhibited mycelial growth, spore germination, and tube elongation of *Foc* TR4 and fermentation broth of H4 significantly decreased the disease index of *Foc* TR4 and improved the growth of banana seedlings in pot test. Zhu *et al*. (2021) exhibited that *Streptomyces morookaensis* strain Sm4–1986 significantly inhibited mycelial growth and spore germination of pathogen *Foc*–TR4 on plate assay. Interestingly, a novel antibiotic 210–A, named as (6S, 8aS, 9S, 11S, 12aR)–6–hydroxyl–9, 10–dimethyldecahydrobenzo[d]azecine–2, 4, 12(3*H*)–trione was isolated from the fermentation broth of *Streptomyces noursei* Da07210 that has strong activity against *Foc* race 4 (Wu *et al*., 2009).

Disease suppressive soils or soil supplements are soils or organic materials which contain a virulent pathogen and do not cause typical levels of disease on the susceptible hosts and plant toxic. Materials were formed as microbial soil inoculation or biofertilizer than can control the disease. It has microbial communities displaying higher richness or heavy inoculum and diversity and a higher number of antagonistic members (Shen *et al*., 2015). Zhang *et al*. (2014) performed the matured compost mixed with antagonists *Paenibacillus polymyxa* SQR–21 and *Trichoderma harzianum* T37 (BIO1), *Bacillus amyloliquefaciens* N6 (BIO2), *Bacillus subtilis* N11 (BIO3), and the combination of N6 and N11 (BIO4), all formula showed

the application of BIOs significantly decreased the incidence rate of Fusarium wilt by up to 80% compared with the control. Shen *et al*. (2015) studied suppressive soils to Fusarium wilt of banana on Hainan Island, China, and found higher richness and diversity indices as well as more operational taxonomic units in the suppressive than in the conductive soils. *Chthonomonas* spp., *Pseudomonas* spp., and *Tumebacillus* genera were significantly enriched in the suppressive soil. *Bacillus amyloliquefaciences* NJN–6 was used as organic fertilizer to reduce the severity of the disease (Xue *et al*., 2015). Fu *et al*. (2017) induced the rhizosphere microbiome by biofertilizer to suppress *Foc* which consists of the microbial genus *Sphingobium*, *Dyadobacter*, *Cryptococcus*, and *Burkholderia*. Köberl *et al*. (2017) studied healthy versus *Foc*–infected banana plants in Central America, and found higher richness and diversity of *Gammaproteobacteria* in healthy plants. In addition, healthy plants also revealed an increase in potential plant–beneficial *Pseudomonas* and *Stenotrophomonas* species.

Table 2.2 Example of biocontrol field experiment against *Fusarium* of banana.

Biocontrol agents	Mode of applications	*Foc* inoculum	References
Endophytes			
Acremonium sp. Q34	Root dripping into the fermentation broth	10^5 conidia/mL	Liu and Lu (2013)
Burkholderia cenocepacia 869T2	Root dipping (OD_{600} = 0.6–0.7)	Natural infestation	Ho *et al*. (2015)
Pseudomonas putida C4r4, *Achromobactrum* sp. Gcr1, *Rhizobium* sp. Lpr2, *Bacillus flexus* Tvpr1	Talc powder formulation of bacterial consortia (10^8 cells/g)	Natural infestation	Thangavelu and Gopi (2015a)
Serratia marcescens ITBBB5-1	Pre-planting soil drenching	Pre-planting soil drenching (10^6 conidia/mL)	Tan *et al*. (2015)
Trichoderma asperellum Prr2 (endophyte), *Trichoderma* sp. NRCB3 (rhizospheric)	Colonized rice chaffy grains	Natural infestation	Thangavelu and Gopi (2015b)
***Pseudomonas* spp.**			
Pseudomonas fluorescens Pf1	Different combinations of paring and pralinage with a *P. fluorescens* formulation ($2.5x10^8$ CFU/g), soil application, and capsule application	Natural infestation	Raguchander *et al*. (2000)
***Bacillus* spp.**			
Bacillus subtilis TRC 54	Soil application combined with *Pseudomonas fluorescens* Pf1 and a plant extract–based fungicide	Natural infestation	Akila *et al*. (2011)
Bacillus spp. (PHC Biopak®)	Soil inoculation in the nursery	No data	Mukhongo *et al*. (2015)
Bacillus amyloliquefaciens W19	Colonized bio–organic fertilizer (10^9 CFU/g)	Natural infestation	Wang *et al*. (2016)
***Serratia* sp.**			
Serratia marcescens	Bentonite and kaolin formulations	Natural infestation	Ting *et al*. (2011)
***Trichoderma* spp.**			
Trichoderma viride	Colonized rice chaffy grains ($1x10^{31}$ CFU/g) + 5% jaggery solution	Colonized sand:maize mixture	Thangavelu and Mustaffa (2010)

Table 2.2 Example of biocontrol field experiment against *Fusarium* of banana (continued).

Biocontrol agents	Mode of applications	*Foc* inoculum	References
Trichoderma viride	Root dipping (10^6 conidia/ml), followed by application of colonized wheat bran:saw dust mixture	Natural infestation	Raguchander *et al*. (1997)
Trichoderma harzianum (ECO–T®)	Soil inoculation in the nursery	Natural infestation	Mukhongo *et al*. (2015)
Arbuscular mycorrhizal fungi			
Glomus mosseae, *Trichoderma harzianum*	Inoculation at transplanting	Colonized sorghum grains ($1.5x10^6$ CFU/g)	Mohandas *et al*. (2010)
Glomus clarum	Soil inoculation in the nursery	No data	Lin *et al*. (2012a)
Rhizatech®	Soil inoculation in the nursery	No data	Mukhongo *et al*. (2015)
Non-pathogenic *Fusarium oxysporum*			
Fusarium oxysporum Ra–1, Ro–3	Colonized sand:maize mixture	Colonized sand:maize mixture	Thangavelu and Jayanthi (2009)

Source: Adapted from Bubici *et al*. (2019)

Table 2.3 Example of biocontrol pot experiment against *Fusarium* of banana.

Biocontrol agents	Mode of applications	*Foc* inoculum	References
Endophytes			
Streptomyces griseorubiginosus S96	Root dipping (10^6 spores/ml)	10^4 conidia/ml	Cao *et al*. (2005)
Burkholderia spp. AB202 and AB213, *Herbaspirillum* spp. BA227 and BA234	Root dipping ($5x10^7$ CFU/ml)	No data	Weber *et al*. (2007)
Pseudomonas fluorescens Pf1, *Bacillus subtilis* EPB10 and EPB 56	*In vitro* co-culturing of plants with bacteria	*In vitro* co-culturing of plants with *Foc* after bacteria inoculation	Kavino *et al*. (2014)
Erwinia chrysanthemi E353	No data	No data	Yin *et al*. (2009)
Fusarium oxysporum BRIP 29089, 29093 and 45952	Colonized ground millet	Colonized ground millet	Forsyth *et al*. (2006)
Penicillium citrinum	Soil drenching (10^6 Conidia/ml)	10^6 CFU/ml	Ting *et al*. (2012)
Lasiodiplodia theobromae TDC029, *Ceratobasidium* sp. TDC037, TDC241, TDC474, *Trichoderma asperellum* TDC075	Root dipping ($1x10^6$ CFU/ml)	10^6 spores/ml	Catambacan and Cumagun (2021)
Trichoderma sp. TJ5	Root dipping ($1x10^6$ CFU/ml)	Soil inoculation (10^6 CFU/ml)	Caballero Hernández *et al*. (2013)
Trichoderma asperellum	No data	No data	Chaves *et al*. (2016)
***Streptomyces* spp.**			
Streptomyces violaceusniger	Soil drenching (10^8 CFU/ml)	10^4 or 10^6 conidia/ml	Getha *et al*. (2005)
Streptomyces lunalinharesii B-03	Fermentation broth	10^6 spores/ml	Zhou *et al*. (2017)
Streptomyces sp. YYS–7	Soil inoculation with 10^6 CFU/g soil	10^6 spores/ml	Wei *et al*. (2020)
Eight actinomycetes	Fermentation broth	$1.85x10^6$ conidia/ml	Qin *et al*. (2010)
***Pseudomonas* spp.**			
Pseudomonas fluorescens Pfcp	Root dipping (10^8 CFU/ml)	10^8 conidia/ml	Sivamani and Gnanamanickam (1988)
Pseudomonas fluorescens	Colonized charcoal (10^8 CFU/ml)	Colonized sorghum seeds ($9.2x10^4$ CFU/ml)	Mohandas *et al*. (2004)
Pseudomonas fluorescens Pf10	Soil drenching (10^9 CFU/ml)	Colonized sand:maize mixture	Thangavelu *et al*. (2003)

Table 2.3 Example of biocontrol pot experiment against *Fusarium* of banana (continued).

Biocontrol agents	Mode of applications	*Foc* inoculum	References
Pseudomonas fluorescens Pfm	Talc powder formulation (10^8 CFU/g)	10^6 conidia/ml	Saravanan *et al*. (2004)
***Bacillus* spp.**			
Bacillus thuringiensis	Root dipping (10^6 CFU/ml)	No data	Shamarao *et al*. (2001)
Bacillus licheniformis C–4	Root dipping	No data	Sun and Wang (2009)
Bacillus subtilis KY–21	Soil drenching ($5x10^5$ CFU/ml)	Soil drenching ($5x10^5$ CFU/ml)	Sun *et al*. (2011)
Bacillus spp. RZ 1, 3, 10, 34, 35, 60, 69, and 76	Root dipping (OD_{540} = 0.5)	Soil drenching (10^5 CFU/ml)	Ribeiro *et al*. (2012)
Bacillus amyloliquefaciens W19	Colonized bio–organic fertilizer (10^9 CFU/g)	Naturally infested field soil ($1.5x10^4$ CFU/g)	Wang *et al*. (2013)
Bacillus amyloliquefaciens WJ22	Colonized bio-organic fertilizer ($3x10^8$ CFU/g)	Naturally infested field soil ($1x10^3$ CFU/g)	Wang *et al*. (2015b)
***Trichoderma* spp.**			
Trichoderma harzianum	Soil inoculation	No data	Ting *et al*. (2003)
Trichoderma viride	Colonized corn grits	Colonized corn–meal:sand	Bastasa and Baliad (2005)
Trichoderma harzianum A34	Soil inoculation ($8x10^9$ CFU/g)	Naturally infested soil	Pérez–Vicente *et al*. (2009)
Trichoderma asperellum PZ6	Root-injury irrigating method	Root–injury irrigating method	Qin *et al*. (2017)
Arbuscular mycorrhizal fungi			
Glomus intraradices, *Glomus* spp.	Soil inoculation in the nursery	No data	Jaizme–Vega *et al*. (1998)
Glomus fasciculatum	Soil culture (500 chlamydospores)	No data	Habeeba *et al*. (2003)
Gigaspora margarita	Soil inoculation in the nursery	No data	Borges *et al*. (2007)
Native arbuscular mycorrhizal fungi	$3.5x10^3$ or $7x10^3$ per kg	10^6 conidia/ml	Sampaio *et al*. (2012)
Non-pathogenic *Fusarium oxysporum*			
Fusarium oxysporum CAV 255 and CAV 241	Soil drench (10^7 spores/ml)	Colonized millet seeds	Nel *et al*. (2006)
Other microorganisms			
Rhizospheric strains FB5, FB2, T2WF, T2WC, and W10	Root dipping	No data	Yang *et al*. (2006)
Paenibacillus spp. RZ–17 and RZ–24	Root dipping (OD_{540} = 0.5)	Soil drenching (10^5 CFU/ml)	Ribeiro *et al*. (2012)

Source: Adapted from Bubici *et al*. (2019)

However, integrated practices are required including improved farming technologies like integrated cultural, physical, and biological control, reduction of chemical usage and the practices depend on cost, labor, land, and site–specific management.

2.2 Actinomycetes

Actinomycetes are filamentous Gram–positive bacteria that have a high G+C genomic G+C content of over 55%. These bacteria are aerobic and some facultative anaerobes or aerobes. The name “Actinomycetes” was originally derived from the Greek word “attacks” means (a ray) and “mykes” means (fungus), so having a related characteristic in both bacteria and fungi. Moreover, they possess sufficient characteristic details that use to determine them into “Kingdom of bacteria” (Das *et al*., 2008). The Bergey’s Manual of Systematic Bacteriology Volume five (Second edition): The Actinobacteria (Whitman *et al*., 2012) is divided into six classes, namely *Actinobacteria*, *Acidimicrobiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria* and *Thermoleophilia* with 19 orders, 50 families and 221 genera (Ludwig *et al*., 2012). Actinomycetes are widespread environmental organisms and are found in both aquatic and terrestrial habitats such as soil, sediment, plant tissue, animal tissue, freshwater, seawater, etc. Actinomycetes produce a variety of secondary metabolite such as bioactive and agroactive compounds for pharmacological and commercial interest.

2.2.1 Classification of actinomycetes

Actinomycetes are in the domain of eubacteria that grow as filamentous mycelia and form spores like fungi. They differ from fungi by having no nuclear membrane in the cell and hyphae size is smaller (0.5 to 1.0 μm in diameter) than fungi (3 to 8 μm in diameter). Furthermore, the cell wall type of actinomycetes is peptidoglycan without chitin or cellulose (Avery and Blank, 1954). They produce asexual spores such as conidiospore or conidia (non–motile asexual spore), sporangiospores (motile asexual spore), and a fragment of hypha (arthrospore) on aerial or substrate mycelia. Actinomycetes are not capable of photosynthesis. Most actinomycetes are saprophytes that are growing by decomposing organic matter. Some actinomycetes are parasitic or mutualistic related to animals or plants as pathogens (Goodfellow and Williams, 1983). Actinomycetes are widespread environmental organisms found in the terrestrial environment such as soil, sediment, plant tissue, and animal tissue and in aquatic environments such as freshwater and marine. They were divided into many types such as mesophile (can grow at 20–40 °C), thermotolerance (can survive at 50 °C), moderately thermophile (can grow 45–55 °C), strictly thermophile (can grow at 55–60 °C) (Jiang and Xu, 1993 ; Jensen *et al*., 2005a), acidophile (can grow at pH 3.5–6.5, optimum at 4.5–5.5) (Khan and Williams, 1975 ; Hagedorn, 1976), extreme halophile (can grow at 2.5–5.2 M salt), moderate halophile (can grow at 0.5–2.5 M salt), endophytic actinomycetes,

symbiotic actinomycetes, endosymbiotic actinomycetes and gut actinomycetes (Anandan *et al*., 2016).

Stackebrandt *et al*. (1997) proposed a new hierarchic classification system for the taxa during the taxonomic levels of genus and class for the actinomycete line of descent as defined by analysis of small subunit (16S) rRNA and genes coding for this molecule (rDNA) (Figure 2.6).

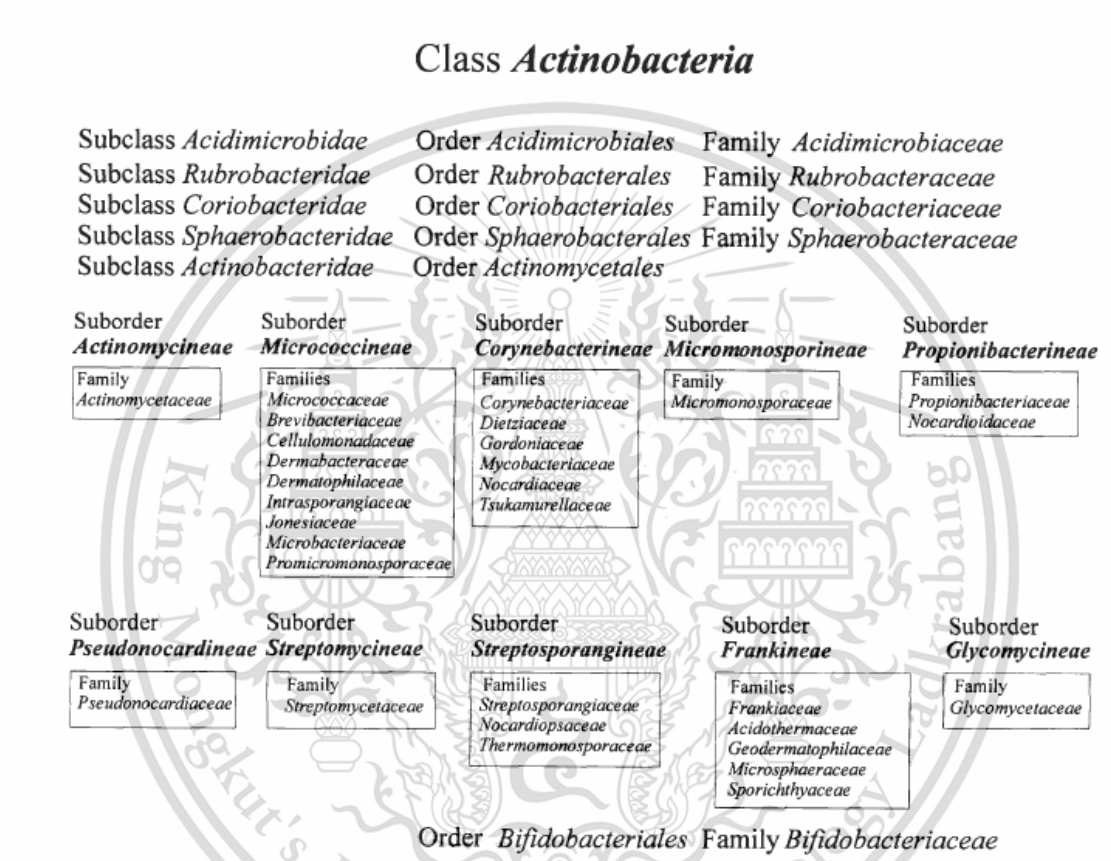


Figure 2.6 Proposed hierarchic classification system of the class *Actinobacteria* based on the phylogenetic analysis of the 16S rDNA/rRNA sequence data.

Source: Stackebrandt *et al*. (1997)

2.2.2 Distribution of actinomycetes in the nature

Actinomycetes are widespread in several habitats in nature. These bacteria are found in basically every natural substrate such as soil, compost, sediment, muds, freshwater, seawater, deep seas, food substances, air, plant tissue, animal tissue, etc. Some habitats are a permanent environment of actinomycetes where they grow and reproduce. In contrast, other habitats act as temporary environments where they are spread out by water and air movements.

2.2.2.1 Soil

Soils are the most important habitat for actinomycetes. More than 90% of actinomycetes genera have been isolated from soil (McCarthy and Williams, 1990). From many reports, *Streptomyces* was interviewed to be the most plentiful genus strains in soil. They were found in organic soil horizon (Hagedorn, 1976), paddy field soil (Priyadharsini and Dhanasekaran, 2015), arid or desert soil (Mansour, 2013 ; Mohammadipanah and Wink, 2016), cool atmospheric soil (Xu *et al*., 1996 ; Raja *et al*., 2010), acidic soil (Zakalyukina *et al*., 2002 ; Poomthongdee *et al*., 2015), alkaline saline soil (Selyanin *et al*., 2005 ; Djebaili *et al*., 2020) or cave soil (Rangseekaew and Pathom–aree, 2019). Soil actinomycetes may relate to plant development by producing plant growth–promoting substances and/or anti–plant pathogens compounds such as antifungal compounds (Hamby and Crawford, 2000). The soil actinomycetes such as *Steptomyces*, *Streptoverticillium*, *Streptosporangium*, *Actinomadura*, *Kitasatosporia*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Kibdelosporangium*, *Micromonospora*, and *Rhodococcus* were found in soil.

2.2.2.2 Compost

Compost contains various populaces of actinomycetes who play a key role in microbial community during composting. Composting process has three steps, the mesophilic or moderate–temperature phase, the thermophilic or high–temperature phase, and maturation phase, which all steps can last from several weeks to months. Therefore, the presence of actinomycetes has more than one group, for example, mesophile, thermophile, thermotolerance, aerobic, alkaline, and acidic pH growth conditions group, etc. Moreover, they have the different properties of degrading organic complexes such as cellulose, hemicellulose, lignin, chitin, carbohydrates, lipids, and proteins by using enzymes that they secrete from the cell. The strains isolated from several composts are *Streptomyces* (Goodfellow and Simpson, 1987 ; Cuesta *et al*., 2010 ; Rojas *et al*., 2015), *Lechevalieria* (Cuesta *et al*., 2010), *Thermasporomyces composti* gen. nov., sp. nov., a thermophilic actinomycete isolated from compost (Yabe *et al*., 2011), *Thermoactinomyces guangxiensis* sp. nov.,

a thermophilic actinomycete isolated from mushroom compost (Wu *et al*., 2015), *Thermomonospora catenispora* sp. nov., isolated from mushroom compost (Wu *et al*., 2019), *Demetria terragena* gen. nov., sp. nov., a new genus of actinomycetes isolated from compost soil (Groth *et al*., 1997) and *Nonomuraea thermotolerans* sp. nov., a thermotolerant actinomycete isolated from mushroom compost (Wu and Liu, 2016).

2.2.2.3 Aquatic environment

Watery habitat highly varies about 70% from earthy natural surroundings. Some watery actinomycetes may be from the terrestrial environments such as soil that can be washed into aquatic habitats where they are rested in sediments, mud, or living cells, and some are indigenous actinomycetes. Two types of the aquatic environments, freshwater and marine, were considered.

Commonly genera present in freshwater are *Actinoplanes*, *Micromonospora*, *Nocardia*, *Rhodococcus*, *Streptomyces*, and the endospore–forming *Thermoactinomyces* (Cross, 1981). Johnston and Cross (1976) showed that micromonosporae were found to be predominant in various lakes while the report of Al–Diwany *et al*. (1978) showed a significant correlation between micromonosporae and thermoactinomycetes isolated from the river Wharfe in West Yorkshire, where an increased number of micromonosporae was found in the adjoining soil. Other inhabitants of freshwater include *Actinomadura*, *Mycobacterium*, *Arthrobacter*, *Corynebacterium*, and *Nocardia* species.

The marine environment including soil, sediments, mud, and living organisms is an untapped source of novel actinomycetes diversity and thus of new secondary metabolite. Firstly, actinomycetes were isolated from the marine environment since 1969 by Weyland (Weyland, 1969). The marine actinomycetes are commonly more problematic for isolation and culture compared with the terrestrial regions due to the special growth conditions, salt minerals, sea water, and unknown factors (Zotchev, 2012). They had to adapt from extremely high pressure and anaerobic conditions at temperatures just below 0–8 ℃ on the deep–sea floor to high acidic conditions at temperatures of over 8–100℃ near hydrothermal vents at the mid–ocean ridges. *Rhodococcus marinonascene* is the first marine actinomycetes to be characterized (Helmke and Weyland, 1984). The indigenous genus was isolated from several composts *Dietzia*, *Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium marinum, Williamsia maris*, *Verrucosispora*, *Micromonospora*, *Microbispora*, *Nocardia* and *Xestospongia* (Shejul, 1998 ; Magarvey *et al*., 2004 ; Stach *et al*., 2004 ; Bull *et al*., 2005 ; Jensen

et al., 2005a ; b ; Jongrungruangchok *et al*., 2008 ; Thawai *et al*., 2008 ; Tanasupawat *et al*., 2010 ; Supong *et al*., 2012 ; 2013a ; b).

2.2.2.4 Air

Actinomycetes spores are known to be important air contaminants in many occupational habitats such as waste composting and agriculture facilities (Lacey, 1989 ; Nielsen *et al*. 1997). Airborne spores of actinomycetes such as *Saccharopolyspora rectivirgula*, *Micropolyspora faeni*, *Micromonospora halophytica*, *Thermoactinomyces vulgaris*, *Streptomyces albus*, and *Nocardia* sp. related to the incidence of allergic alveolitis and other severe health effects (Pepys *et al*., 1963 ; Terho and Lacey, 1979 ; Kagen *et al*., 1981 ; Lacey, 1989 ; Dales *et al*., 1991 ; Lacey and Dutkiewicz, 1994 ; Reponen *et al*., 1998 ; Grigorevski–Lima *et al*., 2006 ; Abdulla *et al*., 2008).

2.2.2.5 Plant Roots

Actinomycetes survive in the rhizoplane, rhizosphere, and endophytes in the roots of various plants. In the case of endophytic actinomycetes, they may exist in both root nodules of leguminous such as *Frankia* strains and non–leguminous plants such as actinomycetes were isolated from *Comptonia* root nodules (Callaham *et al*., 1978). The association between actinomycetes and plants can be formed without harming the plant. They have been demonstrated to promote and improve plant growth, increase yield, enhance soil fertility by producing agroactive compounds, and reduced plant pathogens as well as biotic or abiotic stresses (Vessey, 2003 ; Kumar *et al*., 2014 ; Singh and Dubey, 2018) by producing bioactive compounds or several extracellular enzymes. Actinomycetes inhabit a wide range of plants as symbionts, parasites, or saprophytes. Many researchers isolated and studied the properties of these actinomycetes. Figueiredo de Vasconcellos *et al*. (2010) isolated 130 isolates of actinobacteria by using five different culture media to the comparison their effective. Araújo *et al*. (2000) isolated the genus *Microbispora*, *Streptomyces*, and *Streptosporangium* from the roots and leaves of maize (*Zea mays* L.). *Actinoallomurus liliacearum* and *A. vinaceus* were isolated from the root of *Ophiopogon japonicus* (Koyama *et al*., 2012). Anwar *et al*. (2016) screened rhizospheric actinomycetes for various *in–vitro* and *in–vivo* plant growth–promoting traits and for agroactive compounds. Thawai *et al*. (2018) isolated a novel species of *Jishengella zingiberis* from the root tissue of *Zingiber montanum*. Kuncharoen *et al*. (2019) studied on diversity and antimicrobial activity of endophytic actinomycetes isolated from plant roots in Thailand that the genus *Micromonospora* (21 isolates), *Streptomyces* (14 isolates), *Plantactinospora* (1 isolate) and *Polymorphospora*

(1 isolate) were found. An endophytic novel specie of *Microbispora catharanthi* was found in the root of *Catharanthus roseus* (Klykleung *et al*., 2020). Nammali *et al*. (2021) isolated *Streptomyces coffeae* sp. nov. from the root of *Coffea arabica* (L.).

2.2.2.6 Animals

Actinomycetes have been confirmed to be the causal agents of many human and animal infections such as diphtheria, leprosy, tuberculosis, actinomycosis, cutaneous nocardiosis, and meningitis (Mcneil and Brown, 1994 ; Moniuszko–Malinowska *et al*., 2020 ; Zhou *et al*., 2020). Not only pathogens infecting humans and animals, but also actinomycetes have a rich source of bioactive secondary metabolite or many enzymes. Tan *et al*. (2009) isolated and characterized actinomycetes with probiotic activities from healthy goat feces such as *Streptomyces*, *Nocardiopsis* and *Oerskovia*. Likely, Semwal *et al*. (2018) who identified and screened of actinobacteria from cow feces for industrially important secondary metabolite, and Jiang *et al*. (2013) studied on diversity and bioactivity of cultivable animal fecal actinobacteria. Khucharoenphaisan *et al*. (2012) isolated and identified *Streptomyces niveoruber* from termite's gut against the human pathogen. Interestingly, Parra–Laca *et al*. (2020) isolated seven genera, *Microbacterium*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Kocuria*, *Actinomyces*, and *Micrococcus*, from *Nile tilapia* (*Oreochromis niloticus*) cultivated in semi–intensive systems from Morelos, Mexico central zone.

2.3 Identification of actinomycetes

Currently, the polyphasic approach consisting of phenotypic, chemotaxonomic, and genotypic characteristics that have been used for the identification and classification of actinomycetes.

2.3.1 Phenotypic characteristics

Phenotypic characteristics consist of morphological, cultural, biochemical, and physiological characteristics (Shirling and Gottlieb, 1966 ; Arai, 1975).

2.3.1.1 Morphological and cultural characteristics

Actinomycetes display a different diverse morphology from other bacteria and between species of many genera. The colonization of actinomycetes was firstly developed by the substrate mycelium (primary mycelium) which some hyphae vertically formed aerial mycelium (secondary mycelium), and directly contacted with the air (Figure 2.7). Some genera, *Actinoplanes, Micromonospora*, *Salinispora, Verrucosispora*, etc., not produce an aerial mycelium. The color of substrate, aerial mycelium, and soluble pigments were produced from

actinomycetes. The difference in spore morphology in particular the position, chain, shape, surface, and arrangement of spore were used as key characters to classify the actinomycetes in the genus level (Shirling and Gottlieb, 1966). Moreover, growth and the appearance of colony on various International *Streptomyces* Project (ISP) media (ISP 2–7), czapek's sucrose agar, glucose–asparagine agar (ISP 5 with 1% glucose replacing glycerol), and nutrient agar (Waksman, 1961 ; Shirling and Gottlieb, 1966) were observed.

2.3.1.2 Biochemical and physiological characteristics

Biochemical and physiological properties including the ability to utilize and/or degrade organic materials (carbon sources, nitrogen sources, and insoluble compounds) nitrate reduction, catalase activity, oxidase activity, hydrolysis of starch and casein, urease activity, and gelatin liquefaction were observed. Growth at different temperatures, sodium chloride, and pH are measured (Shirling and Gottlieb, 1966 ; Williams and Cross, 1971 ; Gordon *et al*., 1974 ; Arai, 1975). Additionally, the enzyme activities by using commercial test kits, API ZYM system (bioMérieux, France), are used. All parameters are very useful for the identification of actinomycetes.

2.3.2 Chemotaxonomic characteristics

Chemotaxonomic characteristics are the study of the difference and similarity of chemical properties on the cells present among the microorganisms being classified (Komagata and Suzuki, 1987). Chemotaxonomic properties including the composition of cell wall peptidoglycan, whole–cell sugar, cellular fatty acids, polar lipid composition, and isoprenoid quinones are the key characters for the classification of actinomycetes.

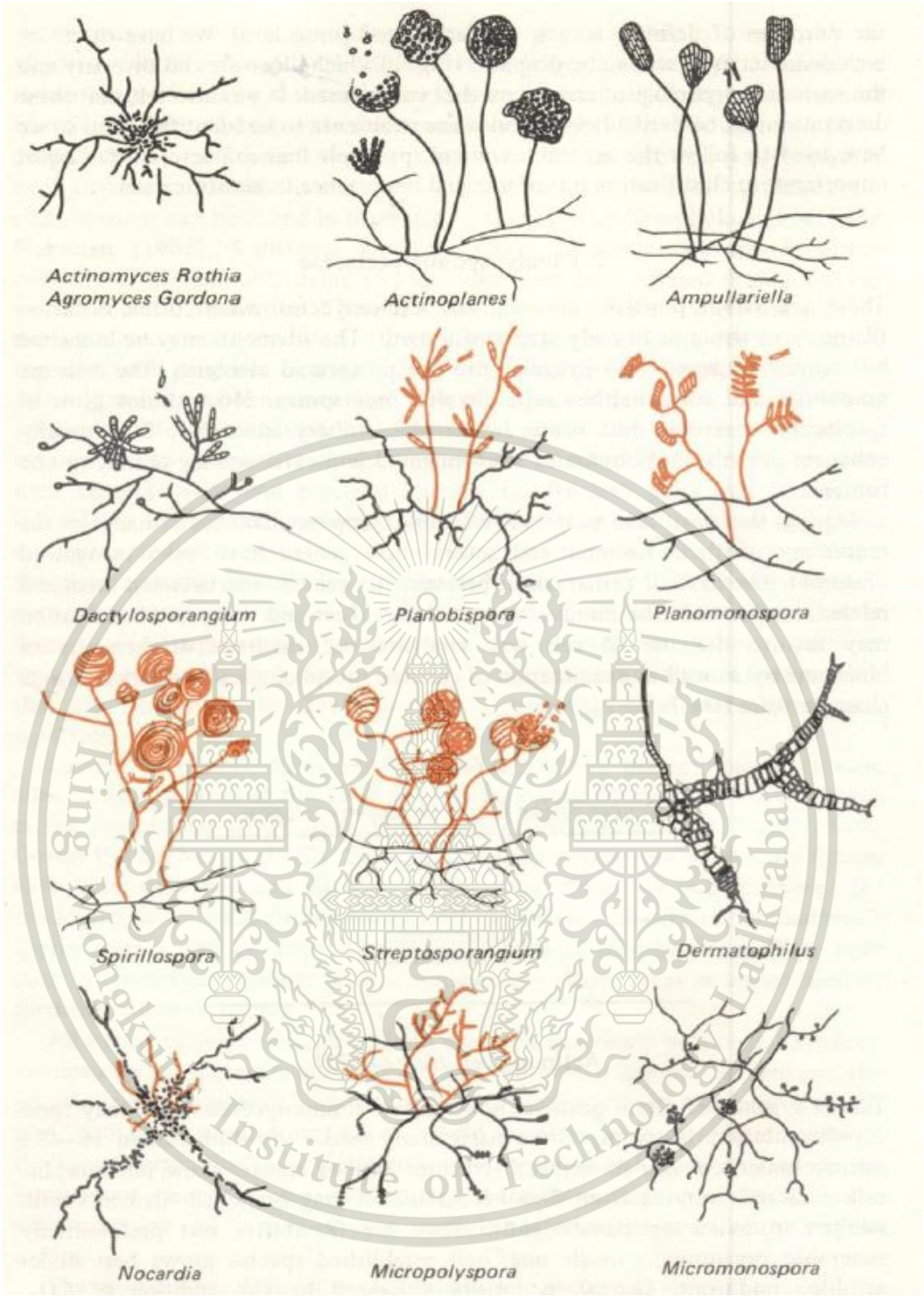


Figure 2.7 Aerial mycelium (orange color) and substrate mycelium (black color) of some actinomycetes on the genus level.

Source: Sykes and Skinner (1973)

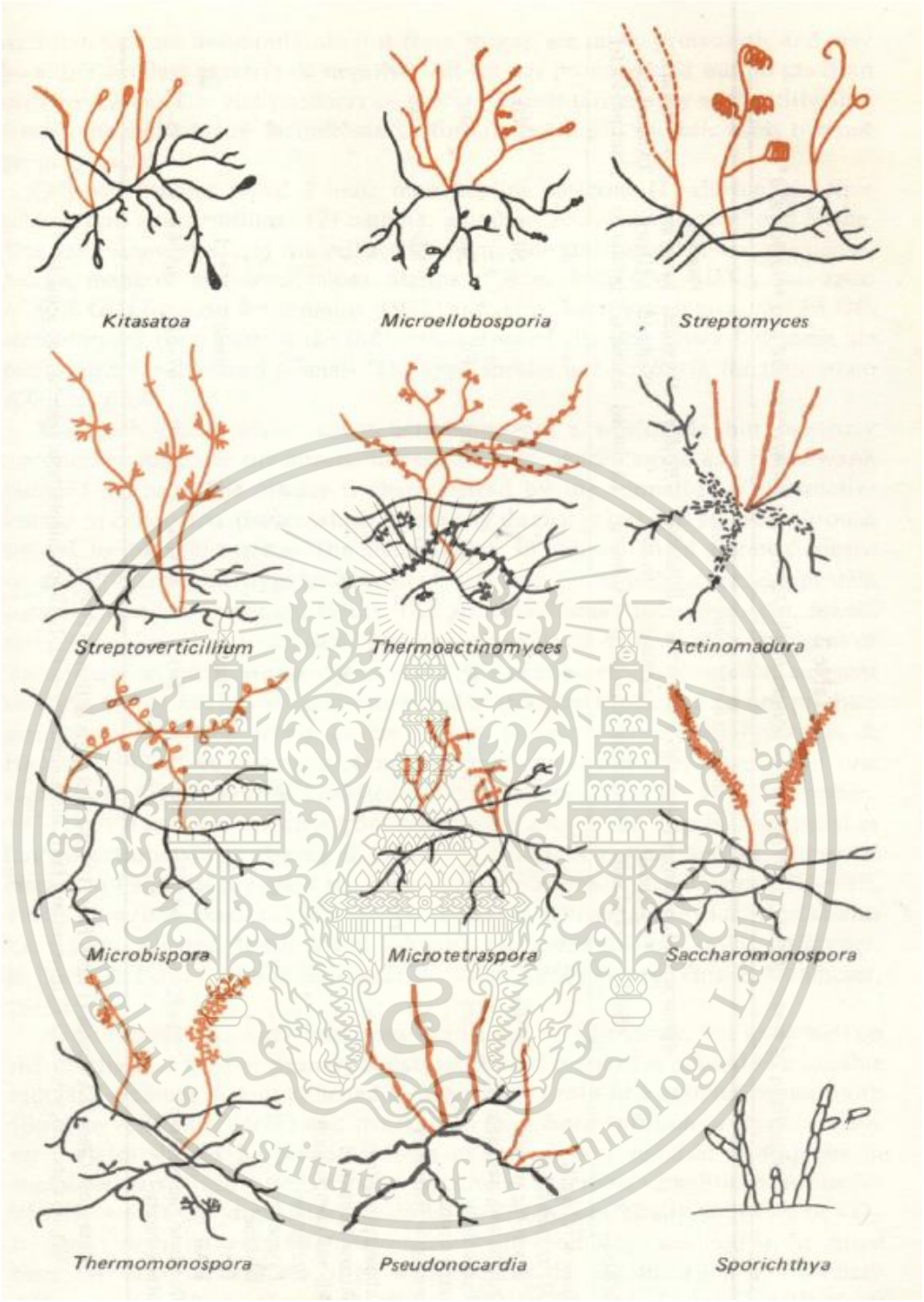


Figure 2.7 Aerial mycelium (orange color) and substrate mycelium (black color) of some actinomycetes on the genus level (continued).

Source: Sykes and Skinner (1973)

2.3.2.1 The composition of cell wall peptidoglycan

The cell wall peptidoglycan type of Gram–positive bacteria is one of the most important and structural indicators of bacterial classification. So, actinomycetes contain cell wall peptidoglycan which consists of two polymers of *N*–actylmuramic acid and *N*–acetylglucosamine, the peptide moiety that links between glycan chains. Three parts of the structure contain the variation of peptide, the isomer of diaminopimelic acid, and *N*–acyl types of muramic acid have been used for the classification of actinomycetes (Figure 2.8). The variations in the peptide moiety are shown in Table 2.4 (Lechevalier and Lechevalier, 1970). All *Streptomyces* species contain only *LL*–diaminopimelic acid isomer, whereas other genera contain *meso*–diaminopimelic acid, 3–OH diaminopimelic acid, 3,4 diaminopimelic acid, 3, 4–dihydroxydiaminopimelic acid and/or the combination of various isomer (Staneck and Roberts, 1974 ; Matsumoto *et al*., 2014). The type of *N*–acyl muramic acid, glycolyl, or acetyl type has been used for actinomyces classification (Uchida and Aida, 1984). Thin-layer chromatography was used to separate the type of diaminopimelic acid isomer that showing the result in Figure 2.9 (Becker *et al*., 1965).

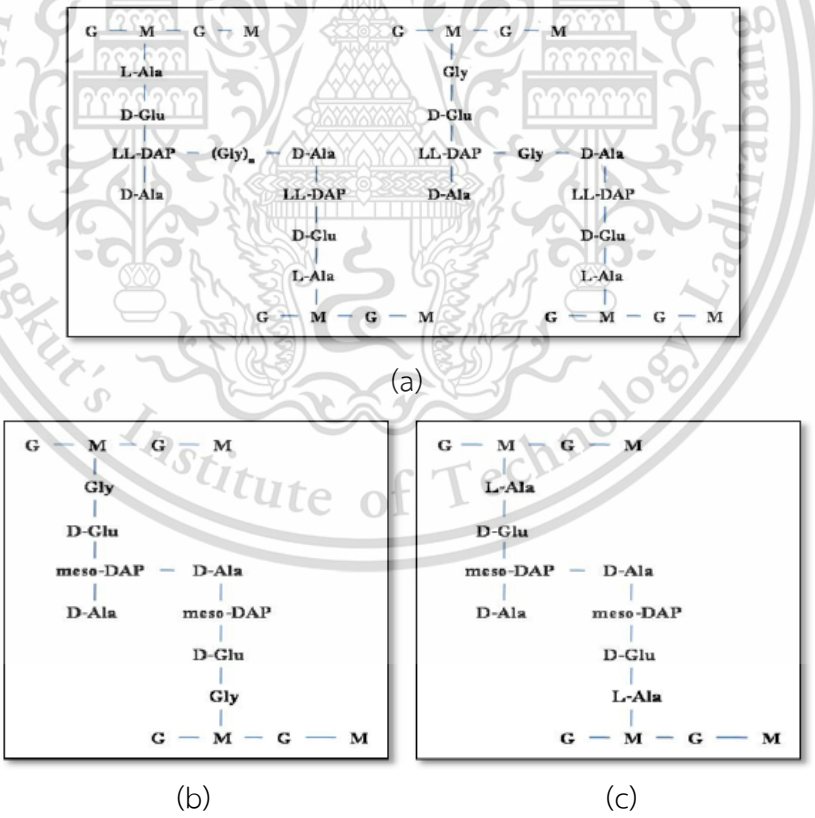


Figure 2.8 Diaminopimelic acid (DAP) structure: *LL*–isomer (a), *meso*-isomer type II (b), *meso*-isomer type III and IV (c) M = NAM, N = NAG.

Source: Kudo (2008)

Table 2.4 Cell wall chemotype on peptidoglycan pattern of the actinomycetes.

Cell wall type	DAB*	Lysine	Ornithine	Aspartic acid	Glycine	*Meso*–DAP	*LL*–DAP	Arabinose	Galactose
I	-	-	-	-	+	-	+	-	-
II	-	-	-	-	+	+**	-	-	-
III	-	-	-	-	-	+	-	-	-
IV	-	-	-	-	-	+	-	+	+
V	-	+	+	-	*	-	-	-	-
VI	-	+	-	+	*	-	-	-	-
VII	+	+	-	+	*	-	-	-	-
VIII	-	-	-	+	*	-	-	-	-

* Glycine is variably present in these groups

** Hydroxyl DAP may be present

+ = present, - = absent

Source: Lechevalier and Lechevalier (1970)

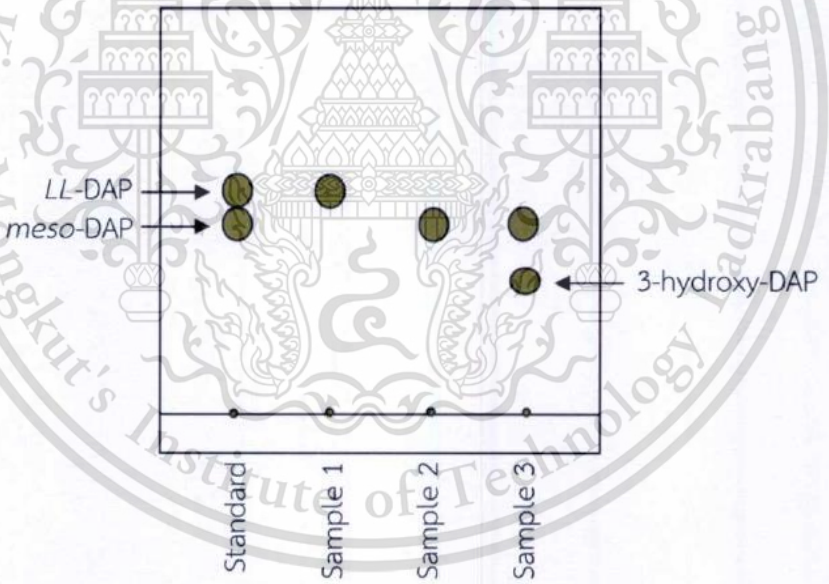


Figure 2.9 The TLC chromatogram of diaminopimelic acid using HPTLC.

Source: Becker *et al*. (1965)

2.3.2.2 Whole–cell sugar

The sugar types in actinomycetes cells have been analyzed for identification and classification of them at the genus level. The patterns were separated into four groups based on the difference in diagnostic sugar in whole–cell hydrolysate as shown in Table 2.5 (Lechevalier and Lechevalier, 1970).

Table 2.5 Whole–cell sugar patterns of actinomycetes with *meso*–diaminopimelic acid.

Pattern	Diagnostic sugar				
	Arabinose	Fuctose	Galactose	Madurose*	Xylose
A	+		+		
B				+	
C	No diagnostic sugars				
D	+				+
E		+			

* Madurose is 3–O–methyl–D–galactose.

Source: Lechevalier and Lechevalier (1970)

2.3.2.3 Cellular fatty acid

The fatty acids are in a form of lipid bilayers of bacterial cell membranes. The variation in cellular fatty acid composition including the number of carbon atoms (12–20 atoms) in a molecule, saturated or unsaturated fatty acids, and *iso*– or *anteiso*–branched fatty acids and 10–methy substituted has been used as a tool for classifying actinomycetes in the family, genus and species levels. Moreover, the presence of mycolic acids (2–hydroxyl–3–alkyl fatty acids with a long alkyl chain) in the genus *Nocardia*, *Mycobacterium*, *Rhodococcus, Coreynebacterium*, and *Gordonia* is used as a key character for classification of these groups. Sasser (1990) found the rapid method for analysis of bacterial cellular fatty acids by using gas chromatography.

2.3.2.4 Polar lipid composition

Actinomycetes phospholipids are located in the cell membrane. Phospholipids are the most common polar lipid types, while glycolipids and amino acids amides are also encountered. Phospholipids are generally derivatives of phosphatidic acid, the most common are phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), acylphosphatidylglycerol (APG), phosphatidylserine (PS),

phosphatidylcholine (PC) and phosphatidylinositol (PI). Actinomycetes also have characteristic glycophospholipids, phosphatidylinositol mannosides (PIMs), which form mono and diacyl dimannosides. In addition, the unknown phospholipids, phosphatidyl glycerol butane–2, 3–diol, were isolated from *Streptomyces olivaceus* (Batrakov and Bergelson, 1978). Glucosamine containing phospholipids of unknown structure has been found in a member of genera *Promicromonospora*, *Microbispora*, and *Streptosporangium* (Lechevalier *et al*., 1977). Lechevalier *et al*. (1977) classified phospholipids composition of actinomycetes into five groups based on the presence and absence of nitrogeneous phospholipids. Type PI shows no nitrogenous phospholipids, PII contains only one nitrogenous phospholipids, PIII has the phosphatidyl choline in phospholipids composition, PIV presence an unidentified phospholipid containing gluocosamine (GluNU), and PV contain phosphatidyl glycerol in addition to GluNU. The difference between phospholipid types is shown in Table 2.6 and phospholipid types of actinomycetes have been used to identify in the genus level shown in Table 2.7.

Two–dimension TLC technique was used to identify the composition of phospholipids by using five reagents detectors including phosphomolybdic acid, Dittmer and Lester, ninhydrin, anisaldehyde, and dragendroff reagents. The positions of all spots are shown in Figure 2.10.

Table 2.6 Phospholipids type of actinomycetes.

Phospholipid type	Polar lipid							
	PIMs	Pi	PC	PG	PE	PME	GluNU	DPG
PI	+	+	-	v	-	-	-	v
PII	+	+	-	v	+	-	-	+
PIII	v	+	+	v	v	+	-	v
PIV	ND	+	-	-	v	v	+	+
PV	ND	+	-	v	v	-	+	+

Abbreviation: PIMs, phosphatidyl inositol mannosides ; PI, phosphatidyl inositol ; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine ; PME, phosphatidyl methyl ethanolamine ; GluNU, glucosamine containing unknown phospholipids ; DPG, diphosphatidyl glycerol ; ND, no data ; v, variable ; -, absent

Source: Lechevalier *et al*. (1977)

Table 2.7 Phospholipid types in actinomycetes to genus level identification.

Phospholipids type	Diagnostic phospholipids	Genera
I	No nitrogenous phospholipids	*Actinomadura, Microtetraspora, Nocardioides, Spirillospora*
II	Phosphatidyl ethanolamine	*Actinoalloteichus, Actinoplanes, Dactylosporangium, Micromonospora, Micropolyspora, Nocardia, Saccharomonospora, Salinispora, Streptomyces, Verrucosispora*
III	Phosphatidyl choline	*Actinomycetospora, Kineosporia, Marinactinospora, Micropolyspora, Nocardiopsis, Nocardia, Pseudonocardia, Saccharopolyspora, Sciscionella, Spinactinospora*
IV	GluNU (unknown glucosamine-containing phospholipids)	*Actinomadura, Streptosporangium, Microbispora, Microtetraspora, Planomonospora, Planobispora*
V	GluNU and phosphatidyl glycerol	*Promicromonospora*

Source: Lechevalier *et al*. (1977)

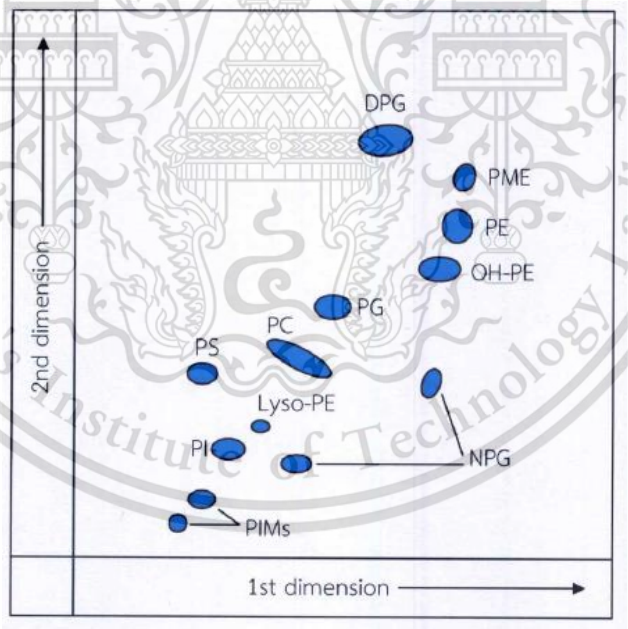


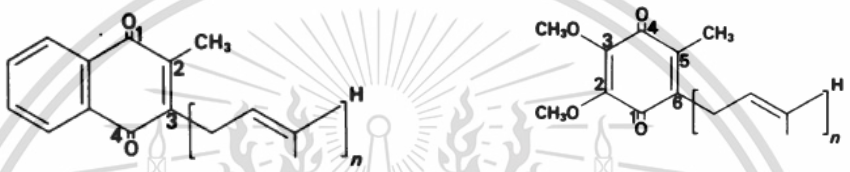
Figure 2.10 The polar lipid position with two–dimensional TLC technique.

Abbreviation: PIMs, phosphatidyl inositol mannosides ; PI, phosphatidyl inositol ; PC, phosphatidyl choline ; PE, phosphatidyl ethanolamine ; PME, phosphatidyl methyl ethanolamine ; OH–PE, hydroxyl-phosphatidyl ethanolamine; Lyso–PE, lyso–phosphatidyl ethanolamine ; DPG, diphosphatidyl glycerol ; PG, phosphatidyl glycerol ; PS, phosphatidylserine ; NPG, ninhydrin-positive phosphoglycolipids

Source: Niemhom (2018)

2.3.2.5 Isoprenoidquinones

Isoprenoidquinones are a component of the bacterial cell membranes that are known to have a function as intermediate hydrogen carriers in the electron transport chain. Isoprenoid quinones are classified by the difference in core structures as ubiquinone and menaquinone (Figure 2.11). According to the report of Collin and Jones (1981), menaquinone was normally observed as the predominant in the cell membranes of actinomycetes. The difference in the number of isoprene units and the degree of hydrogenation with double bonds in the isoprene chain is the important characteristics for the classification of actinomycetes at the genus level (Komagata and Suzuki, 1987).



Menaquinones Ubiquinones

Figure 2.11 chemical structures of menaquinones and ubiquinones.

Source: Collin and Jones (1981)

2.3.3 Genotypic characteristics

Currently, modern microbial taxonomy has been influenced by the improvement of molecular genetic techniques (Tindall *et al*., 2010). Actinomycetes taxonomists generally use 16S rRNA gene sequence analysis, phylogenetic analysis, DNA base composition, whole–genome sequence analysis, phylogenomic analysis, average nucleotide identity analysis, average amino acid identity, and digital DNA–DNA hybridization for identification and classification of them. Moreover, multilocus sequence analysis by using housekeeping genes is used for analyzing of the relationship of actinomycetes species.

2.3.3.1 16S rRNA gene sequences and phylogenetic tree analysis

Ribosomal RNA represents only a small part (0.3–0.4%) of the genome and the citrons coding for it are highly conserved. They have developed less rapidly than the rest of the chromosome. The rRNA consists of three types 23S, 16S, and 5S. Bacterial 16S rRNA sequences are interesting targets for identification methods because they represent conserved regions in all bacteria and species (Stackebrandt and Goebel, 1994). Bacterial identification based on %similarity of

16S rRNA has been using PCR technique, nucleotide sequencing, and similarity analysis of 16S rRNA genes. Basal Local Alignment Search Tool (BLAST) searches and pairwise global sequence alignments (https://www.ezbiocloud.net/) was developed by Kim *et al*. (2012) as a powerful identification function. At the moment, this tool gives an effective taxonomic determination for actinomycetes identification.

2.3.3.2 DNA base composition

The determination of DNA base composition has developed into important criterion in microbial taxonomy (Marmur and Doty, 1962). DNA consists of four nitrogenous bases adenine (A), guanine (G), cytosine (C), and thymine (T). This character has proved useful for both the classification and identification of actinomycetes in the genus and maybe the species levels. The base composition of the bacterial genome ranges from 25–80% G+C. The high G+C content in actinomycetes can determine the members of actinomycetes from other bacterial phyla. Furthermore, the DNA base compositions of a species show a narrow range of about 1–3 mole % G+C). So, the difference in DNA base composition can be assumed to the different in the genome and belong to difference species (Tamaoka, 1994). The use of the phylogenetic and molecular evolutionary approach has highly supported the classification methods (Babalola *et al.,* 2009 ; Hozzein and Goodfellow, 2011).

2.3.3.3 Whole–genome sequence and phylogenomic analysis

Metagenomics is a novel procedure for the identification and classification of bacterial diversity and the next–generation DNA sequencing technologies increase researcher interest in understanding the microbial diversity inhabiting different environments. Whole–genome sequencing is a forceful tool for genomics research and the most extensive method for analyzing the genome such as determining the sequence of individual genes, operons, full chromosomes, or entire genomes of any organisms (Amin *et al*., 2020). This technique provides a high-resolution, base–by–base view of the genome and delivery large volumes of data in a short amount of time to support the assembly of novel genomes (Yoon *et al*., 2017).

The several next–generation sequencing methods, new methods for DNA sequencing, such as reversible dye terminators (Illumina sequencing) (Bentley *et al*., 2008), massively parallel signature sequencing (Brenner *et al*., 2000), 454 pyrosequencing (Margulies *et al*., 2005), polony sequencing (Shendure *et al*., 2005), sequencing by oligonucleotide ligation detection (Mardis, 2008), ion torrent sequencing by synthesis (Rusk, 2010), single–molecule real-time sequencing by

synthesis (Eid *et al*., 2009) and DNA nanoball sequencing (Drmanac *et al*., 2010) are used for determination of whole–cell DNA bases and the production of raw sequence data is only the beginning of its detailed bioinformatics analysis (Severin *et al*., 2014). The use of the phylogenomic evolutionary tree has highly supported the classification methods.

2.3.3.4 Average nucleotide identity, average amino acid identity, and digital DNA–DNA hybridization

The average nucleotide identity (ANI) is a similarity index between a given pair of genomes which can be suitable to prokaryotic organisms separately of their G+C content and cut off score of >95% indicates that they belong to the same species (Goris *et al*., 2007 ; Richter and Rosselló–Mora, 2009). The genome sequencing studies may use different software packages for ANI determination such as JSpecies (http://www.imedea.uib.es/jspecies/) (Richter *et al*., 2016), Gegenees (http://www.gegenees.org/documentation.html) (Ågren *et al*., 2012), ANI calculator (https://www.ezbiocloud.net/tools/ani, http://enve-omics.ce.gatech.edu/ani/index).

The average amino acid identity (AAI) is an index of pairwise genomic relatedness, and multiple studies have proposed its application in prokaryotic taxonomy and related disciplines. The cutoff value for delimiting species boundaries of AAI is 95–96% (Konstantinidis and Tiedje, 2005 ; Thompson *et al*., 2013). This value was calculated using the Kostas Lab AAI calculator (http://enve-omics.ce.gatech.edu/aai/) (Rodriguez–R and Konstantinidis, 2014).

DNA–DNA hybridization is acknowledged as the superior method for the elucidation of relationships between closely related taxa, such as strains and species. Up to present, the DNA–DNA relatedness values of about 70% have been accepted for the threshold for assigning strains to the same species (Wayne *et al*., 1987). The *in silico* method or called “digital DNA–DNA hybridization” is usually used to analyze the DNA–DNA relatedness between two genomes (Auch *et al*., 2010).

2.3.3.5 Multilocus sequence analysis (MLSA)

Currently, multilocus sequence analysis (MLSA) is now established as a powerful phylogenetic approach for defining the taxonomic structure and evolutionary history of diverse bacteria. MLSA uses data retrieved from fragmented nucleotide sequences of five to seven housekeeping genes spread across the chromosome, thereby making it possible to construct a refined framework for resolving population structures and prokaryotic diversity at the genome level (Rong and Huang, 2014). MLSA is a phylogenetic tool to support and clarify the resolution

of bacterial species with a higher resolution as in 16S rRNA gene–based studies (Glaeser and Kämpfer, 2015). After being amplified in each gene, the sequences of all protein–coding loci for each strain were concatenated by joining head–to–tail in a frame and the phylogenetic tree on the concatenated gene sequence was reconstructed. Example of housekeeping gene is *atp*D (ATP synthase F1 beta subunit), *gyr*B (DNA gyrase beta subunit), *rpo*B (RNA polymerase beta subunit), *rec*A (recombinase A), and *trp*B (tryptophan synthase beta subunit) that are used for identification and classification in the genus *Streptomyces* (Rong and Huang, 2014).

2.4 Bioactive and agroactive compounds from actinomycetes

Actinomycetes are known for their capacity to produce secondary metabolite or bioactive compounds with diverse biological activities. Approximately, 22,500 bioactive metabolites have been isolated from microorganisms and it has been estimated that over two–thirds of compounds were isolated from actinomycetes (Challis and Hopwood, 2003 ; Berdy, 2005 ; Dharmaraj, 2010). The major of these compounds are derived from the genus *Streptomyces*. The bioactive compounds of some actinomycetes showed a broad range of biological activities such as antibacterial, antifungal, antiviral, antioxidants, anticancer, antimalarial, antitubercular, neuroprotective, and immunosuppressive (Quinn *et al*., 2020).

2.5 Actinomycetes in agriculture

In the last decade, there has been reached in the world's population at a dangerous rate, and is forecasted to rise of about nine billion by 2050 (Godfray *et al*., 2010) which will require high levels of yield from agricultural systems. Nowadays, plant growth and productivity are limited by environmental stress such as salinity, drought, cold, and heat, limited nutrient availability, heavy metals and industrial chemicals contamination, and hypoxia. The global climate change is subsequently going to affect agriculture worldwide owing to an increase in surface temperature that in turn will reduce soil moisture. Moreover, the increase in the world's temperature could impact crop pest insect and plant pathogens populations in several complex ways. Consequently, the use of microorganisms that primarily colonizes the rhizosphere or endorhizosphere of plants and thus favors growth directly or indirectly is gaining priority in stress management. Because of several beneficial effects, actinomycetes are used in agriculture systems. They produced plant growth hormones like indole acetic acid, gibberellins, cytokinins, or other unknown determinants which primarily relate to increased uptake of nutrients leading to improved plant health (Egamberdieva and Kucharova, 2009). For plant disease pathogens and pest control, actinomycetes have been produced as the

secondary metabolite as an antibiosis mechanism (Cooper and Chilton, 1950 ; Barakate *et al*., 2002 ; Getha *et al*., 2005), hyperparasitism mechanisms (Sabaou *et al*., 1983 ; Upadhyay and Rai, 1987 ; Khan *et al*., 1993) or cell–wall degrading enzymes production (Gomes *et al*., 2000 ; Nawani *et al*., 2002 ; El–Tarabily, 2003 ; 2006). Figure 2.12 represent possible plant–microbe interactions favoring plant growth and/or biocontrol of phytopathogens by streptomycetes.

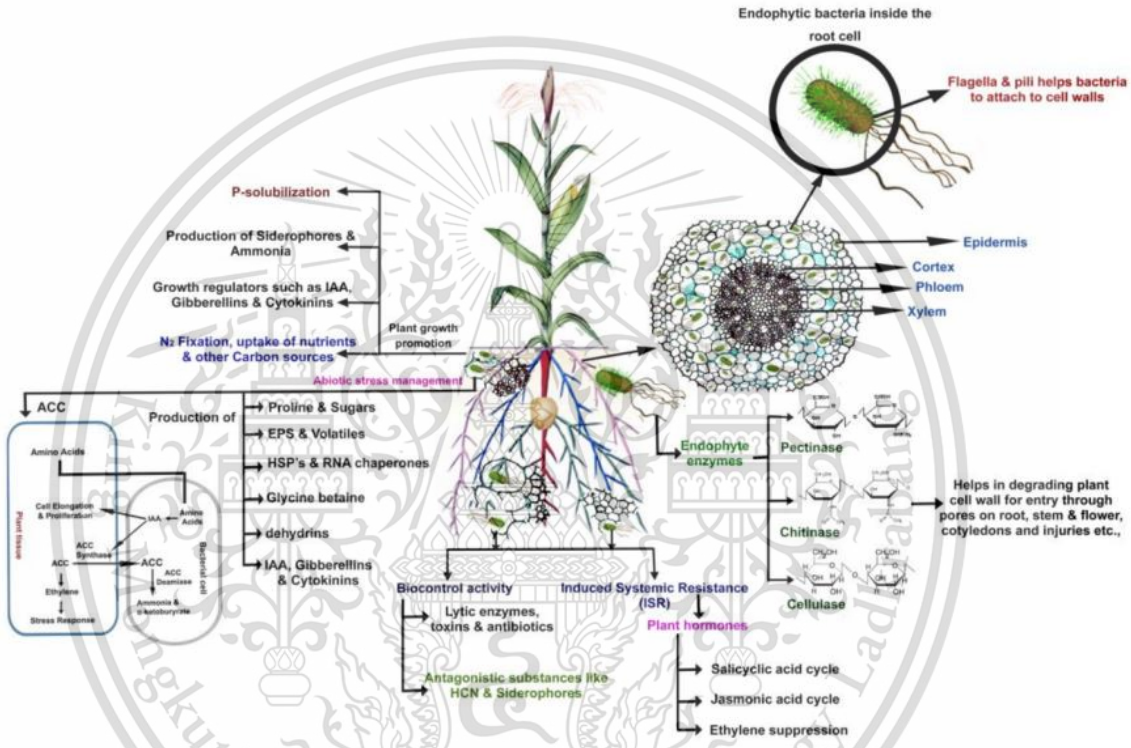


Figure 2.12 Representation of possible plant–microbe interactions favoring plant growth and/or biocontrol of phytopathogens by streptomycetes as rhizosphere competent microorganisms and/or endophytes.

Source: Vurukonda *et al*. (2018)

2.5.1 Actinomycetes as plant growth promoters and plant nutrition improvement

Actinomycetes can also promote plant growth directly through the production of plant growth promoter substances (Merckx *et al*., 1987). Several mechanisms are involved such as the production of indole acetic acid, gibberellins, cytokinins, siderophores, nutrient solubilization, or beneficial synergistic effects.

2.5.1.1 Indole acetic acid production

Indole acetic acid (IAA) is a general plant hormone and active form of auxins. It plays an important role in plant development through its life cycle. Bacteria synthesize IAA mainly by alternate tryptophan–dependant pathway, through indole pyruvic acid. IAA enhances the development of lateral roots and divisions of the apical meristem that derives from root elongation (López *et al*., 2004) and seed germination. It has an advantage for young seedlings as it gains their ability to mainstay themselves in the soil and to obtain water and nutrients from the environment. Several actinobacterial genera can be produced IAA such as *Streptomyces* (El–Sayed *et al*., 1987 ; El–Shanshoury *et al*., 1991 ; Manulis *et al*., 1994 ; Myo *et al*., 2019), *Kitasatospora* (Shrivastava *et al*., 2008), *Nocardia* (El–Tarabily and Sivasithamparam, 2006 ; Ghodhbane–Gtari *et al*., 2019), *Frankia* (Nouioui *et al*., 2019).

2.5.1.2 Gibberellins production

Gibberellins (GAs) are a large family of tetracyclic diterpenoid which functions as plant growth–promoting. There are two pathways for the synthesis of gibberellin, mevalonic acid–dependent and mevaloni acid–independent cycle (Lange and Lange, 2006). Mevalonic acid–dependent pathway relates synthesis of sterol and mevaloni acid–independent relates synthesis of carotenoids. GAs regulate and promote cell division and elongation, seed germination, stem and hypocotyl elongation, root growth and flowering induction (Sun and Gubler, 2004 ; Shukla *et al*., 2005 ; Lucas *et al*., 2008 ; Sun, 2011). Endophytic *Actinoplanes campanulatus* and *Streptomyces spiralis* (El–Tarabily *et al*., 2008), *Streptomyces neopeptinius* from sand truffles (Goudjal *et al*., 2016a), marine *Streptomyces* sp. (Rashada *et al*., 2015) and *Nocardia* sp. from soil (Brown, 1972) produced gibberellins.

2.5.1.3 Cytokinins production

Cytokinins are adenine derivative phytohormones that regulate cell division activity in mature plants by altering the size and activity of meristems

(Werner *et al*., 2001). Cytokinins stimulate the growth of root and shoot as well as branching, control the apical dominance in the shoot, chloroplast development, and leaf senescence (Oldroyd, 2007). *Rhodococcus fascians* (Pertry *et al*., 2009), *Streptomyces turgidiscabies* (Joshi and Loria, 2007), and *Streptomyces rochei* ERY1 (Suwitchayanon *et al*., 2018) were detected as cytokinins production.

2.5.1.4 Siderophore production

Siderophores, water soluble–compounds, are low–molecular weight secondary metabolite with iron–chelating potential. Siderophores are produced by various microorganisms and are generally classified into four types, hydroxamates, catecholates, carboxylates, and mixed types, based on their structural features, functional groups, and types of ligands (Ali and Vidhale, 2013 ; Kumar *et al*., 2017). Hydroxamates contain a hydroxamic acid functional group, which is carbonyl oxygen combined with an amino group. Catecholate ligands have adjacent hydroxyl oxygens on an aromatic ring. Most the siderophores that are produced by bacteria are hydroxamate and catecholate types and few bacteria produce carboxylates (Drechsel *et al*., 1995). Actinomycetes are one the most important groups in terms of siderophore production, especially genus *Streptomyces* have been reported that inhibit the growth of phytopathogens by competition for iron in plant rhizosphere soils (Müller and Raymond. 1984 ; Müller *et al*., 1984 ; Tokala *et al*., 2002). Additionally, the genus *Thermobifida* has been reported as the producer of a siderophore known as fuscachelin A (Dimise *et al*., 2008).

2.5.1.5 Nitrogen fixation

Nitrogen is the most critical for plant growth and metabolisms. It is a major component of chlorophyll, the most important pigment needed for photosynthesis. All important processes in the plant are related to proteins, of which nitrogen is an essential constituent. Nitrogen plays a key role in agriculture by increasing crop yields but it represents about 2% of the total plant dry matter that enters the food chain. The plant can not use directly access dinitrogen gas (N_2) from the atmosphere. It first must absorb the available nitrogen in the soil through its root in the form of ammonia (NH_3) or nitrate (NO_3). The optimum rate of nitrogen increases photosynthetic processes, leaf area production, leaf area duration as well as net assimilation rate (Ahmad *et al*., 2009), and plant biomass. The deficiency of nitrogen causes reduced growth, appearances of chlorosis, and appearances of red and purple spots on the leaves that restrict lateral bud growth (Bianco *et al*., 2015).

Only some prokaryotes can use atmospheric nitrogen through a process known as biological nitrogen fixation (BNF) in the nitrogen cycle, which is the

conversion of atmospheric N_2 to NH_3 and nitrite (NO_2) to NO_3 nitrification process, a form that can be used by plants (Lam *et al*., 1996 ; Franche *et al*., 2009). The bacteria responsible for nitrogen fixation are called diazotrophs, they encode nitrogenase, the enzyme complex that catalyzes the conversion of N_2 gas to ammonia. The nitrogenase complex is highly conserved in free–living and symbiotic diazotrophs. Two types of nitrogen–fixing bacteria including actinomycetes are recognized. The first type is free–living (non–symbiotic) bacteria. For example, *Streptomyces thermoautotrophicus* (Gadkari *et al*., 1992 ; Ribbe *et al*., 1997), *Streptomyces* sp. (Dahala *et al*., 2017), *Micromonospora lupine*, *Micromonospora coriariae*, *Micromonospora saelicesensis* (Carro *et al*., 2013) and *Nocardia cakureu*, *Nocardia cetluluns* (Metcalfe and Brown, 1957). The second type is the mutualistic (symbiotic) bacteria that are associated with leguminous plants (various members of the pea family). *Frankia* sp., actinorhizal symbioses, has an outstanding feature related to vesicle specialized in nitrogen fixation (Wall, 2000 ; Franche and Bogusz, 2011 ; Pawlowski and Demchenko, 2012).

2.5.1.6 Phosphate solubilization

Phosphorus (P), a non–renewable macronutrient, plays an essential role in plants that is the second–largest agricultural fertilizer limiting crop yields (Hameeda *et al*., 2008). The majority of soluble organic P is rapidly immobilized by soil fixation and turns unavailable for plant uptake, leading to low P–use efficiency and potentially excess P (Kochian, 2012). Soil P must thus be managed to minimize its loss and increase its use efficiency. P enhance root development, stem and stalk strength, flower and seed formation, crop maturity and production, N_2–fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition (Khan *et al*., 2009).

Microorganisms able to solubilize and mineralize P pools in soils are considered to be vital in promoting P bioavailability. Bacteria are the predominant microorganisms that solubilize mineral P in soils, as compared to fungi (Kucey, 1983). Inorganic–phosphate–solubilizing bacteria (iPSBs) are particularly effective in releasing P from pools of inorganic P. iPSBs can also prevent the liberated P from being fixed again (Richardson and Simpson 2011). *Streptomyces griseus* and *Micromonospora aurantiaca* from Moroccan phosphate-mining centers are abled to release soluble phosphate from rock phosphate (Hamdali *et al*., 2008). Franco–Correa *et al*. (2010) reported that *Streptomyces* sp. MRC26 has the capacity of secreting acid phosphatase for mineralizing sources of organic phosphorus. Prada–Salcedo *et al*. (2014) showed seven actinomycetes isolated from the rhizosphere of wild plants from the eastern Cordillera of the Colombian Andes were

the best phosphate solubilizing strains. Zheng *et al*. (2018b) examined seven *Streptomyces* sp. and three *Rhodococcus* sp. as inorganic–phosphate–solubilizing actinobacteria. Aallam *et al*. (2021) reported that *Streptomyces* sp. from sugar beet fields was able to use natural rock phosphate (RP) and tricalcium phosphate (TCP) as sole phosphate sources.

2.5.1.7 Potassium solubilization

Potassium (K) is one of the most important macronutrients required for plant growth and development. It is related to plant metabolisms such as adjustment of plant cellular osmotic pressure and transport of compounds in plants via potassium channel (Dreyer and Uozumi 2011), cells synthesis, promote the activation of enzymes, protein and sugar synthesis, vitamin production, and utilization of nitrogen. It also boosts plant photosynthesis (Sparks, 1987). Moreover, K increases plant resistance to abiotic and biotic stressors (Epstein and Bloom, 2005). In plants, K deficiency causes slow growth in the plant, yellowing of the leaf edges and giving them a burned appearance, producing smaller seeds, and finally lower yields (Gupta *et al*., 2015). Generally, K in soils is very low (K^+, 2% of total K in the soil which can be directly uptake by plants and more than 90–98% of K in the soil exists as insoluble mineral K, exchangeable and non–exchangeable forms (Shanware *et al*., 2014).

Therefore, introducing alternative sources of fertilizer from microbial activation such as potassium solubilizing bacteria (KSB) can be an effective way to reach sustainable agriculture. The KSB were capable of solubilizing rock K, and synthetic K mineral powder through the production and excretion of some organic acids and enzymes (Friedrich *et al*., 1991). For potassium solubilizing actinobacteria, few studies showed that *Microbacterium foliorum* from rhizospheric soil (Zhang and Kong, 2014), *Streptomyces rochei*, and *Streptomyces sundarbansensis* rhizosphere of *Mikania micrantha* Kunth (Han *et al*., 2018) and *Streptomyces alboviridis* P18, *Streptomyces griseorubens* BC3, *S.griseorubens* BC10, and *Nocardiopsis alba* BC11 from soil (Boubekri *et al*., 2021) solubilized K.

2.5.1.8 1–aminocyclopropane–1–carboxylate deaminase production

1–aminocyclopropane–1–carboxylate (ACC) deaminase catalyzes the cleavage of the plant ethylene precursor, ACC, into ammonia and α–ketobutyrate (Honma and Shimomura 1978). Microorganisms containing ACC deaminase attach to plant cells, act as a sink for plant ACC to uptake and cleave the ACC secreted by plant cells and reduce plant ACC concentration, ethylene evolution and extent of ethylene inhibition of plant growth (Dubois *et al*., 2018), particularly,

under a variety of abiotic and biotic stresses (Glick, 2014). The schematic model of this reaction is shown in Figure 2.13.

The only enzyme shown in this scheme is ACC deaminase. SAM is converted to ACC by the enzyme ACC synthase; ACC is converted to ethylene by ACC oxidase. IAA biosynthesis, both in bacteria and in plants is a complex multi–enzyme/protein process as is IAA signal transduction.

Morever, bacteria containing ACC deaminase can down–regulate the plant genes involved in ethylene–induced stress responses and defense signaling pathways and up–regulate the plant gene involved in growth protein production (Hontzeas *et al*., 2004). Actinomycetes containing ACC deaminase that are used as a key marker for identifying the plant growth-promoting actinomycetes were isolated from nature such as *Streptomyces filipinensis*, *Streptomyces atrovirens*, and *Streptomyces albovinaceus* from soil (El–Tarabily, 2008), *Micrococcus yunnanensis* RS222 from the soil of barren fields (Siddikee *et al*., 2010), *Arthrobacter pokkalii* sp. nov. from saline tolerant Pokkali rice (Krishnan *et al*., 2016), *Streptomyces* sp. GMKU 336 from roots of *Clerodendrum serratum* (L.) Moon (Jaemsaeng *et al*., 2018), *Streptomyces levis*, *Streptomyces* sp., *Streptomyces diastaticus*, *Streptomyces griseorubiginosus*, *Streptomyces longispororuber* and *Nocardiopsis dassonvillei* from the root of tea plants (*Camellia sinensis*) (Shan *et al*., 2018) and *Streptomyces thinghirensis*, *Nocardiopsis dassonvillei* subsp. *dassonvillei* and *Streptomyces ambofaciens* from a saline site (Djebaili *et al*., 2021).

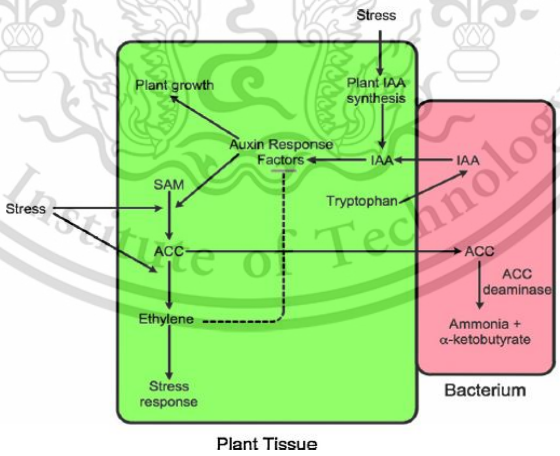


Figure 2.13 A schematic model of how plant growth–promoting bacteria that both produce ACC deaminase and synthesize IAA may facilitate plant growth.
Abbreviations: ACC, 1–aminocyclopropane–1–carboxylate; IAA, indole–3–acetic acid; SAM, S–adenosyl methionine

Source: Glick (2014)

2.5.1.9 Other growth promoters

Brassinolide enhances the content of chlorophyll, stimulate protein synthesis, activate certain enzymes, and regulates cellular differentiation. Jasmonic acid induces systemic resistance against necrotrophs, and activates phylloptosis, tuber formation, fruit ripening, and pigment formation. An example of actinomycetes that can produce two hormones are *Streptomyces* sp. (Merzaeva and Shirokikh, 2010). Salicylic acid from *Streptomyces* sp. (Lin *et al*., 2012b) can induce systemic resistance, prolong the life of flowers, inhibit ethylene biosynthesis, and facilitate pollination of certain plants. Serotonin from *Streptomyces* sp. (Tsavkelova *et al*., 2005) is a structural analog of auxins and plants metabolize serotonin to IAA. Abscisic acid from *Streptomyces* sp. (Bach and Rohmer, 2012) relate to phyllopsis and closure of stomata and aging.

2.5.2 Actinomycetes in plant protection against biotic stresses

Biotic stress in plants is caused by living organisms, specifically bacteria, fungi, viruses, nematodes, insects, arachnids, and weeds. The biotic stress agents directly deprive their host of its nutrients leading to reduced plant vigor, yield and quality loss, and death of the host plant depending on the environment and thus varies region. It can become major because of pre– and postharvest losses. Due to lacking the adaptive immune system in plants or the ability to adapt to new diseases and memorize past infections, plants have evolved a plethora of complicated strategies to counteract biotic stresses via plant genetic code. Many biotic stresses affect photosynthesis such as chewing insects and virus infections reducing leaf area.

The use of biological organisms to control or protect plant disease (biocontrol) or other biotic agent could potentially augment the use of synthetic pesticides, herbicides, or nematicide. Biocontrol research has a lot of attention in recent years and there are many well–documented examples of biocontrol activity (Junaid *et al*., 2013 ; Bardin *et al*., 2015). Many actinomycetes can protect the plant using bioactive or agroactive compounds and/or other mechanisms that act as biocontrol properties. The mechanisms of action for biocontrol properties are antibiosis, hyperparasitism, and cell–wall degrading enzyme production.

2.5.2.1 Antibiosis

Antibiosis is an association between two microorganisms that is detrimental to at least one of them and that is caused by the release of metabolite or cell components (Haggag *et al*., 2007). Normally, this mechanism of action of actinomycetes has focused mainly on *in vitro* screens. In routine screening tests, antibiosis is determined by pairing colonies on agar plates (Johnson and Curl, 1972). It

has been several modifications method including Herr's triple agar–layer plate technique (Herr, 1959), agar–ring method (Williams and Willis, 1962), reversed layer method (Hasegawa *et al*., 1990), and mycelial spray technique (Alvarez *et al*., 1995), all of which indicate the level of effectiveness of the antagonist to inhibit the vegetative growth of a pathogen. These are usually followed by tests involving culture filtrates or purified fractions of the filtrates (Smith, 1957 ; Rothrock and Gottlieb, 1984 ; El–Abyad *et al*., 1993 ; Trejo–Estrada *et al*., 1998). The results of effective actinomycetes or other microorganisms were developed for antibiotics production.

2.5.2.2 Hyperparasitism

Hyperparasitism is another plant protection strategy in which endophytes directly attack the plant pathogen and kill the pathogen and its inoculum (Dutta *et al*., 2014). Many studies showed hyperparasitism such as Sabaou *et al*. (1983) reported that a strain of *Nocardiopsis dassonvillei* showed parasitic activities against the vegetative hyphae of *Fusarium oxysporum* f.sp. *albedinis* and Upadhyay and Rai (1987) found that a strain of *Micromonospora globosa* parasitized the hyphae of *Fusarium udum in vitro*. Soil–borne *Actinoplanes* sp. parasitized oospores of *Phytophthora megasperma* f.sp. *glycinea* (Sutherland *et al*., 1984) and *Pythium* spp. (Khan *et al*., 1993) *in vitro* studies. *Actinoplanes missouriensis* was found to parasitize the oospores of *Phytophthora megasperma* var. *sojae*, *Phytophthora cactorum*, *Pythium* sp., and *Aphanomyces euteiches* in natural soil (Sneh *et al*., 1977). *Streptomyces albus* parasitized on *Nectria inventa* (Tu, 1986) and *Streptomyces alni* parasitized on *Fusarium oxysporum* caused root-rot of grapevine (Ziedan *et al*., 2010).

2.5.2.3 Cell–wall degrading enzymes

Actinomycetes have been recorded to produce cell wall degrading enzymes including β–1,3, β–1–4, β–1–6–glucanases (Gacto *et al*., 2000 ; El–Tarabily, 2006), and chitinase (Nawani *et al*., 2002 ; El–Tarabily, 2003) that lysed the fungal cell wall composition (glucan and chitin). For β–1,3, β–1–4, β–1–6–glucanases production, Valois *et al*. (1996) reported that *Nocardioides* capable of producing β–1,3, β–1–4, and β–1–6–glucanases hydrolyzed glucans from *Phytophthora fragariae* cell walls, caused hyphal lysis and reduced the severity of the root-rot disease of rasberry, El–Tarabily (2006) reported that isolates of *Microbispora rosea*, *Micromonospora chalcea*, and *Actinoplanes philippinensis* produced β–1,3, β–1–4 and β-1-6-glucanases *in vitro* caused lysis of *Phytophthora aphanidermatum* hyphae *in vitro* and reduced damping-off disease of cucumber

under controlled glasshouse conditions. Many *Streptomyces* species have potentially produced enzymes glucanase (Koishizawa and Kamei 2007 ; Shi *et al*., 2010 ; Sakdapetsiri *et al*., 2016 ; Edison and Pradeep, 2020). In the case of chitinase production, *Actinoplanes missouriensis* produced the enzyme for the cell wall lysis of *Plectosporium tabacinum* the causal agent of lupin root rot in Egypt (El–Tarabily, 2003). *Streptomyces thermoviolaceus* (Tsujibo *et al*., 1993), *Streptomyces violaceusniger* (Shekhar *et al*., 2006), *Streptomyces rimosus* (Brzezinska *et al*., 2013), *Streptomyces rubiginosus* (Jha *et al*., 2016). *Streptomyces albus* (Santhi, 2016), *Microbispora* sp. (Nawani *et al*., 2002), and *Micromonospora* sp. (Gasmi *et al*., 2019) detected in chitinase production.

In the case of pesticides, much research showed only tests of culture–dependent actinomycetes cells. For example, *Streptoverticillum* sp. isolated from soil showed a lethal effect on pupae of *Spodoptera liltoralis* (Bream *et al*., 2001), *Actinoplanes philippinensis*, *Actinoplanes missouriensis*, *Streptomyces clavuligerus* from United Arab Emirates soil exhibited against *Drosophila melanogaster* pupal formation (Gadelhak *et al*., 2005), *Streptomyces rochei*, *Streptomyces minutiscleroticus, Streptomyces phaeoluteigrisseus* produced the larvicidal against 3rd instar larvae of *Tribolium castaneum* (red flour bettle) (Anwar *et al*., 2014), Kaur *et al*. (2014) isolated *Streptomyces hydrogenans* DH16 from soil which exhibited larvicidal and growth inhibitory activities to *Spodoptera litura*, Chen *et al*. (2018a) found *Streptomyces albidoflavus* from neem tree that showed insecticidal activity against the green peach aphid and *Streptomyces pactum*, *Streptomyces rochei*, root–zone microbes, controlled the cause of root–knot nematode disease (Ma *et al*., 2017). Table 2.8 shows some actinomycetes in plant protection against biotic stresses.

For weed control, *Nocardiodes* sp. and *Actinomadura* sp. showed 60% reduction in seed germination of billygoat weed (*Ageratum conyzoides*), *Saccharopolyspora* sp. exhibited 80% reduction in seed germination of congress grass (*Parthenium hysterophorus*) (Singh *et al*., 2018), *Streptomyces anulatus* showed phytotoxic against hairy crabgrass (*Digitaria sanguinalis*) and *Sorghum bicolor* (Bo *et al*., 2019), *Streptomyces olivochromogenes* showed phytotoxic against southern crabgrass (*Digitaria ciliaris*) (Kim *et al*., 2020).

Table 2.8 Some biological control of plant disease caused by biotic stresses using actinomycetes.

Species/Strains	Diseases	Pathogens/Agents	Host plants	References
Anti–virus				
Streptomyces albovinaceus, *Streptomyces sparsogenes*	Zucchini yellow mosaic virus	Potyvirus	Cucumber	Askora (2005)
Streptomyces sp.	Tobacco mosaic virus	Tobamovirus	*Datura* plant	Ara *et al*. (2012)
Strepcomces noursei var·*xichangensisn*	Tobacco mosaic virus	Tobamovirus	Tobacco	Han *et al*. (2014)
Streptomyces kanasensis	Tobacco mosaic virus	Tobamovirus	Tobacco	Zhang *et al*. (2016)
Streptomyces olivaceus	Cucumber mosaic virus	Tobamovirus	Cucumber	Latake and Borkar (2017)
Streptomyces ahygroscopicus	Tobacco mosaic virus	Tobamovirus	*Nicotiana glutinosa*	Chen *et al*. (2019)
Streptomyces sp.	*Odontoglossum* ringspot virus, *Cymbidium mosaic* virus	Tobamovirus, Potexvirus	Orchid	Lee *et al*. (2019)
Streptomyces pactum	Tomato yellow leaf curl virus	Begomovirus	Tomato	Li *et al*. (2019)
Streptomyces cellulosae	Tobacco mosaic virus	Tobamovirus	Tomato	Abo–Zaid *et al*. (2020)
Anti–bacteria				
Streptomyces scabies, *Streptomyces lavendulae*, *Streptomyces griseolus*	Crown gall	*Agrobacterium radiobacter*	Many	Schreven (1964)
Streptomyces plicatus	Soft rot disease	*Erwinia carotovora* subsp. *carotovora*	Many	Zamanian *et al*. (2005)
Streptomyces cinereoruber	Soft rot disease	*Erwinia chrysanthemi*	Many	Karkouri *et al*. (2010)
Streptomyces sp.	Leaf blight	*Xanthomonas campestris* pv.*glycines*	Soybean	Mingma *et al*. (2014)
Streptomyces sp.	Peach crown gall	*Agrobacterium tumefaciens*	Peach	Wang *et al*. (2014)
Streptomyces sp.	Rice leaf blight	*Xanthomonas oryzae* pv. *oryzicola*	Rice	Hata *et al*. (2015)
Streptomyces violaceusnige	Oily spot disease	*Xanthomonas axonopodis* pv. *punicae*	Pomegranate	Chavan *et al*. (2016)
Streptomyces spp.	Many	*Pectobacterium carotovorum*	Many	Promnuan *et al*. (2020)
Streptomyces physcomitrii	Wilt	*Ralstonia solanacearum*	Tomato	Zhuang *et al*. (2020)
Anti–Fungi				
Actinoplanes spp.	Damping-off	*Pythium ultimum*	Table beet	Khan *et al*. (1997)
Actinoplanes missouriensis	Root rot	*Phytophthora megasperma* f. sp. *glycinea*	Soybean	Sutherland and Lockwood (1984)
Actinoplanes philippinensis	Cavity spot	*Pythium coloratum*	Carrots	El–Tarabily *et al*. (1997)
Actinomadura sp.	Root rot	*Phytophthora cinnamomi*	Snapdragon	You *et al*. (1996)
Actinomadura rubra	Cavity spot	*Phytophthora coloratum*	Carrots	El–Tarabily *et al*. (1997)
Amorphosporangium auranticolor	Root rot	*Phytophthora megasperma* f. sp. *glycinea*	Soybean	Filonow and Lockwood (1985)
Microbispora rosea	Damping-off	*Phytophthora aphanidermatum*	Cucumber	El–Tarabily (2006)
Micromonospora sp.	Wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Tomato	Smith (1957)
Micromonospora sp.	Leaf infection	*Botrytis cinerea*	*Medicago sativa*	Martínez–Hidalgo *et al*. (2015)

Table 2.8 Some biological control of plant disease caused by biotic stresses using actinomycetes (continued).

Species/Strains	Diseases	Pathogens/Agents	Host plants	References
Micromonospora carbonacea	Basal drop	*Sclerotinia minor*	Lettuce	El–Tarabily *et al.* (2000)
Nocardia globerula	Silver scurf	*Helminthosporium solani*	Potato	Elson *et al.* (1997)
Saccharothrix algeriensis	Wilt	*Fusarium oxysporum* f. sp. *lycopersici*	*Solanum lycopersicum*	Merrouche *et al.* (2017)
Streptomyces sp.	Seed infection	*Aspergillus* sp.	*Zea mays*	Bressan (2003)
Streptomyces violaceusniger	Wood rot	*Gloeophyllum trabeum*	Many	Shekhar *et al.* (2006)
Streptomyces vinaceusdrappus	Blast	*Pyricularia oryzae*	*Oryza sativa*	Ningthoujam *et al.* (2009)
Streptomyces sp.	Powdery mildew	*Oidium* sp.	*Lathyrus odoratus*	Sangmanee *et al.* (2009)
Streptomyces sp.	Brown spot	*Alternaria* spp.	*Nicotiana tabacum*	Gao *et al.* (2014)
Streptomyces indiaensis	Wilt	*Fusarium oxysporum*	*Capsicum*	Jalaluldeen *et al.* (2014)
Streptomyces ambofaciens	Anthracnose	*Colletotrichum gloeosporioides*	*Capsicum*	Heng (2015)
Streptomyces cyaneus	Fungal infection	*Sclerotinia sclerotiorum*	*Lactuca sativa*	Kunova *et al.* (2016)
Streptomyces humidus	Leaf spot	*Alternaria brassicicola*	*Brassica oleracea*	Hassan *et al.* (2017)
Streptoverticillium netropsis	Cavity spot	*Phytophthora coloratum*	Carrots	El–Tarabily *et al.* (1997)
Anti–*Meloidogyne*				
Streptomyces sp.	Root-knot	*Meloidogyne incognita*	Chilli	Ruanpanun and Chamswarng (2016)

Chapter 3
Research methodology

3.1 Sample collection and isolation of actinomycetes

3.1.1 Source of sample

Thirteen samples were collected from several locations in Thailand (Table 3.1). The four samples were collected from aerobic bio–sludge compost, two samples from anaerobic bio–sludge compost, five samples from agricultural waste compost, and two samples from the soil. All samples were collected randomly at the depth of 15–30 cm from the surface.

Table 3.1 Samples, sites, codes, and geographical locations of the sample.

Samples	Sites	Codes	Geographical locations
Aerobic bio–sludge compost	Micro Biotec Company, Ltd., Nikhompattana, Rayong Province	RBST1	12°50’49”N 101°08’08”E
	Micro Biotec Company, Ltd., Nikhompattana, Rayong Province	RBST2	12°50’45.6”N 101°08’14.7”E
	Micro Biotec Company, Ltd., Don Chedi, Suphanburi Province	SBST1	14°38’04”N 99°50’47.2”E
	Micro Biotec Company, Ltd., Don Chedi, Suphanburi Province	SBST2	14°38'06" N 99°50'45"E
Anaerobic bio–sludge compost	KEWPIE (THAILAND) Company, Ltd., Mueang, Ratchaburi Provice	RBCP1	13°35’14”N 99°48’37”E
	KEWPIE (THAILAND) Company, Ltd., Mueang, Ratchaburi Provice	RBCP2	
Agricultural waste compost	Suthanee Nunthawat cassod tree farming area, Nikhompattana, Rayong Province	RCPT1	12°52'17"N 101°08'25"E
	Suthanee Nunthawat Integrated farming area, Nikhompattana, Rayong Province	RCPT2	12°52'15"N 101°08'28"E
	Nichanart Onsai Integrated farming area, Nikhompattana, Rayong Province	RCPT3	12°52'06"N 101°08'10"E
	Lawan’s broiler chicken farm, Thap Khlo, Pichit Province	PCP1	16°07’24”N 100°32’46”E
	Lawan’s broiler chicken farm, Thap Khlo, Pichit Province	PCP2	
Soil	Khao Cha-Ngoom royal soil remediation study center, Photharam, Ratchaburi Province	CH	13°42’43”N 99°41’42”E
	Ornic Integrated farming area, Lat Yao, Nakhon Sawan Province	NKS	15°50’16”N 99°48’44”E

3.1.2 Isolation and maintenance of actinomycetes

The samples were dried out at room temperature and then were heated at 100 °C in a hot air oven for 1 h. Then, 10 g of each sample was ground and homogenized, and then suspended in 90 ml sterile distilled water with 0.01% sodium dodecyl sulfate and shaken the suspension for better release and distribution of soil particles and actinomycetes structure. After that, 1 ml of the suspension was transferred to another 9 ml same solution tube and this step was repeated to set up a 10–fold dilution series. Of the final dilution step (10^{-3}), aliquots (100 µl) were spread onto Zhang's starch soil extract (ZSSE) plate (Appendix A) supplemented with nalidixic acid (20 µg/ml) and nystatin (50 µg/ml) and the plates were incubated at 30 °C for 7–14 days. A single colony was picked up and cross–streaked for purification on yeast extract malt extract agar (ISP 2) (Appendix A). The purified isolate was preserved on slants of ISP 2 and in glycerol 20% (w/v) suspension at –80 °C at the Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang.

For the measuring of pH, a dried sample (10 g) was put into a 250 ml erlenmeyer flask and then 25 ml of distilled water was added and shaken for the 30s. The soil solution was stood for 30 min. Finally, the soil solution was gently mixed for 2–3 sec, measured pH value using pH glass electrodes recorder by put the pH glass electrode in erlenmeyer flask at the 2/3 depth from the surface and hold it for more than 30s to stabilize electric potential.

3.2 Isolation, pathogenicity, and identification of *Fusarium oxysporum* f.sp. *cubense* (*Foc*) from banana

3.2.1 Isolation of *Foc*

Sampling locations were identified in two provinces of Thailand, Nakhon Sawan (15°50'16"N 99°48'44"E) and Lampang (18°42'05.3"N 99°33'59.3"E). From Nakhon Sawan province, contaminated soil samples from *Musa* (AAA) 'Kluai Hom Thong' banana plantations were collected from the first 25 cm depth, stored in a paper bag, and then air–dried sample was ground and homogenized with mortar. Ten grams of sample was suspended in 90 ml sterile distilled water with 0.01% sodium dodecyl sulfate and shook the suspension for better release and distribution of soil particles and fungal structure. After that, one milliliter of suspension was spread onto potato dextrose agar (PDA) (Appendix A) supplemented with 0.5% streptomycin solution (1 g of streptomycin sulfate powder to 100 ml distilled water) for bacterial elimination (Vicente *et al*., 2014) and the plates were incubated at 30 °C for 72 h.

From Lampang province sample, diagnostic specimens were collected from a disease of *Musa* (AAA) 'Kluai Hom Thong' banana plants display typical Fusarium wilt symptoms (light yellow coloring of the lower leaves, most prominent around the margins, collapsed leaves at the petioles, and pseudostem discoloration and splitting) (Daly and Walduck, 2006 ; Maryani *et al*., 2019). The tissue samples were cut into 10–15 cm lengths, washed in running tap water and sterile distilled water, and then cut into small cube shape pieces (≤1 cm). After that, tissue samples were cleaned with 70% ethanol for the 30s followed by three rinses with sterile distilled water and placed on sterile filter paper to dry. Tissue samples were placed onto the center of PDA and the plates were incubated at 30 °C for 72 h.

Different fungal colonies were observed on the medium by examination of morphological and microscopic observation of hyphae and conidia characteristics (Nelson *et al*., 1983 ; Leslie and Summerell, 2006). Pure cultures were purified and fungal isolates were stored on PDA plates. Mycelial fragments were collected with the tip of an inoculation needle and placed on the slide and stained with lactophenol cotton blue stain and observed under a light microscope (ECLIPSE E200, Nikon) with 10X and 40X magnifications.

3.2.2 Pathogenicity testing

3.2.2.1 *In vitro* testing

The four varieties of banana leaves, *Musa* (AAA) 'Kluai Hom Thong', *Musa* (AA) 'Kluai Khai', *Musa* (ABB) 'Kluai Namwa', and *Musa* (AA) 'Kluai Lep Mue Nang' were dissociated from healthy plant and the leave surface was cleaned with 70% ethanol. Sterile leaves were cut into 5 x 5 cm pieces and placed on an upper surface of a sterile plate containing moist filter paper to maintain humidity. Leave pieces were made would at the center using a sterile needle. Seven days old of mycelial fungi on PDA were cut to 6 mm disc with a cork borer and placed onto the would on leave pieces. Negative controls were placed with an agar plug without fungus. The plates were inoculated at 30 °C for 7 days. The positive results showed the leaves turned yellow and the negative results showed no symptoms (Udompongsuk and Soytong, 2016).

3.2.2.2 In pot testing

Musa (AAA) 'Kluai Hom Thong' tissue culture plantlets were obtained from a commercial company. Tissue cultured plantlets were acclimatized for 4 weeks in the nursery. Identified *Foc* were grown in potato dextrose broth (PDB) (Appendix A) for 7 days without shaking. *Fusarium* inoculums were tested at high (10^6

spore/ml) concentrations following the experiment of Getha *et al*. (2005). The experiment was tested in a pot that consisted of a mixture of sand and clay (4 : 1, v/v) with commercial compost in 9 : 1 ratio and was autoclaved at 121 °C for 30 min and air–dried at least 72 h before being used. Twenty–seven plantlets after sterilization with 70% ethanol and washed with sterile distilled water were grouped into nine treatments with three replications. For eight treatments of *Foc*, the root of the plantlets were immersed in a suspension identified *Foc* isolate 1 to 8 for 30 min before replanting. For negative control, the root of the plantlets was immersed in sterile distilled water. All plantlets in each pot were watered once a day. Six weeks later, all plantlets were uprooted. Disease evaluation was followed by the experiment of Brake *et al*. (1995). The external examination for chlorosis of leaves is leveled according to the leaf symptom index (LSI) scale for every week and the internal examination for the extent of rhizome discoloration is leveled at the last week according to the rhizome discoloration index (RDI) scale. LSI scale had five levels (1 = no streaking or yellowing of leaves and plant appears healthy, 2 = slight streaking and/or yellowing of lower leaves, 3 = streaking and/or yellowing of most of the lower leaves, 4 = extensive streaking and/or yellowing on most or all of the leaves and 5 = dead plant), while RDI level had eight levels (1 = no discoloration of tissue of a stellar region of rhizome or surrounding tissue, 2 = no discoloration of stellar region of rhizome; discoloration at the junction of most and rhizome, 3 = trace to 5% of stellar region discolored, 4 = 6–20% of stellar region discolored, 5 = 21–50% of stellar region discolored, 6 = more than 50% of stellar region discolored, 7 = discoloration of the entire rhizome stele and 8 = dead plant). After recording LSI and RDI scales in the last week, the overall disease severity index (DSI) for leaf symptoms and rhizome discoloration for each treatment was calculated as follows:

$$\text{Disease Severity Index (DSI)} = \frac{\sum (\text{Number of scale} \times \text{Number of seedlings on that scale})}{\sum (\text{Number of treated seedling})}$$

The DSI scales were translated into designations in Table 3.2.

Table 3.2 Translation of disease severity index (DSI) scales.

DSI Scales for LSI	DSI Scales for RDI	Translation
1	1	Resistant
Between 1.1 – 2.0	Between 1.1 – 3.0	Tolerant
Between 2.1 – 3.0	Between 3.1 – 5.0	Susceptible
Between 3.1 – 4.0	Between 5.1 – 8.0	Highly susceptible

Source: Brake *et al*. (1995)

3.2.3 Identification of *Foc* by internal transcribed spacer (ITS) region sequencing

Mycelial fungus of 7 days old culture grown on PDB was extracted from the genomic DNA according to the applied method of Möller *et al*. (1992). Fungal mycelium was harvested by filtration through Whatman No.1 filter paper. Mycelium was ground into a fine powder in liquid nitrogen with a mortar. Fifty milligrams of powdered mycelium were put into a microtube with 500 µl TES solution (Appendix B). The samples were incubated at 55–60 °C for 1 h. Then, the mixture was adjusted the salt concentration to 1.4 M with 5 M NaCl and 1/10 vol 10% cetyltrimethylammonium bromide (CTAB) and incubated for 10 min at 65 °C. The homogenized mixture of samples was subjected to 1 vol chloroform : isoamylalcohol (24 : 1, v/v) and incubated at 0 °C for 30 min, and then centrifuged at max speed for 10 min. The supernatant was transferred to a fresh tube and added 225 µl of 5 M ammonium acetate (NH_4Ac) (Appendix B) and placed on ice for 30 min, then centrifuged at max speed for 10 min. The upper aqueous layer was collected and transferred to 1.5 ml microtube and 0.55 vol of isopropanol was added into the solution to precipitate DNA and immediately centrifuged for 5 min. The DNA pellet was air–dried overnight to remove traces of ethanol and dissolved in 40–100 µl of TE buffer (Appendix B).

The genomic DNA was amplified using ITS1 and ITS4 primers. PCR amplification was carried out in a 50 µl reaction mixture containing 25 µl master mix (Bioneer, South Korea), 3 µl of 10 mM primer ITS1 (5'–TCC GTA GGT GAA CCT GCG G–3') and ITS4 (5'–TCC TCC GCT TAT TGA TAT GC–3'), 17 µl of deionized water and 2 µl of genomic DNA template. The polymerase chain reaction (PCR) ran in the T100™ thermal Cycler (USA) under the following Aguilar–Hawod *et al*. (2020) conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, 60 °C annealing for 1 min, extension at 72 °C for 3 min and completed with a final extension at 72 °C for 10 min. PCR products were measured by 1.2% (w/v) agarose gel electrophoresis in 0.5X TAE buffer and visualized in UV transilluminator. The amplified ITS gene was purified with FavorPrep™ Gel/PCR purification kit (Fisher Biotec, Australia) and sequenced using the ITS1 and ITS4 primer (1Base, Malaysia).

3.3 Screening of actinomycetes against *Foc*

Screening of anti–*Foc* activity by actinomycetes was evaluated using the agar plug technique following the experiment of Bredholdt *et al*. (2007). All *Foc*s were grown on PDA at 30 °C for 7 days and actinomycetes were grown on ISP 2 agar at 30 °C for 7 days. Each fungus was cut into 6 mm disc with a sterile cork borer and placed at the center of the PDA plate. Agar plugs of a single actinomycetes colony

were placed around the fungal plug at a distance of 30 mm. Negative control was ISP 2 agar plug without inoculation. The plates were incubated at 30 °C and the inhibition zone around the plug was observed as an antifungal compound after 7 days. The control plate was incubated with only fungal plug at the center of the plate. The radial growth of fungal mycelium was measured and the percentage of inhibition of radial growth (PIRG) was calculated using the Pandey *et al.* (1982) formula:

$$PIRG = ((R1-R2)/R1) \times 100$$

When R1 = Radial growth of fungus in control plates and R2 = Radial growth of fungus interacting with antagonistic actinomycetes.

Actinomycetes against *Foc* with PIRG value of more than 21% (Khucharoenphaisan *et al*., 2013) were selected for evaluation of plant growth–promoting ability.

3.4 Screening of plant growth–promoting ability of the selected actinomycetes

3.4.1 Nitrogen

3.4.1.1 Nitrogen fixation

A preliminary test for nitrogen fixation was done by growing on nitrogen–free bromothymol blue (NFb) solid plate and semi–solid medium (Appendix A) (Döbereiner and Day, 1976 ; Döbereiner, 1997). The selected actinomycete isolates were streaked on NFb agar plate and incubated at 30 °C for 7–14 days and the appearance of actinomycetes growth was observed as qualitative evidence of atmospheric nitrogen fixation (Döbereiner, 1997). For confirmation of the nitrogen fixation results, the selected actinomycetes were cultured in the subsurface of NFb semi–solid medium and inoculated at 30 °C for 7–14 days. The pellicle formation was observed every day after 2–3 days or more of inoculation.

3.4.1.2 Ammonia production

The freshly grown culture of actinomycetes was inoculated into 5 ml of peptone water (Appendix A) and inoculated at 30 °C for 7 days. After the actinobacterial growth, 0.5 ml of Nessler's reagent (Appendix B) was added to each tube. The development of yellow to brown color was observed as a positive result

for ammonia production (Cappuccino and Sherman, 2002). The uninoculated medium was used as a blank comparison.

3.4.1.3 Nitrate reduction

Peptone potassium nitrate broth (Appendix A) was used for nitrate reduction of selected actinomycetes. A freshly grown culture of actinomycetes was inoculated at 30 °C for 14 days. Each culture broth was added with two drops of sulfanilic acid and three drops of *N, N*–dimethyl–1–naphtylamine reagent (Appendix B) and mixed well. Development of pink to red color was observed as a positive result for nitrate reduction.

3.4.2 Phosphate solubilization

Phosphate solubilization activity was screened by growing the strains on Pikovskaya's agar (Pikovskaya, 1948) and NBRIP–BPB (NBRIP media with 0.025 g/L bromophenol blue) (Appendix A) (Metha and Nautiyal, 2001). Each strain was point inoculated on an agar plate and incubated at 30 °C for 7–21 days. Every seven days, the zone of digestions was shown halo zone around the colonies and calculated the phosphate solubilization efficiency (SE) followed Nguyen *et al*. (1992) formula:

$$SE = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

3.4.3 Potassium solubilization

Potassium solubilization activity was screened by growing the strains on Aleksandrov agar (Appendix A) (Hu *et al*., 2006). Each strain was point inoculated on an agar plate and incubated at 30 °C for 7–21 days. Every seven days, the zone of digestions was shown halo zone around the colonies and determined the potassium solubilization efficiency (PSE) followed Sarikhani *et al*. (2019) formula:

$$PSE = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

3.4.4 Indole acetic acid production

The selected actinomycetes were quantified by growing in ISP 2 liquid medium supplemented with 0.5% of L–tryptophan at 30 °C at 180 rpm for 7 days. Culture media was centrifuged at 6,000 rpm for 30 min. The supernatant (1 ml) was mixed with 2 ml of Salkowski's reagent (Appendix B). The appearance of a pink to red color after incubation 30 min at room temperature in the dark conditions

indicated a positive result. Optical density was read at 535 nm using a spectrophotometer (Ali and Hasnain, 2007 ; Anwar *et al*., 2016). The level of IAA production was measured with a standard curve (Appendix C, Figure 1).

3.4.5 Gibberellic acid production

The supernatant culture from section 3.4.4 was extracted with phosphate buffer (pH 8) (Appendix B) and centrifuged at 6,000 rpm for 30 min. The supernatant was adjusted to pH 2.5 with 5 N hydrochloric acid and partitioned with equal volumes of ethyl acetate five times. Fifteen milliliters of ethyl acetate fractions were added with 2 ml of 10% (w/v) zinc acetate solution and 2 ml of 1% (w/v) potassium ferrocyanide solution and mixed well. The solutions were centrifuged at 6,000 rpm 20 min and 5 ml of the supernatant was added to 5 ml of 10% (w/v) hydrochloric acid and then the mixture was inoculated at 20 $^{\circ}$C for 75 min. The absorbance was measured at 254 nm and compared with a gibberellic acid standard curve (Appendix C, Figure 2) (Goudjal *et al*., 2016b).

3.4.6 Cytokinin production

The selected strains were grown on M9 medium supplemented with 0.2% casamino acids, 0.01% thiamine, and 2 pg of biotin per liter (Appendix A) (Akiyoshi *et al*., 1987) and were incubated 30 °C at 180 rpm for 7 days. Culture media was centrifuged at 6,000 rpm for 30 min. The supernatant was extracted with ethyl acetate three times and evaporated to dryness. The residues were re–dissolved with methanol. Thin–layer chromatography (TLC silicate gel 60 F_{254} plates, Merck, USA) was spotted with the samples and standard cytokinin using kinetin on the baseline at 1.0 cm. The spot was left to dry for a minute. The TLC plate was placed vertically in a through containing the mixture of solvent with isopropanol : ammonia : distilled water (10 : 1 : 1, v/v/v) ratio. When the solvent moved up to the front line of TLC plates, the plate was taken out and dried. UV light at 254 nm lamp was used for visualization of the bands (Hussain and Hasnain, 2009 ; Sabat *et al*., 2013).

3.4.7 Siderophore production

The selected strains were grown on the Chrome Azurol S (CAS) agar (Appendix A) according to the method of Alexander and Zuberrer (1991). Each strain was spotted on CAS agar and incubated at 30 °C for 7–14 days. Siderophore production was indicated by a change in color from blue to yellow or orange halos around the colonies were considered positive. Further, the siderophores production index (SPI) was calculated by Alexander and Zuberrer (1991) formula:

$$SPI = \frac{\text{Halo zone diameter}}{\text{Growth diameter}}$$

3.4.8 Hydrogen sulfide production

The selected strains were inoculated on triple sugar iron (TSI) agar (Appendix A) by stabbing into the medium in the butt of the tube and then streaked back along the surface of the slant. After incubation at 30 °C for 14 days, the black precipitate was observed as a positive result for hydrogen sulfide production (Microbugz, 2021).

3.4.9 Hydrocyanic acid production

Hydrocyanic acid (HCN) production was evaluated according to the method of Anwar *et al*. (2016). Each selected actinomycete was streaked on ISP 2 agar supplemented with 4.4 g/L of glycine. Whatman filter paper No.1 was dipped in 2% sodium carbonate in 0.5% picric acid for a minute and placed underneath the plate lids. All plates were sealed with parafilm and incubated at 30 °C for 7–14 days. The rating scales for HCN production were used: 0 = no color change (yellow) (no HCN production), 1 = light reddish brown, 2 = medium reddish brown and 3 = dark reddish brown (Sreevidya *et al*., 2016).

3.5 Measurement of extracellular enzyme production of the selected actinomycetes

3.5.1 1–aminocyclopropane–1–carboxylate (ACC) deaminase production

Primary screening of ACC utilization was checked on nitrogen–free Dworkin and Foster's salt minimal agar (Dworkin and Foster, 1958) with 3 mM ACC as the sole nitrogen source (DF–ACC medium, Appendix A). Each strain was streaked on DF–ACC agar and incubated at 30 °C in the dark condition for 7–14 days. Growth of the strain on DF–ACC agar indicated the efficiency to utilize ACC and produce ACC deaminase (El–Tarabily, 2008).

3.5.2 Amylase production

The selected isolates were point inoculated onto inorganic salt starch agar (ISP 4) (Appendix A) and incubated at 30 °C for 3–7 days. The iodine solution (Appendix B) was flooded onto the surface culture media. The results were qualitatively expressed as levels of enzymatic activity (LEA) (González *et al*., 2020) using the formula:

$$\text{LEA} = \frac{\text{Clearance zone diameter}}{\text{Growth diameter}}$$

3.5.3 Gelatinase production

The selected isolates were point inoculated onto gelatin agar (Arai, 1975) (Appendix A) and incubated at 30 °C for 7–14 days. The acid mercuric chloride solution (Appendix B) was flooded onto the surface culture media. The halo zone around the colonies was measured and calculated the same manner as described in section 3.5.2.

3.5.4 Urease production

The selected isolates were inoculated onto Christensens's urea agar (Christensen, 1946) (Appendix A) and incubated at 30 °C for 7–14 days. The development of the pink color indicated a positive result.

3.5.5 Caseinase production

The selected isolates were point inoculated onto skimmilk agar (Adinarayana *et al*., 2003) (Appendix A) and incubated at 30 °C for 3–7 days. Zone of digestion was measured and calculated the same manner as described in section 3.5.2.

3.5.6 Lipase production

Tributyrin agar (Sztajer *et al*., 1988) and tween 80 agar (Kumar *et al*., 2012) (Appendix A) was used to detect lipase enzyme production. The selected actinomycetes were point inoculated onto the agar plates and incubated at 30 °C for 3–7 days. The clear zone on tributyrin agar and the insoluble crystals (fatty acids bind with the calcium) around the colony on tween 80 agar were measured and calculated the same manner as described in section 3.5.2.

3.6 Measurement of stress, chemical fertilizer, and fungicide tolerance properties of the selected actinomycetes

3.6.1 Measurement of salt tolerance

The tolerance of the actinomycetes to salinity was performed according to Amaresan *et al*. (2014). The selected actinomycetes were streaked onto ISP 2 agar medium amended with various concentrations of NaCl (1–16 g/100 ml) and incubated at 30 °C for 7–14 days. Actinobacterial growth on NaCl amended media was recorded.

3.6.2 Measurement of pH tolerance

The selected actinomycetes were streaked on ISP 2 medium in various pH (4–12) and incubated at 30 °C for 7–14 days and recorded the actinobacterial growth.

3.6.3 Measurement of thermo tolerance

The selected actinomycetes have tested for the ability to tolerate heat stress. Actinomycetes were grown in ISP 2 medium at 30 °C with shaking 180 rpm for 7 days. After that, ten milliliters of actinobacterial samples were transferred into the new sterile test tube and then placed at different temperatures of 4, 25, 30, 37, 45, 55, 60, 65, 70, 80, 90, and 100 °C for 1 h. After incubation, a small volume of broth was streaked on ISP 2 agar plate and observed for the growth of actinomycetes after incubation for 7–14 days at 30 °C.

3.6.4 Measurement of polyethylene glycol (PEG) tolerance

The osmotic pressure resistance of the selected actinomycetes was performed by observing the growth on ISP 2 medium amended with various concentrations of PEG6000 (1–35 g/100 ml). All samples were incubated at 30 °C for 7–14 days and recorded the growth capacity.

3.6.5 Measurement of chemical fertilizer and fungicide tolerance

The selected actinomycetes were streaked onto ISP 2 agar supplemented with 0.4% chemical fertilizer related banana planting such as the formula of 46–0–0, 0–0–60, 13–13–21, 25–7–7, 8–8–24 and 16–16–16. The selected actinomycetes were streaked onto ISP 2 agar supplemented with 2.0% benzimidazole (carbendazim). All plates were incubated at 30 °C for 7–14 days and recorded the growth capacity.

3.7 Growth measurement of the selected actinomycetes

3.7.1 Inoculum preparation

All twenty–four actinomycetes were grown on a modified soil extract agar (A9) plate (Appendix A) for 7 days at 30 °C. The actinobacterial spores were scraped and flooded with 0.01% (v/v) of tween 80 solutions. The solution with spores was counted using a haemacytometer and diluted to 10^6 spore/ml as an inoculum.

3.7.2 Submerged fermentation

Growth of actinomycetes was carried out using ISP 2 broth. The substrate with two replications per isolate was contained to 250 ml erlenmeyer flask with 50

ml working volume. The substrate was inoculated with 2% of spore suspension and incubated at 30 ℃ for 7 days (manually mixed 2–3 times per day). One milliliter of the suspension was transferred to another 9 ml same solution tube and this step was repeated to set up a 10–fold dilution series. Aliquots (100 µl) were spread onto ISP 2 agar and the plates were incubated at 30 ℃ for 7 days. Growth of actinomycetes in colony forming unit per one milliliter of sample were compared between strains.

3.7.3 Solid–state fermentation

Growth of actinomycetes was carried out using red sorghum (55%), water (35%), fine rice bran (7%), bat guano (2.7%) and supplement (0.3%) (glucose : yeast extract : malt extract : magnesium chloride 0.4 : 0.4 : 1.0 : 0.2) as a substrate. Substrate with two replications per isolate was contained in a plastic bag (8 x 12 $inch^2$) and covered with cotton and then sterilized for 15 min at 121 ℃. The substrate was inoculated with 2% of spore suspension and incubated at 30 ℃ for 7 days (manually mixed every 3 days). Ten grams of each sample was weighed, ground, and homogenized, and then suspended in 90 ml sterile distilled water with 0.01% tween 80 solutions and shake the suspension for better release and distribution of actinobacterial structure for 10 min. After that, the suspension was ten folds diluted to 10^{-2}–10^{-6} and 0.1 ml of 10^{-4}–10^{-6} dilutions was spread onto ISP 2 agar and the plates were incubated at 30 ℃ for 7 days. Growth of actinomycetes in colony forming unit per one gram of the sample was compared between strains.

3.8 Banana toxicity of the selected actinomycetes

3.8.1 Preparation of banana tissue culture

The cultivar used was *Musa* (AAA) 'Kluai Hom Thong' tissue culture plantlets that were obtained from a commercial company. The plantlets were carefully washed with tap water for eliminated the agar and acclimatized for 4 weeks in the nursery. After that, tested plantlets were carefully uprooted and washed with tap water, soaked with 70% ethanol for 10 min, and sterile distilled water for 3 times. The plantlets with healthy white roots were selected for the experiments.

3.8.2 Preparation of tested actinomycetes

Twenty–four actinomycetes were grown on a modified soil extract agar (A9) agar plate for 7 days at 30 ℃. The actinomycetes spores were scraped and flooded with 0.01% (v/v) of tween 80 solutions. The solution with spores was counted using a haemacytometer and diluted to 10^8 spores/ml as a test solution.

3.8.3 The experiment

The experiment was tested using a small pot (8 cm high x 10 cm in diameter, one plantlet per pot) and prepared according to the "double–tray technique" (AbdulKadir, 2001). Seventy–five pots were each filled with soil that consisted of sand and clay (4 : 1, v/v) and was autoclaved at 121 °C for 30 minutes and air–dried at least 72 h before use. The compositions of soil were measured by the Land Development Department of Thailand (Appendix D, Figure 1). Seventy–five plantlets were numbered and grouped into twenty–five treatments with three replications (24 isolates and without inoculation or control). The root of the tested plantlets was immersed in an actinobacterial suspension for 30 min before planting. Dead and survivor of plantlets were recorded until seven weeks. Toxicity evaluation was applied using the DSI index (Brake *et al*., 1995).

3.9 Antagonistic activity among the selected actinomycetes

ISP 2 agar plates were inoculated by streaking one strain at 1.5 cm distance from the petri dish edge. Actinobacterial target antagonists were streaked at 90° angle to the main isolate (Rahman *et al*., 2011). The inhibition distances were recorded after 7 days at co–inoculation at 30 °C.

3.10 Taxonomic characterization of the selected actinomycetes

3.10.1 Morphological characteristics

Twenty–four actinomycetes were grown on ISP 2 agar for 14 days at 30 °C, and a spore was observed under a light microscope (ECLIPSE E200, Nikon) with total 400X magnifications. The color of the aerial and substrate mycelium and soluble pigment was determined by NBS/IBCC color system (Kelly, 1964).

3.10.2 Identification of the selected actinomycetes by 16S rRNA gene sequencing

Twenty–four actinomycetes were grown on ISP 2 agar for 2–4 days at 30 °C. The fresh colony was collected by a sterilized toothpick and suspended in sterilized deionized water. For amplification of the 16S rRNA gene, the PCR was performed in a total volume of 50 µl containing 1 µl of DNA template, 25 µl of master mix (i–taq, iNtRON Biotechnology, Korea), 5 µl of 10 pmol/µl of 9F (5'–GAGTTTGATCCTGGCTCAG–3') and 1541R (5'–GTTACCTTGTTACGACTT–3') primers and 14 µl of MilliQ water. The PCR ran in the T100™ thermal Cycler (USA) under the following conditions: initial denaturation at 94 °C for 3 min; 40 cycles of 94 °C for 30s, 56 °C for the 30s and 72 °C for 90s; and final extension at 72 °C for 5 min. The PCR

product was purified with FavorPrep™ Gel/PCR purification kit (Fisher Biotec, Australia) and sequenced using the 27F (5'–AGAGTTTGATCCTGGCTCAG–3'), 518F (5'–CCAGCAGCCGCGGTAATACG–3'), 800R (5'–TACCAGGGTATCTAATCC–3') and 1492R (5'– GGTTACCTTGTTACGACTT–3') primers. The 16S rRNA gene sequence was aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by using the CLUSTAL W program within BioEdit program version 7.1.3.0 (Hall, 1999). The alignment was manually verified and adjusted before the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor–joining (Saitou and Nei, 1987) and maximum–likelihood (Felsenstein, 1981), and the MEGA program version X (Kumar *et al*., 2018). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings. The values for sequence similarity among all actinomycetes were first determined using the EzBioClound server (http://www.ezbiocloud.net/) (Yoon *et al*., 2017). Gaps and ambiguous nucleotides were eliminated from the calculations.

3.11 Product formation from the selected actinomycetes

3.11.1 Criteria of product formation

In this study, the concept of product formation is for anti–*Foc* and promoting banana growth. Firstly, the selection of actinomycetes isolated came from these two purposes. Other important factors were extracellular enzyme production to degrade the natural nutrients for growth and produced some biological materials, tolerance, fast growth, and non–toxic to host plant or non–pathogenic to human and environmentally safety. Moreover, the combination between actinomycetes isolates was not an antagonist. Finally, the industrial application of the microbial products will require a low–cost production but high efficiency (Parnell *et al*., 2016).

3.11.2 Actinomycete products formulation

Eight actinomycetes (RBST1–4, RBST2–21, RBST2–54, RCPT1–4, RCPT3–28, RCPT3–40, CH5–8, and CH9–7) were prepared and cultured on solid medium using red sorghum and others components same the manner as described in the section 3.7.3. Seven days later, actinomycetes on the solid substrate were mixed with sterilized well compost in ratio 1 : 5 (w/w) and air–dried under shade until the moisture was below 10%. All samples were taken for growth evaluation before mixing. Each actinomycete was equally weighted and set in three formulas based on their different properties as follows: ATU-Bio 1 (RBST1–4, RCPT3–28, CH9–7), ATU-Bio 2 (CH5–8, RCPT1–4, RBST2–54) and ATU-Bio 3 (RCPT1–4, RCPT3–40,

RBST2–21), respectively. Each isolate and mixture was filled in an aluminum foil bag with a zip lock and stored at 30 ℃ before being used.

3.11.3 Shelf–life determination

Actinobacterial total plate count technique was used for shelf–life determination and measured every 0, 4, 7, 9, 12, and 16 month, respectively. Ten grams of each actinomycete isolate and the product with 2 replications were weighed and then suspended in 90 ml sterile distilled water with 0.01% tween 80 solution and shaken the suspension for better release and distribution of actinobacterial structure for 10 min. After that, the suspension was ten folds diluted to 10^{-1}–10^{-6} and 0.1 ml was spread onto ISP 2 agar in duplication and the plates were incubated at 30 ℃ for 7 days. The growth of actinomycetes was calculated in colony forming unit per one gram of sample.

3.12 Field experiment from actinomycete products

3.12.1 Plant growth promotion experiment

The cultivar used was *Musa* (AAA) 'Kluai Hom Thong' tissue culture plantlets that were obtained from a commercial company. The plantlets were carefully washed with tap water for eliminated the agar and acclimatized for 4 weeks in the nursery. After that, tested plantlets were carefully uprooted and washed with tap water, soaked with 70% ethanol for 10 min, and sterile distilled water for 3 times. The plantlets with healthy white roots were selected for the experiments.

For the experimental design, a completely randomized design (CRD) (Getha *et al*., 2005) was arranged into seven treatments with five replications. The "double–tray technique" (AbdulKadir, 2001) with a small pot (17 cm high x 21 cm of diameter, one plantlet per pot) was used for the experiment. Twenty–eight pots were each filled with soil that consisted of sand and clay (4 : 1, v/v) and was autoclaved at 121 ℃ for 30 min and air–dried at least 72 h before use. The compositions of soil were measured by the Land Development Department of Thailand (Appendix D, Figure 1). Twenty–eight plantlets after sterilization with 70% ethanol and washing with sterile distilled water were grouped into seven groups (treatments) as follows:

I. T1: No supplement (control)
II. T2: Carrier (well compost)
III. T3: Chemical fertilizer (16-16-16 formula)
IV. T4: Commercial plant growth–promoting rhizobacteria 2 (PGPR–2)
V. T5: ATU–Bio 1
VI. T6: ATU–Bio 2
VII. T7: ATU–Bio 3

Each material (except T1) was added into the soil with 0.3% for T2–T3, the final concentration of 10^6 CFU/g for T4–T7, and mixed well. One banana plantlet was planted into the center of each pot. All pots were watered once a day and placed on opened shade greenhouse that received 12 h sunlight each day. The experiment was performed for 12 weeks. Plant growth measurements were recorded as follows:

I. Plant height (using a scale) every week
II. Root length (using scale) before planting and the last week
III. Stem diameter (using vernier caliper) every week
IV. Fresh weight (using a digital weight scale) before planting and the last week.
V. Dry matter (oven–dried at 103–105 °C for 1–2 h to constant weight and using digital weight scale) the last week.

For statistical analysis, the mean of all parameters were analyzed using analysis of variance (ANOVA) and compared by Duncan's new multiple range test (DMRT) at *P=0.05* using SPSS for window Ver.25.

3.12.2 Plant growth promotion and disease control experiment

3.12.2.1 Protective experiment

The banana plantlets and soils were prepared same manner as described in the plant growth promotion experiment.

For the experimental design, 7 x 5 factorial in completely randomized design (CRD) (Getha *et al*., 2005) was arranged with two factors (factor A: tested material with seven levels and factor B: *Foc* strain with five levels) and four replications. The "double–tray technique" (AbdulKadir, 2001) with a small pot (17 cm high x 21 cm of diameter, one plantlet per pot) was used for the experiment. One hundred and forty pots were each filled with soil that consisted of sand and

clay (4 : 1 v/v). One hundred and forty plantlets after sterilization with 70% ethanol and wash with sterile distilled water were grouped into thirty–five treatment combinations as follows:

Table 3.3 Protective experiment treatment combinations.

Factor A	Factor B
A1: No supplement (control)	B1: *Foc* 1[b]
A2: 3% Carrier (well compost)	B2: *Foc* 6[b]
A3: Fungicide (2% carbendazim)	B3: *Foc* 7[b]
A4: Commercial *Trichoderma harzianum*	B4: *Foc* 8[b]
A5: ATU-Bio 1[a]	B5: No *Foc*
A6: ATU-Bio 2[a]	
A7: ATU-Bio 3[a]	

[a, b] Final concentration at 10^6 CFU/g (Getha *et al*., 2005)

All factors A was added into each pot filled with soil and mixed, and then one banana plantlet was planted into the center of each pot. All pots were watered once a day and placed on opened shred greenhouse that received 12 h sunlight each day. After three weeks, all factors B was added into the pot nearly the root of banana plantlets. The experiment was performed for 12 weeks. Plant growth measurements and disease signal were recorded as follows:

I. Plant height (using a scale) every week

II. Root length (using scale) before planting and the last week

III. Stem diameter (using vernier caliper) every week

IV. Fresh weight (using a digital weight scale) before planting and the last week.

V. Dry matter (oven–dried at 103–105 °C for 1–2 h to constant weight and using digital weight scale) at the last week.

VI. Disease evaluation was followed by the experiment of Brake *et al*. (1995)

For statistical analysis, the mean of all parameters were analyzed using analysis of variance (ANOVA) and compared by Duncan's new multiple range test (DMRT) at *P=0.05* using SPSS for window Ver.25.

3.12.2.2 Curative experiment

The banana plantlets and soils were prepared same manner as described in the plant growth promotion experiment. The experiment design and technique were performed same manner describes in the protective experiment.

Banana plantlets were immersed with factor B for 30 min and planted into the center of each pot that was filled with soil. All pots were watered once a day and placed on opened shade greenhouse that received 12 h sunlight each day. When the banana plantlets showed the light yellow coloring of the lower leaves or wilting, factors A were added into the pot nearly banana root and soil, and gently mixed. The experiment was performed for 12 weeks. Plant growth measurements and disease signals were recorded as follow the protective experiment.

3.13 Evaluation of the mechanism against *Foc*

3.13.1 Extracellular enzyme production

3.13.1.1 Chitinase production

The selected actinomycetes were screened the chitinase production using the medium according to Nawani *et al*. (2002) using chitin agar (Appendix A). Each strain was inoculated on an agar medium and incubated at 30 °C for 7–28 days. The halo zone around the colonies was measured and calculated same manner as described in section 3.5.2.

3.13.1.2 Carboxymethyl cellulase (CMCase) production

The selected actinomycetes were point inoculated onto caboxymethy cellulose (CMC) agar (Ariffin *et al*., 2006) (Appendix A) and incubated at 30 °C for 14 days to allow hydrolyzing of the CMC substrate. All plates were flooded with 1 mg/ml congo red dye for 15 min and destaining with 1 M NaCl for 15 min. The halo zone around the colonies was measured and calculated same manner as described in section 3.5.2.

3.13.1.3 Cellulase production

ISP 9 agar medium with 1% Avicel® PH–101 (Appendix A) was applied to use for detection cellulase production from all strains. After incubation at 30 °C for 7–28 days, the halo zone around the colonies was measured and calculated same manner as described in section 3.5.2.

3.13.2 Secondary metabolite production

3.13.2.1 Fermentation and extraction

For the starter preparation, two selected isolates (CH5–8 and CH 9–7) were cultured on ISP 2 agar for 10–14 days at 30 °C and then the agar was cut into pieces (1 x 1 cm^2). The pieces were added into 250 ml erlenmeyer flasks, each containing 50 ml of ISP 2 broth. The strain was incubated at 30 °C for 10–14 days at 180 rpm on a rotary shaker. For the production scale (20 L), 10% of the starter was added into 1 L erlenmeyer flask which contained 250 ml of ISP 2 broth and the culture was shaken (200 rpm) at 30 °C for 14 days.

After an incubation period, fermentation broth and cells were extracted three times with an equal volume of ethyl acetate. The ethyl acetate was dried over Na_2SO_4 and evaporated to dryness to obtain a crude extract.

3.13.2.2 Isolation and purification

The crude extract was preliminary analyzed the chemical profile using the analytical HPLC technique. The crude extract was dissolved with 100% methanol and analyzed using HPLC (Utimate 3000, DIONEX) equipped with C–18 column (Puropher® Star; Merck 5 µm, 2.1 x 50 mm) with the linear gradient system (0–100% CH_3CN in water + 0.05% formic acid), flow rate 0.5 ml/min for 15 min. The detector was UV/UV-VIS. The chromatograms obtained from the system were compared with the in–house database of BIOTEC, NSTDA, Thailand.

For secondary metabolite isolation, the crude extract was dissolved with methanol, filtered, and then the solution was isolated using a Sephadex LH20 column chromatography (4.5 x 40 cm, as a stationary phase). The active fractions with anti–*Foc* activity were isolated using preparative–HPLC (Sunfire C18 column, 19 x 250 mm). The details for the isolation and purification of the selected isolates were shown in Figures 3.1 and 3.2.

3.13.2.3 Anti–*Foc* activity of the fractions

Anti–*Foc* activity of the crude extract, the selected fractions, and pure compounds were performed using the agar disc diffusion method (Nanthan *et al*., 1978). Eight *Foc*s were cultured on PDA at 30 °C for 7 days. The spore was scraped and adjusted the concentration to $1x10^6$ spore/ml using a haematocytometer. The test organisms were swabbed on the top of PDA and were allowed to dry for 10 min. The crude extract and the selected fractions were dissolved in methanol at the maximum concentration of 5 mg/ml and then 20 µl was dropped on the sterile paper disc (6 mm) and allowed to dry for 10 min. Each

disc was placed on the top of swabbed PDA. A paper disc with methanol was used as negative control. Cycloheximide, benzimidazole (carbendazim), and nystatin were used as positive controls. All tests were incubated at 30 °C for 2–3 days and antagonism of test organisms was recorded.

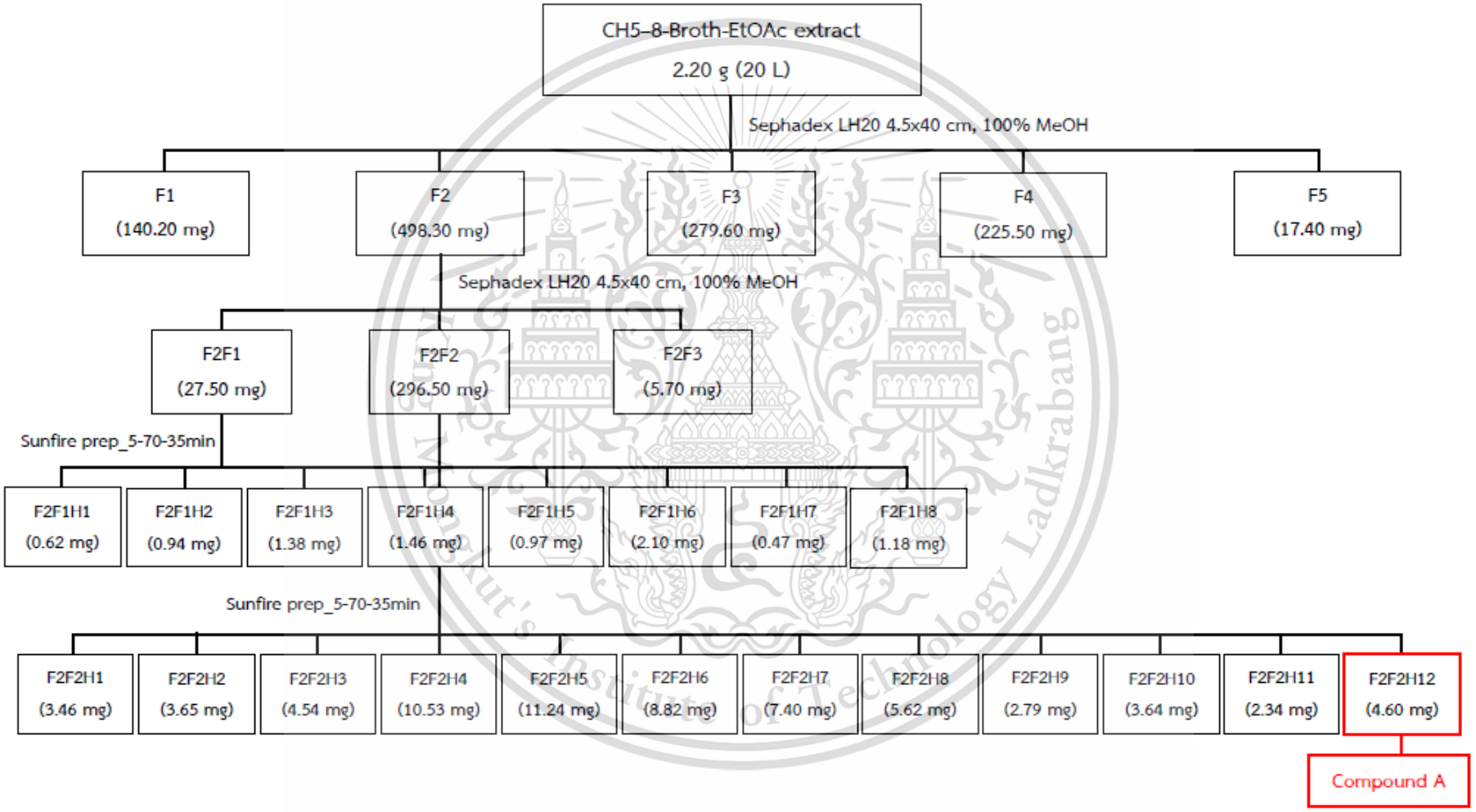


Figure 3.1 Isolation of the ethyl acetate extract of the strain CH5–8.

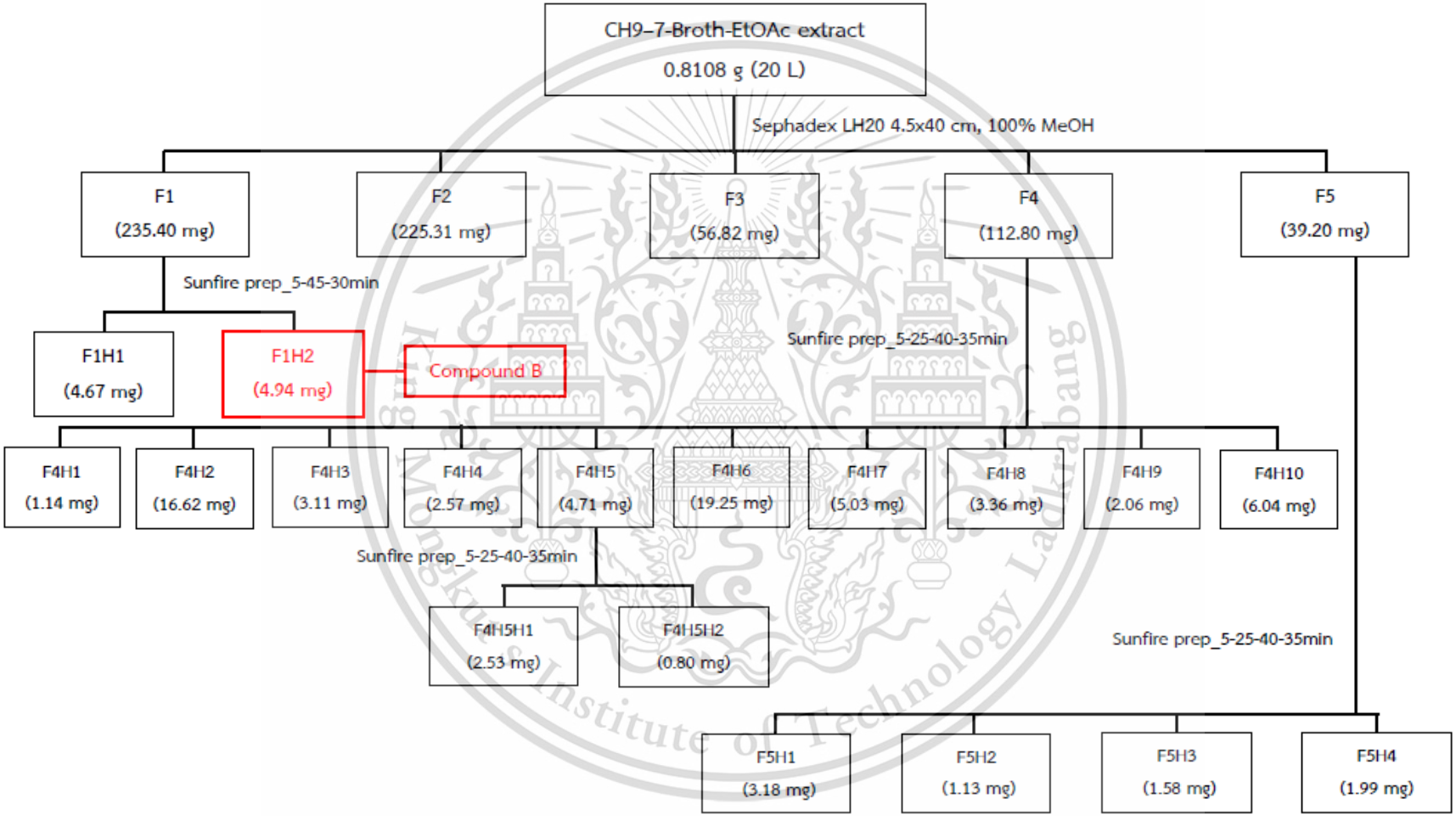


Figure 3.2 Isolation of the ethyl acetate extract of the strain CH9–7.

3.14 Polyphasic taxonomic characterization of the selected actinomycetes

Three selected actinomycetes, RCPT1–4, CH5–8, and CH9–7, were identified using a polyphasic taxonomy approach including phenotypic, chemotaxonomic, and genotypic characteristics.

3.14.1 Phenotypic characteristics

3.14.1.1 Morphological and cultural characteristics

Morphological characteristics of the selected actinomycetes were grown on ISP 2 at 30 °C for 14 days and determined using light and scanning electron microscope (model JSM-6610 LV; JEOL). For SEM, the samples were prepared by the method of Itoh *et al*. (1989). Cultural characteristics were studied on 14–day old culture at 30 °C on nine different agar media including yeast extract–malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt–starch agar (ISP 4), glycerol–asparagine agar (ISP 5), peptone–yeast extract iron agar (ISP 6), tyrosine agar (ISP 7), Czapek's sucrose agar, glucose–asparagine agar (Shirling and Gottlieb, 1966), and nutrient agar (Appendix A). The color description of the colony including aerial and substrate mycelium and soluble pigment was determined by NBS/IBCC color system (Kelly, 1964).

3.14.1.2 Physiological characteristics

The selected actinomycetes were inoculated on ISP 2 agar with various NaCl concentration (0, 1, 2, 3, 4, 5, 6, 7% (w/v)), pH (4, 5, 6, 7, 8, 9, 10, 11, 12) at 30 °C and incubation temperature (4, 15, 20, 25, 30, 37, 40, 42, 45, 47, 50, 55 °C) after 14–day incubation. The maximum NaCl growth concentration was recorded.

3.14.1.3 Biochemical characteristics

1 Carbon utilization

The carbon utilization was performed on ISP 9 medium (Appendix A) and a carbon source was added to give the concentration of approximately 1% (Shirling and Gottlieb, 1966). After autoclaving at 110 °C for 10 min, the melted mixture was poured into the sterile petri dish.

Carbon sources and controls used for the test were no carbon source (negative control), D–glucose (positive control), L–arabinose, L–rhamnose, D–xylose, adonitol, dextran, salicin, lactose, D–ribose, D–melezitose,

sucrose, D–mannose, xylitol, D–cellobiose, D–galactose, trehalose, inulin, *myo*–inositol, D–raffinose, D–melibiose, D–mannitol, D–fructose and glycerol, respectively.

A loopful of fresh actinomycete isolates were streaked on the agar surface and incubated at 30 ℃ for 10–14 days. Actinomycetes growth was recorded by comparing with two controls (negative and positive) using the criteria as follows:

I. Positive utilization (+): when actinobacterial growth on the test agar plate is significantly better than on the negative plate but somewhere less than on the positive control.

II. Doubtful utilization (±): when actinobacterial growth on the test agar plate is only slightly better than on the negative plate and significantly less than on the positive plate.

III. Negative utilization (-): when actinobacterial growth on the test agar plate is similar to or less than growth on the negative plate.

2 Acid production from carbohydrate

The acid production from carbon sources was tested using the basal inorganic nitrogen medium (Appendix A), adjusted the pH to 7.0 before the addition of 0.04% bromocresol purple solution with 15 ml per liter. The agar medium was added to the test tube and sterilized at 110 ℃ for 10 min. Each carbon source (sterilized separately) was added to the test tube at the final concentration of 1%. A few amounts of each actinomycete suspension was dropped into an agar tube and incubated at 30 ℃ for 7–14 days. The positive result showed the color changed from purple to yellow.

3 Nitrogen utilization

The nitrogen utilization was performed on a basal medium for nitrogen source (Appendix A) and nitrogen source was added to give the concentration of approximately 1%. After autoclaving at 121 ℃ for 15 min, the melted mixture was poured into the sterile petri dish.

Nitrogen or amino acid sources and controls used for the test were no nitrogen source (negative control), L–asparagine, L–histidine, 4–hydroxyproline, L–methionine, L–phenylalanine, L–threonine, DL–2–aminobutane, L–arginine, L–cysteine, L–proline, L–serline and L–valine, respectively.

A loopful of fresh actinomycetes isolates were streaked on the agar surface and incubated at 30 ℃ for 10–14 days. Actinomycetes growth was recorded by comparing with negative control (+ = growth on the test agar plate is significantly better than on the negative plate, w = growth on the test agar plate is

slightly better than on the negative plate, and - = growth on the test agar plate is similar to or less than growth on the negative plate).

4 Starch hydrolysis

The selected actinomycetes were streaked onto the surface of inorganic salt–starch agar (ISP 4) (Shirling and Gottlieb, 1966) (Appendix A) and incubated at 30 °C for 14 days. The halo zone around colony after flooding with 1% (v/v) iodine solution indicated the positive result of starch hydrolysis.

5 Gelatin liquefaction

The selected actinomycetes were inoculated into the test tube of Bouillon gelatin broth (Arai, 1975) (Appendix A) and incubated at 30 °C for 7–14 days. The uninoculated control was used to compare and observed both tubes when placed at 4 °C for 30 min. The positive result showed gelatin became too liquid or did not solidify.

6 Urease production

The selected actinomycetes were streaked onto the surface of Christensen's urea agar (Christensen, 1946) (Appendix A) and incubated at 30 °C for 7–14 days. The development of the pink color indicated a positive result.

7 Coagulation and peptonization of milk

The selected actinomycetes were inoculated into 10% skim milk broth (Appendix A). If milk was peptonized, the broth would become clear, and its coagulation would be converted to a solid form. Peptonization test medium (Appendix I) was used for confirmation of milk peptonized. The clear zone around colony after incubation at the same temperature and time indicated a positive result.

8 Nitrate reduction

Peptone potassium nitrate broth (Appendix A) was used for nitrate reduction of the selected actinomycetes. A freshly grown culture of actinomycetes was inoculated at 30 °C for 14 days. Each culture broth was added two drops of sulfanilic acid and three drops of *N, N*–dimethyl–1– naphtylamine reagents (Appendix B) and mixed well. The development of the pink to red color was observed as a positive result for nitrate reduction.

9 Oxidase and catalase activity

Catalase activity was observed by bubble production after applying 3% (v/v) hydrogen peroxide solution onto a fresh cells. Test for oxidase activity was carried out by oxidation of 1% *N, N, N', N'*–tetramethyl–p–phenylenediamine dihydrochloride. The filter paper was immersed with the solution and then fresh actinomycetes were scraped using a sterile toothpick and smeared on wet filter paper. The development of brown to the blue color indicated a positive result.

10 Hydrogen sulfide production

The selected actinomycetes were inoculated on triple sugar iron (TSI) agar (Appendix A) by stabbing into the medium in the butt of the tube and then streaked back along the surface of the slant. After incubation at 30 °C for 14 days, the present of black precipitate indicated a positive result.

11 Decomposition of an insoluble compound

The selected actinomycetes were streaked on ISP 2 agar supplemented with 1% insoluble compound (autoclaved separately) such as cellulose, xanthine, adenine, hypoxanthine, and tyrosine, and incubated at 30 °C for 10–14 days. The digestion zone around the colony indicated a positive for decomposition of insoluble compounds.

12 Enzyme activity

The enzyme activities were determined using commercially available test kits, API ZYM® system (bioMérieux, France). The Api strip was incubated following by manufacturer's manual. Sixty–five microliter of actinomycetes suspension with McFarland No. 6 was added into each dried substrate cupule on the strip, covered, and incubated at 37 °C for 5 h. After incubation, each drop of ZYM A and ZYM B reagents was added to the cupule, incubated at room temperature for 5–10 min, and observed and recorded the intensity of color change.

3.14.2 Chemotaxonomic characteristics

Biomass used for chemotaxonomic analyses was obtained from freeze–dried cells from the cultures grown in ISP 2 broth on a rotary shaker at 200 rpm at 30 °C for five days.

3.14.2.1 Isomers of diaminopimellic acid analysis

Ten milligrams of freeze–dried cells were hydrolyzed with 1 ml of 6 N HCl at 100 °C for 18 h. The whole–cell hydrolysate was filtered and evaporated. The dried extracts were dissolved with 400 µl of distilled water. The DAP was determined using the standard TLC method (Staneck and Robert, 1974). The solution was loaded into a cellulose HPTLC plate (no.5716, Merck, Germany) and developed with methanol : water : 6 N HCl : Pyridine (80 : 26 : 4 : 10, v/v). The standard 2, 6 DAP (contained *meso*–DAP and *LL*–DAP) was used as the control. After the second development, the cellulose HPTLC plate was sprayed and detected with 0.4% ninhydrin in water saturated n–butanol solution (Appendix B) and dry heated at 100 °C for 5 min. The DAP spot were seen as grey green fading to yellow. *LL*–DAP spot appeared above *meso*–DAP spot (Staneck and Robert, 1974).

3.14.2.2 Whole–cell sugars analysis

Fifty milligrams of freeze–dried cells were hydrolyzed with 1 ml of 1 N H_2SO_4 at 100 °C for 2 h. The pH of whole–cell hydrolysate was adjusted to 5.2–5.5 with saturated $Ba(OH)_2$ solution. The solution was centrifuged at 4,500 rpm for 10 min to remove the precipitate. The upper solution was evaporated to dryness and 400 µl of distilled water was added to dissolve the dried sample. The solution was loaded into a cellulose HPTLC plate (no.5716; Merck, Germany) and developed with n–butanol : water : pyridine : toluene (10 : 6 : 6 : 1, v/v). After the second development, the cellulose HPTLC plate was sprayed and detected with aniline phthalate solution (Appendix B) and dry heated at 100 °C for 4 min (Komagata and Suzuki, 1987). Two sets of mixed sugar were used as a standard solution. Set I was galactose, mannose, xylose, and rhamnose and set 2 was glucose, arabinose, and ribose.

3.14.2.3 Polar lipids analysis

An analysis of polar lipids was performed in three steps as follows:

1 Extraction

One hundred and fifty milligrams of freeze–dried cells were suspended in 3 ml of methanol : 0.3% NaCl (100 : 10, v/v) and 3 ml of petroleum ether and mixed for 15 min. The cell suspensions were centrifuged at 3,000 rpm for 10 min. The precipitate was added 1 ml of petroleum ether and mixed again for 2–5 min. This lower layer was heated at 100 °C for 5 min and immediately cooled with tap water. The sample was added with 2.3 ml of chloroform : methanol : water (90 :

100 : 30, v/v) solution and mixed for 1 h. After centrifugation at 3,000 rpm for 10 min, the upper layer was transferred to a new test tube (tube no.2). The precipitate was extracted twice with 2.3 ml of chloroform : methanol : water (50 : 100 : 40, v/v) solution and mixed for 30 min. After centrifugation at 3,000 rpm for 10 min, the upper layer was transferred to the previous tube (tube no.2). Mixed upper layer solution (tube no.2) was added with 1.3 ml of chloroform and 1.3 ml of water and mixed for 5 min. After centrifugation at 3,000 rpm for 10 min, the final lower layer was collected into a new vial and dried with nitrogen gas (Minnikin *et al.*, 1984).

2 Analysis of polar lipids

The polar lipids were determined using two–dimensional TLC methods (Minnikin *et al.*, 1984). The dried sample was dissolved with 60 µl of chloroform : methanol (2 : 1, v/v) and applied to the corner of TLC silica–gel plate (no. 1.05633 Kieselgel 60 F_{254}; Merck, Germany). The first dimension of TLC development was done in chloroform : methanol : water (65 : 25 : 4 v/v) and the second dimension was developed in chloroform : acetic acid : methanol : water (40 : 7.5 : 6 : 2, v/v).

3 Detection

To compare the chromatogram patterns, five specific reagents were sprayed onto each TLC plate as follows:

I: Molybdenum blue spray reagent. For all phospholipid detection (blue spot)

II. Phosphomolybdic acid reagent (Appendix B). After spraying, TLC plates were heated at 120 ℃ for 10 min. For all lipid detection (blue spot)

III. Ninhydrin reagent (Appendix B). After spraying, TLC plates were heated at 110 ℃ for 10 min. For phosphatidylethanolamine (PE) and its derivatives (lyso–PE, OH–PE and methyl–PE) detection (pink spot)

IV. Anisaldehyde reagent (Appendix B). After spraying, TLC plates were heated at 110 ℃ for 10 min. For glycolipids (Green-yellow spot) and other lipids (blue spot)

V. Dragendroff's reagent (Appendix B). For choline–containing phospholipids (phosphatidyl choline) detection (orange spot)

3.14.2.4 Cellular fatty acids analysis

Cellular fatty acids were prepared as follow the method of Sasser (1990). The method is divided into four steps as follows:

1 Saponification

Forty milligrams of freeze–dried cells were added into the capped test tube and 1 ml of saponification reagent (Appendix B). The tube was mixed well, heated at 100 °C for 30 min, and cooled down at room temperature in water.

2 Methylation

Methylation reagent (Appendix B) with 2 ml was added to a cooled tube and mixed for 5–10 sec. After mixing, the suspension was heated at 80 °C for 10 min and cooled down at room temperature in water.

3 Extraction

The suspension was added with 1.25 ml of extraction solvent (Appendix B) and mixed well for 10 min. The tube was centrifuged at 4,500 rpm for 10 min and the supernatant was transferred to the new test tube.

4 Base washing

The supernatant was added with 3 ml of base wash (Appendix B) and mixed well for 5 min. If the solution became emulsion form, added the saturated sodium chloride. After centrifugation at 4,500 rpm for 10 min, the 2/3 of the supernatant was transferred to a new vial. The fatty acid methyl ester sample was analyzed using GLC according to the instructions of the Microbial Identification System (MIDI, version 6.0) (Sasser, 1990 ; Kämpfer and Kroppenstedt, 1996). The composition of fatty acids was detected by gas chromatography (GC) (6890; Hewlett 107 Packard) with Sherlock Aerobic Bacterial Database (TSBA6) in the Microbial Identification software package.

3.14.2.5 Isoprenoidquinones analysis

One hundred to five hundred milligrams of freeze–dried cells were extracted with 20 ml of chloroform : methanol (2 : 1, v/v) and stirred overnight with a rotary shaker. Cells were removed using filter paper and the filtrates were evaporated to dryness. The dried sample was dissolved with 1 ml of acetone and spotted on a TLC silica–gel plate (no. 1.05633 Kieselgel 60 F_{254}; Merck, Germany). A TLC plate was developed using benzene. The band of menaquinone was

presented under UV lamp at 254 nm, scraped off, and dissolved with a small amount of acetone (HPLC grade). The suspension was filtrated with 0.45 µm membrane and dried using N_2 gas. The isoprenoidquinone was analyzed by HPLC using methanol : 2-propanol (2 : 1, v/v) as a mobile phase.

3.14.3 Genotypic characteristics

3.14.3.1 16S rRNA gene analysis and phylogenetic tree construction

The selected actinomycetes were grown on ISP 2 agar for 2–4 days at 30 °C. The fresh colony was collected by a sterilized toothpick and suspended in sterilized deionized water. For amplification of the 16S rRNA gene, the PCR was performed in a total volume of 50 µl containing 1 µl of DNA template, 25 µl of master mix (I–taq, iNtRON Biotechnology, Korea), 5 µl of 10 pmol/µl of 9F (5'–GAGTTTGATCCTGGCTCAG–3') and 1541R (5'–GTTACCTTGTTACGACTT–3') primers and 14 µl of MilliQ water. The PCR ran in the T100™ thermal Cycler (USA) under the following conditions: initial denaturation at 94 °C for 3 min; 40 cycles of 94 °C for the 30s, 56 °C for 30s and 72 °C for 90s; and final extension at 72 °C for 5 min. The PCR product was purified with FavorPrep™ Gel/PCR purification kit (Fisher Biotec, Australia) and sequenced using the 27F (5'–AGAGTTTGATCCTGGCTCAG–3'), 518F (5'–CCAGCAGCCGCGGTAATACG–3'), 800R (5'–TACCAGGGTATCTAATCC–3') and 1492R (5'–GGTTACCTTGTTACGACTT–3') primers. The 16S rRNA gene sequence was aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by using the CLUSTAL W program within BioEdit program version 7.1.3.0 (Hall, 1999). The alignment was manually verified and adjusted before the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor–joining (Saitou and Nei, 1987) and maximum–likelihood (Felsenstein, 1981), and the MEGA program version X (Kumar *et al*., 2018). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings. The values for sequence similarity among all actinomycetes were first determined using the EzBioClound server (http://www.ezbiocloud.net/) (Yoon *et al*., 2017). Gaps and ambiguous nucleotides were eliminated from the calculations.

3.14.3.2 Whole–genome sequencing

1 Genomic DNA extraction

The selected actinomycetes were extracted the genomic DNA for genome sequencing using the modified method of William *et al*. (2012). Each

isolate was extracted from 3–day old cultures grown in ISP 2 broth at 180 rpm at 30 °C. Cells were washed five times with sterile saline–EDTA pH 8 (Appendix B) and broke cells pellet using a micro pestle. The suspension (740 µl) was transferred to 1.5 ml microtube and added 20 µl of lysozyme solution (100 mg/ml), mixed well, and incubated at 37 °C for 30–60 min. The lysed cell was added with 40 µl of 10% SDS, mixed well and 8 µl of proteinase K (10 mg/ml) and mixed again. After that, the mixture was incubated at 56 °C for 1–3 h or overnight (the mixture must become to clear solution). The clear solution was added 100 µl of 5 M NaCl, mixed well, and added 100 µl of CTAB/NaCl solution (heated at 65 °C) (Appendix B) and incubated at 65 °C for 10 min, then 0.5 ml of phenol : chloroform (1 : 1, v/v) was added and gently mixed. The tube was spun at 13,500 rpm for 10 min at room temperature and the upper layer was transferred to the new microtube. The solution was twice processed with the same solution, then added 0.6 volume of absolute ethanol or isopropanol (–20 °C) and incubated at –20 °C for 30–60 min. The microtube was centrifuged at 13,500 rpm for 10 min, the upper solution was discarded and the pellet was dried at room temperature for several hours (depending on the humidity).

2 Purification of genomic DNA

A dried pellet of genomic DNA was suspended with 100–200 µl of TE buffer or deionized water and then 20–100 µl, based on the concentration of RNA in DNA, RNase A solution (Appendix B), and incubated at 37 °C for 1 h or more. The debris was removed by gently mixed with 0.5 ml of phenol : chloroform (1 : 1, v/v), centrifuged at 7,000 rpm for 7–10 min, and then discarded the upper solution. Air–dried genomic DNA was resuspended with a small amount of TE buffer or deionized water and loaded on 1% agarose gel to check the quality.

3 Sequencing and data analysis

The genomic DNA was sequenced using an Illumina HiSeq 4000 platform. The resulting 2x150 bp paired–end sequencing reads of selected actinomycetes were *de novo* assembled by using SPAdes (Bankevich *et al*., 2012). The genome was annotated using the PROKKA pipeline (Seemann, 2014). The ANI–blast (ANIb) and ANIMUMmer (ANIm) algorithms in the JSpecies Web Server (Richter *et al*., 2016) were used to calculate the average nucleotide identity (ANI) values. Correlation indexes of tetra-nucleotide signature (Tetra) were applied within the same Web Server (Richter and Rosselló-Móra, 2009 ; Richter *et al*., 2016). The digital DNA–DNA hybridization (dDDH) values between the genome and the most closely related species were calculated using the genome–to–genome distance calculator (GGDC 2.1; blast+method) in which formula 2 (identities/HSP length) was

applied to the incomplete draft genome (Meier–Kolthoff *et al*., 2013). The average amino acid identity (AAI) was calculated using the Kostas Lab AAI calculator (http://enve-omics.ce.gatech.edu/aai/) (Rodriguez–R and Konstantinidis, 2014)

The phylogenomic tree was reconstructed using the Type (strain) Genome Server (TYGS) (Meier–Kolthoff and Göker, 2019). The tree inferred with FastME version 2.1.6.1 from GBDP distances calculated from genome sequences (Lefort *et al*., 2015). The branch lengths are scaled in terms of GBDP distance formula *d5*. The numbers above branches are GBDP pseudo–bootstrap support values >60% from 100 replications, with average branch support. The tree was rooted at the midpoint (Farris, 1972).

Chapter 4
Main results and discussion

4.1 Isolation of actinomycete strains

One hundred and seventy–three actinomycetes were isolated from the compost types: four samples from aerobic bio–sludge compost, two samples from anaerobic bio–sludge compost, five samples from agricultural waste compost, and two samples from the soil collected from Rayong, Suphanburi, Ratchaburi, Pichit and Nakhon Sawan provinces. Eighty–two actinomycetes (47.40%) were isolated from the aerobic bio–sludge composts. Nine actinomycetes (5.20%) were isolated from anaerobic bio–sludge composts. Sixty–eight actinomycetes (39.31%) were isolated from agricultural waste composts. In addition, fourteen actinomycetes (8.09%) were isolated from soil samples (Table 4.1).

Table 4.1 Actinomycetes from the different compost types and soil.

Samples	Codes	pH	Total	Isolate No.
Aerobic bio–sludge compost	RBST1	7.15	21	RBST1-1–RBST1-4, RBST1-6, RBST1-14–RBST1-17, RBST1-19–RBST1-21, RBST1-25–RBST1-27, RBST1-32, RBST1-37, RBST1-38, RBST1-40, RBST1-42, RBST1-43
	RBST2	7.25	39	RBST2-1, RBST2-2, RBST2-4, RBST2-5, RBST2-9, RBST2-11, RBST2-20–RBST2-28, RBST2-30, RBST2-35, RBST2-36, RBST2-40–RBST2-42, RBST2-46, RBST2-51–RBST2-57, RBST2-65, RBST2-66, RBST2-68, RBST2-69, RBST2-71, RBST2-75, RBST2-78, RBST2-79, RBST2-82, RBST2-83
	SBST1	6.98	12	SBST1-1–SBST1-5, SBST1-12, SBST1-13, SBST1-15, SBST1-19. SBST1-21, SBST1-23, SBST1-27
	SBST2	7.04	10	SBST2-1–SBST2-7, SBST2-11–SBST2-13
Anaerobic bio–sludge compost	RBCP1	7.00	8	RBCP1-1, RBCP1-2, RBCP1-8, RBCP1-9, RBCP1-11, RBCP1-13–RBCP1-15
	RBCP2	6.89	1	RBCP2-3

Table 4.1 Actinomycetes from the different compost types and soil (continued).

Samples	Codes	pH	Total	Isolate No.
Agricultural waste compost	RCPT1	6.58	20	RCPT1-1–RCPT1-13, RCPT1-15, RCPT1-19–RCPT1-22, RCPT1-26, RCPT1-27
	RCPT2	7.07	5	RCPT2-2, RCPT2-6, RCPT2-9, RCPT2-13, RCPT2-15
	RCPT3	6.13	26	RCPT3-2, RCPT3-4–RCPT3-7, RCPT3-10, RCPT3-12–RCPT3-17, RCPT3-21, RCPT3-22, RCPT3-25– RCPT3-28, RCPT3-32, RCPT3-33, RCPT3-35, RCPT3-38–RCPT3-42
	PCP1	7.50	14	PCP1-3–PCP1-5, PCP1-8, PCP1-11, PCP1-31, PCP1-32, PCP1-34, PCP1-41, PCP1-43–PCP1-45, PCP1-47, PCP1-48
	PCP2	8.00	3	PCP2-1, PCP2-17, PCP2-40
Soil	CH	7.00	7	CH1-1, CH1-7, CH2-1, CH5-5, CH5-8, CH7-1, CH9-7
	NKS	5.50	7	NKS1–NKS2, NKS4, NKS9, NKS15–NKS16, NKS18

4.2 Isolation, pathogenicity, and identification of *Foc* from banana

Eight fungal strains were isolated and identified to *Foc*. One strain (12.5%) was isolated from the soil of Nakhon Sawan province. Seven strains (87.5%) were isolated from infected fungal banana pseudostem of Lampang province. The colonies have variable morphology on PDA. It could be hairy to cottony and color from whitish to yellow; pink or purple (Table 4.2). The macroconidia were nearly straight, slender, and thin–walled with 3–4 septa, a foot–shaped basal cell, and a curved tapered apical cell. Microconidia were 1 or 2 cells of oval to kidney–like shape and were produced in false heads (Figures 4.1–4.8).

Pathogenicity tests showed that the *Foc* caused symptoms in banana leaves. As reported by Hermanto *et al*. (2011) in Indonesia, this *Foc* is pathogenic to banana. In addition, in pot testing of 8 isolated *Foc* strains was also tested using *Musa* (AAA) 'Kluai Hom Thong' banana cultivar. Firstly, the *Foc* strain numbers 2, 3, 4, and 5 showed the disease severity index (DSI) ranging from 1.67 to 2.00 for leaf symptom index (LSI) and 1.10 to 1.67 for rhizome discoloration index (RDI), however, DSI values of LSI and RDI were translated to tolerant (Brake *et al*., 1995). The *Foc* strain numbers 6 and 7 showed the DSI were 2.67 for LSI and 2.67 to 3.33 for RDI, however, these values were translated as susceptible. Lastly, the *Foc* strain number 1 and 8 showed the DSI were 4.00 for LSI and 5.67 to 6.00 for RDI, however, these values

were translated as highly susceptible (Brake *et al*., 1995). For the identification of *Fusarium* strains through ITS sequencing, all eight of the strains were identified as *F. oxysporum* (Table 4.2).

Table 4.2 Cultural characteristics of eight *Foc* strains on PDA.

Foc strains/ Accession number	Colony color	Colony morphology	Total base pairs
Foc 1 (LC554430)	White	Cottony	553
Foc 2 (LC554431)	Whitish to pink	Cottony	518
Foc 3 (LC554432)	Whitish to purple	Cottony	517
Foc 4 (LC554433)	Whitish to yellow	Hairy	512
Foc 5 (LC554434)	Yellow	Hairy	517
Foc 6 (LC554435)	White	Hairy	489
Foc 7 (LC554436)	Yellow to orange	Cottony	521
Foc 8 (LC554437)	Whitish to purple	Cottony	520

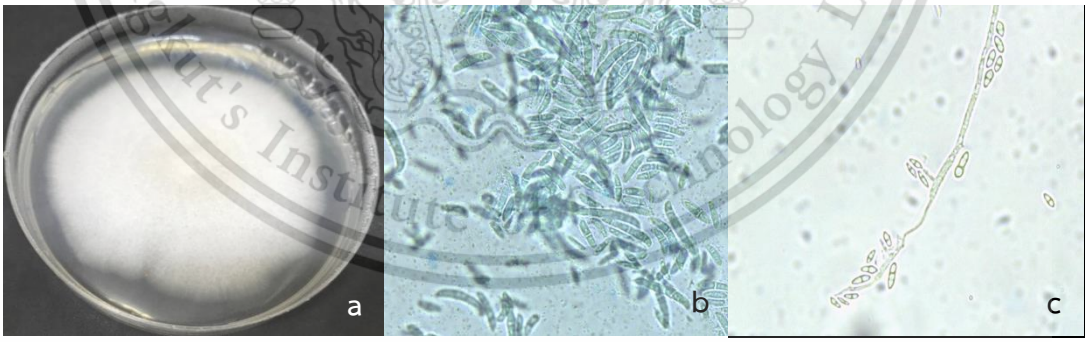


Figure 4.1 Reproductive structures of *Foc* 1 on PDA media (a), macroconidia, and microconidia (c).

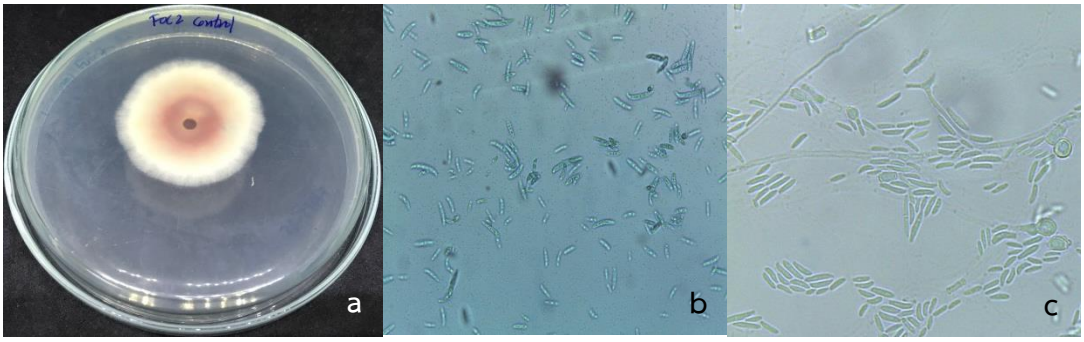


Figure 4.2 Reproductive structures of *Foc* 2 on PDA media (a), macroconidia, and microconidia (c).

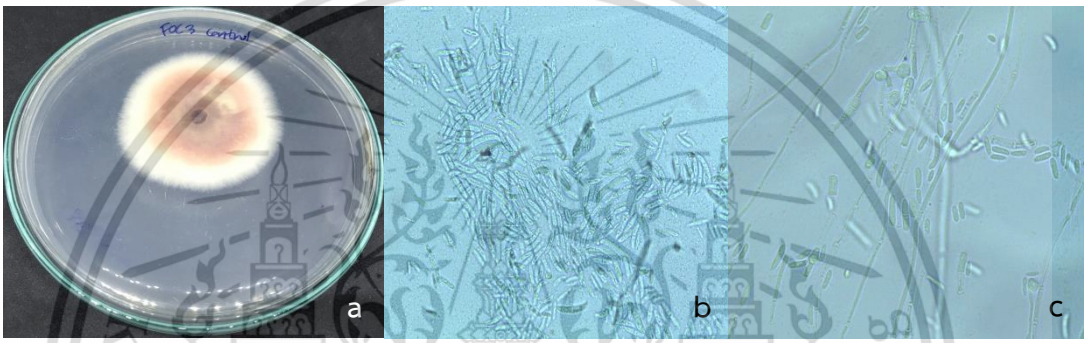


Figure 4.3 Reproductive structures of *Foc* 3 on PDA media (a), macroconidia, and microconidia (c).

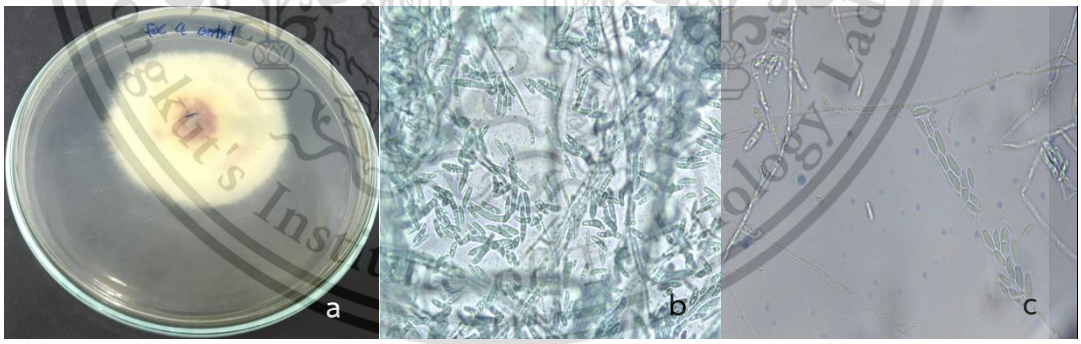


Figure 4.4 Reproductive structures of *Foc* 4 on PDA media (a), macroconidia, and microconidia (c).

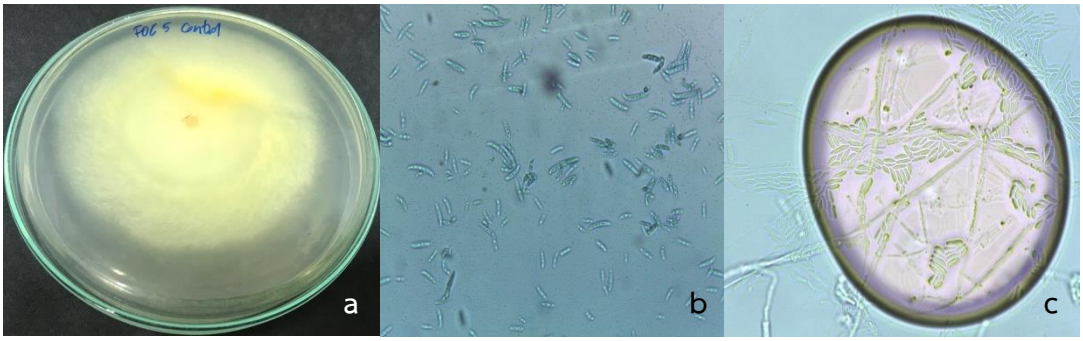


Figure 4.5 Reproductive structures of *Foc* 5 on PDA media (a), macroconidia, and microconidia (c).

Figure 4.6 Reproductive structures of *Foc* 6 on PDA media (a), macroconidia, and microconidia (c).

Figure 4.7 Reproductive structures of *Foc* 7 on PDA media (a), macroconidia, and microconidia (c).

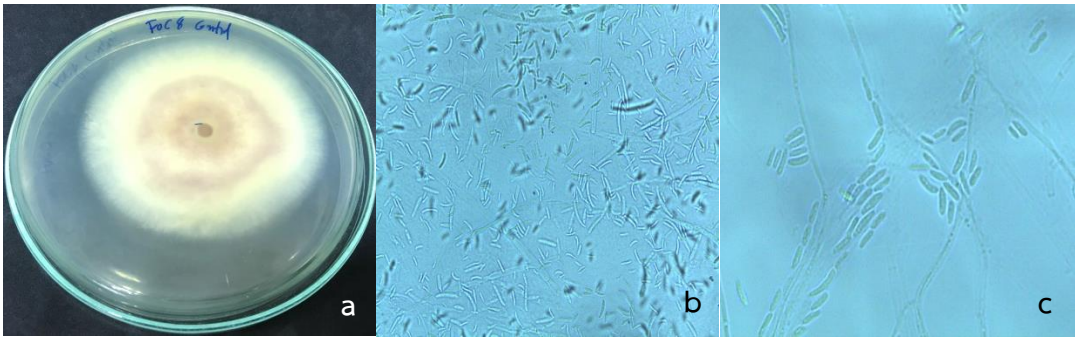


Figure 4.8 Reproductive structures of *Foc* 8 on PDA media (a), macroconidia, and microconidia (c).

4.3 Screening of actinomycetes against *Foc*

One hundred and seventy–three actinomycetes were screened for the anti–*Foc* activity. Among 173 isolates, only 24 isolates (13.87%) showed antagonistic ability with PIRG values of more than 21% (Khucharoenphaisan *et al*., 2013) and at least 50% of *Foc* strain (4 of 8 *Focs*). Only four strains, designated RBST2–54, SBST1–23, RCPT3–35, and CH9–7, had a strong antagonistic activity to all *Foc* strains. Strain CH5–8 showed the highest PIRG value against *Foc* 7 with 78.33% (Table 4.3).

Table 4.3 The percentage inhibition of radial growth of *Foc* by actinomycetes through agar plug technique.

Isolates	*Foc* 1	*Foc* 2	*Foc* 3	*Foc* 4	*Foc* 5	*Foc* 6	*Foc* 7	*Foc* 8
RBST1–4	0.00±0.00	23.61±2.41	26.39±2.41	26.67±3.34	24.51±1.70	8.08±1.75	27.78±4.81	23.33±3.34
RBST2–5	13.54±1.81	34.29±2.90	51.11±1.92	22.92±4.78	52.22±5.09	49.52±4.37	34.44±1.93	20.00±2.82
RBST2–9	49.17±1.44	24.24±2.62	9.09±0.00	40.40±1.75	64.76±4.36	48.89±1.92	24.07±3.21	0.00±0.00
RBST2–11	34.31±1.70	23.93±1.48	9.88±2.14	25.56±1.93	22.92±1.80	3.60±1.57	8.00±0.00	0.00±0.00
RBST2–20	0.00±0.00	24.79±1.48	33.27±2.92	24.43±1.93	30.67±2.31	8.34±1.81	9.91±1.56	0.00±0.00
RBST2–21	0.00±0.00	23.93±1.48	36.95±3.57	24.00±0.00	24.00±0.00	26.13±1.57	33.33±2.31	0.00±0.00
RBST2–41	21.93±1.52	24.32±2.71	0.00±0.00	17.78±1.92	37.15±4.95	36.19±1.65	9.91±1.56	0.00±0.00
RBST2–53	7.29±1.81	66.67±3.30	18.33±2.89	31.38±1.75	56.19±4.36	65.56±1.93	24.32±2.79	49.52±1.65
RBST2–54	56.67±5.77	25.76±2.62	38.94±3.41	32.22±1.92	60.00±2.86	48.89±1.92	27.51±4.78	60.00±2.86
RBST2–65	0.00±0.00	56.19±1.65	9.09±4.55	40.40±1.75	64.76±7.19	35.56±1.93	27.54±4.83	46.66±1.65
SBST1–1	0.00±0.00	22.52±1.56	22.92±1.80	26.67±2.31	33.33±1.70	27.78±1.92	46.38±2.51	44.79±1.81
SBST1–23	49.12±4.02	53.33±2.23	28.33±2.89	35.56±1.93	50.48±1.65	56.00±4.00	43.21±2.14	34.67±2.31
SBST1–27	19.30±1.52	58.52±2.56	25.00±5.00	31.11±3.85	39.05±1.65	46.67±2.31	18.52±3.71	32.00±4.00
SBST2–5	0.00±0.00	43.87±4.46	0.00±0.00	36.67±5.77	25.00±5.00	24.17±1.40	0.00±0.00	22.50±2.50

Note: Mean ± Standard deviation, N = 3

Table 4.3 Percentage inhibition of radial growth of *Foc* by actinomycetes through agar plug technique (continued).

Isolates	*Foc* 1	*Foc* 2	*Foc* 3	*Foc* 4	*Foc* 5	*Foc* 6	*Foc* 7	*Foc* 8
RCPT1–4	50.00±2.63	55.86±1.57	29.76±2.06	19.26±2.56	43.81±1.65	43.81±1.65	25.93±3.71	44.76±1.65
RCPT3–25	0.00±0.00	54.29±2.86	36.67±2.89	36.67±3.34	58.33±3.82	57.78±3.85	18.52±3.20	37.50±3.13
RCPT3–26	4.63±1.61	35.83±2.89	26.39±6.36	28.33±2.89	37.14±2.86	24.44±1.93	28.89±1.92	24.44±1.93
RCPT3–27	17.54±1.52	11.11±2.22	43.33±5.77	0.00±0.00	40.95±1.65	17.33±2.31	49.38±2.14	49.33±2.31
RCPT3–28	14.04±1.52	12.59±1.28	43.33±5.77	0.00±0.00	42.86±2.86	10.67±2.31	48.15±0.00	41.33±2.31
RCPT3–35	47.37±2.63	54.95±1.57	50.00±3.57	42.22±1.92	59.93±2.76	46.66±1.65	49.38±2.14	30.48±1.65
RCPT3–40	28.57±2.86	53.33±4.36	46.67±2.84	35.56±1.93	61.67±2.89	56.67±3.34	18.52±3.20	34.38±3.13
CH5–8	47.78±1.92	58.82±5.89	66.67±5.56	26.26±1.75	54.67±4.62	51.52±2.63	78.33±7.64	18.67±2.31
CH9–7	57.78±1.92	52.94±5.88	31.48±3.20	48.33±2.89	72.00±4.00	71.23±2.64	68.33±5.77	58.67±2.31
NKS15	0.00±0.00	12.22±3.85	46.67±2.89	56.00±4.00	47.78±1.92	46.67±2.31	53.33±6.11	45.45±4.55

Note: Mean ± Standard deviation, N = 3

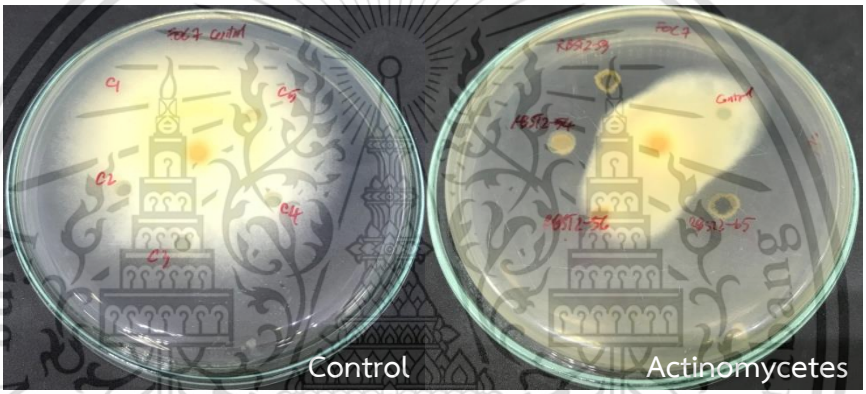


Figure 4.9 Sample of antagonistic effect of the selected actinomycetes against *Foc*.

4.4 Screening of plant growth–promoting ability of the selected actinomycetes

Twenty–four isolates were selected for further analyses based on plant growth promoting traits.

4.4.1 Macronutrients production

For nitrogen–fixing ability and ammonia production, all isolates were able to grow on Nfb agar and form the pellicle in Nfb semi–solid medium, and produce ammonia. Among all isolates, fifteen actinomycete strains were able to produce nitrate reductase (Table 4.4).

Table 4.4 Qualitative properties of nitrogen fixation, ammonia production, and nitrate reduction of the selected actinomycetes.

Isolates	Nitrogen fixation	Ammonia production	Nitrate reduction
RBST1–4	+	+	+
RBST2–5	+	+	-
RBST2–9	w	+	-
RBST2–11	w	+	-
RBST2–20	+	+	w
RBST2–21	+	+	w
RBST2–41	w	+	-
RBST2–53	+	+	+
RBST2–54	+	+	w
RBST2–65	+	+	w
SBST1–1	+	+	+
SBST1–23	+	+	-
SBST1–27	+	+	-
SBST2–5	w	+	-
RCPT1–4	+	+	+
RCPT3–25	+	+	-
RCPT3–26	+	+	w
RCPT3–27	+	+	+
RCPT3–28	+	+	+
RCPT3–35	+	+	+
RCPT3–40	+	+	+
CH5–8	+	+	w
CH9–7	+	+	-
NKS15	+	+	+

Note: + = positive, w = weakly positive, - = negative, N = 3

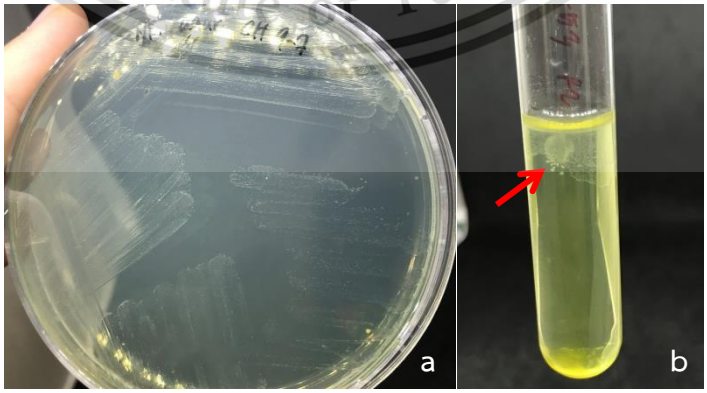


Figure 4.10 Growth of actinomycetes on Nfb agar (a) and Nfb semi–solid medium (b), the red arrow in (b) indicates the characteristic pellicle formation.

Of all the two tested media for phosphate solubilization (Pikovskaya's and NBRIP–BPB agar), four actinomycete strains, RCPT1–4, CH5–8, CH9–7, and NKS15, showed clear halo zone with phosphate solubilization efficiency (SE) ranging from 102.55 to 132.73 (Table 4.5). Strain RCPT1–4 showed the highest SE value in all tested media.

A total of 11 isolates were found to be capable of solubilizing K, and the potassium solubilization efficiency (PSE) ranging from 115.59 to 166.30 (Table 4.5). Strain RCPT1–4 showed the most pronounced ability to solubilize K (116.30).

Table 4.5 Phosphate solubilization and potassium solubilization efficiency index of the selected actinomycetes.

Isolates	Phosphate solubilization efficiency		Potassium solubilization efficiency
	Pikovskaya's agar	NBRIP–BPB agar	Aleksandrov agar
RBST1–4	0.00±0.00	0.00±0.00	119.90±14.90
RBST2–5	0.00±0.00	0.00±0.00	0.00±0.00
RBST2–9	0.00±0.00	0.00±0.00	0.00±0.00
RBST2–11	0.00±0.00	0.00±0.00	0.00±0.00
RBST2–20	0.00±0.00	0.00±0.00	118.41±8.17
RBST2–21	0.00±0.00	0.00±0.00	116.42±9.96
RBST2–41	0.00±0.00	0.00±0.00	0.00±0.00
RBST2–53	0.00±0.00	0.00±0.00	130.69±12.79
RBST2–54	0.00±0.00	0.00±0.00	117.97±10.37
RBST2–65	0.00±0.00	0.00±0.00	115.99±6.59
SBST1–1	0.00±0.00	0.00±0.00	0.00±0.00
SBST1–23	0.00±0.00	0.00±0.00	128.57±6.19
SBST1–27	0.00±0.00	0.00±0.00	115.59±15.55
SBST2–5	0.00±0.00	0.00±0.00	0.00±0.00
RCPT1–4	130.43±3.99	132.73±12.60	166.30±20.20
RCPT3–25	0.00±0.00	0.00±0.00	136.87±11.77
RCPT3–26	0.00±0.00	0.00±0.00	0.00±0.00
RCPT3–27	0.00±0.00	0.00±0.00	0.00±0.00
RCPT3–28	0.00±0.00	0.00±0.00	0.00±0.00
RCPT3–35	0.00±0.00	0.00±0.00	0.00±0.00
RCPT3–40	0.00±0.00	0.00±0.00	0.00±0.00
CH5–8	102.55±2.83	107.56±5.63	123.93±12.10
CH9–7	108.16±5.91	102.68±0.94	131.32±16.75
NKS15	113.79±6.83	124.65±7.04	144.13±8.72

Note: Mean ± Standard deviation, N = 3

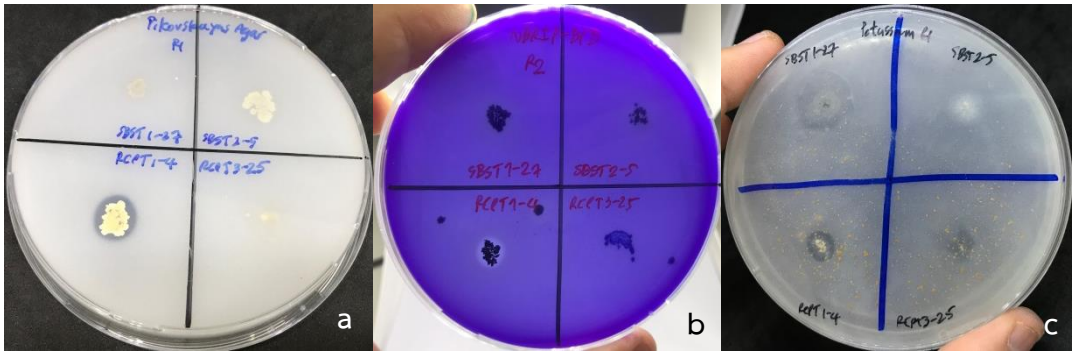


Figure 4.11 Halo formations around colonies of phosphate–solubilizing actinomycete isolates on Pikovskaya's agar (a) and NBRIP–BPB agar (b), and potassium–solubilizing actinomycete isolates on Aleksandrov agar (c).

Nitrogen is the most critical for plant growth and metabolisms. It is a major component of chlorophyll, the most important pigment needed for photosynthesis. All important processes in the plant are related to proteins, of which nitrogen is an essential constituent. Non–symbiotic nitrogen fixation bacteria including actinomycetes were found in several reports such as *Streptomyces thermoautotrophicus* isolated from a burning charcoal pile (Gadkari *et al*., 1992), *Streptomyces* sp. from the soil of the badlands of South Dakota (Dahal *et al*., 2017), and *Nocardia cakureu* and *Nocardia cetluluns* from the soil of chalk grassland (Metcalfe and Brown, 1957).

Marquesetal (2010) recommended that bacteria can synthesize ammonia and supply nitrogen to the host plant. Moreover, overproduction of ammonia serve as a prompting factor for the virulence of opportunistic plant pathogens. Our results are in agreement with those reported for ammonia production from actinomycetes (Passari *et al*., 2015 ; Anwar *et al*., 2016).

Nitrate reductase is a key enzyme in the nitrogen reduction and assimilation pathway. It catalyzes the reduction of nitrate to nitrite, which is itself reduced to ammonia, before being assimilated into the amino acids and the nitrogen compounds of the cell (Beevers and Hageman, 1969 ; Campbell, 1999).

Phosphorus (P) is an essential macronutrient for plant growth. A great portion of phosphorus from chemical fertilizers becomes insoluble by its conversion into calcium or magnesium salts in soils and thereby become unavailable to plants. Certain soil microorganisms transform the insoluble forms of phosphorus into soluble forms and thus influence the subsequent availability of phosphate to plant roots (Illmer and Schinner, 1995). Phosphate–solubilizing bacteria are important components of soil and directly or indirectly influence the soil's health through their useful activities. It is known that rhizospheric microbes mediate many soil processes

such as decomposition, nutrient mineralization, and nitrogen fixation (Pradhan and Sukla, 2009). Many reports showed phosphate solubilizing ability from actinomycetes (Hamdali et al., 2008 ; Franco–Correa *et al*. 2010 ; Prada-Salcedo *et al*., 2014 ; Zheng *et al*., 2018b ; Aallam *et al*., 2021).

Potassium (K) is one of the macronutrients for plant growth. Most of the K in the soil exists in various insoluble rocks, minerals, and sedimentary materials. Soil K may be solubilized and absorbed by plants under certain circumstances. Microbes play an important role in natural K. Potassium–solubilizing bacteria decompose silicate minerals such as K–feldspar, and mica. They transform solid K in the soil in to available K that can be directly absorbed by plants, and they secrete active substances that promote plant growth (Sheng *et al*., 2001). Few studies showed potassium solubilizing actinomycetes such as *Microbacterium foliorum* isolated from rhizospheric soil (Zhang and Kong, 2014), *Streptomyces rochei*, and *Streptomyces sundarbansensis* from the rhizosphere of *Mikania micrantha* Kunth (Han *et al*., 2018) and *Streptomyces alboviridis* P18, *Streptomyces griseorubens* BC3, *Streptomyces griseorubens* BC10 and *Nocardiopsis alba* BC11 from soil (Boubekri *et al*., 2021).

4.4.2 Phytohormones, siderophore, and toxicants production

Quantitative analysis of culture supernatant of the selected actinomycete isolates revealed the production of variable amounts of IAA and GA ranging from 6.40 to 40.11 µg/ml for IAA and 31.53 to 226.60 µg/ml for GA (Table 4.6). Strain SBST2–5 produced the highest amount of IAA and SBST1–23 produced the highest amount of GA. For cytokinin production, quantitative analysis showed that all strains were able to produce cytokinin (Figure 4.12).

The siderophore production was detected in all 24 actinomycete isolates on CAS agar media, forming a clear yellow or orange halo zone around the colonies (Figure 4.13). Siderophore production index (SPI) of all strains ranging from 1.10 to 2.28. Strain NKS15 showed the highest amount of SPI (2.28).

Among all selected isolates, no presence of actinomycetes produced HCN. Only one strain (SBST2–5) was found to produce H_2S.

The majority of the PGPR actinomycetes synthesize IAA, GA, and cytokinin which are responsible for increased plant growth. IAA enhances the development of lateral roots and divisions of the apical meristem that derives from the root elongation (López *et al*., 2004) and seed germination. GA regulates and promote cell division and elongation, seed germination, stem and hypocotyl elongation, root growth, and flowering induction (Sun and Gubler, 2004 ; Shukla *et al*., 2005 ; Lucas *et al*., 2008 ; Sun, 2011). Cytokinins stimulate the growth of root and shoot as well as branching, control the apical dominance in the shoot, chloroplast development, and

leaf senescence (Oldroyd, 2007). Several actinomycetes genera, such as *Streptomyces* (El–Sayed *et al*., 1987 ; El–Shanshoury *et al*., 1991 ; Manulis *et al*., 1994 ; Joshi and Loria, 2007 ; Rashada *et al*., 2015 ; Goudjal *et al*., 2016b ; Suwitchayanon *et al*., 2018; Myo *et al*., 2019), *Kitasatospora* (Shrivastava *et al*., 2008), *Nocardia* (Brown, 1972 ; El–Tarabily and Sivasithamparam, 2006 ; Ghodhbane–Gtari *et al*., 2019), *Actinoplanes* (El–Tarabily *et al*., 2018), *Rhodococcus* (Pertry *et al*., 2009) and *Frankia* (Nouioui *et al*., 2019) can produce IAA, GA, and cytokinin.

Siderophore production is one more feature that stimulates plant growth by forming a complex with iron form (Fe^{3+}) in the rhizosphere making iron unavailable to the phytopathogens (Tan *et al*., 2009). Genus *Streptomyces* have been reported to inhibit the growth of phytophatogens by competition for iron in plant rhizosphere soils (Müller and Raymond, 1984 ; Müller *et al*., 1984 ; Tokala *et al*., 2002). Additionally, the genus *Thermobifida* has been reported as the producer of a siderophore known as fuscachelin A (Dimise *et al*., 2008).

HCN is produced by actinobacteria as a biocontrol agent based on its toxicity to phytopathogens such as *Streptomyces* spp. isolated from roots of *Solanum nigrum* (Goudjal *et al*., 2016b), *Streptomyces* spp. from rhizospheric soil (Anwar *et al*. 2016). H_2S was reported to be toxic to plants and to inhibit the growth (Zhang *et al*., 2017).

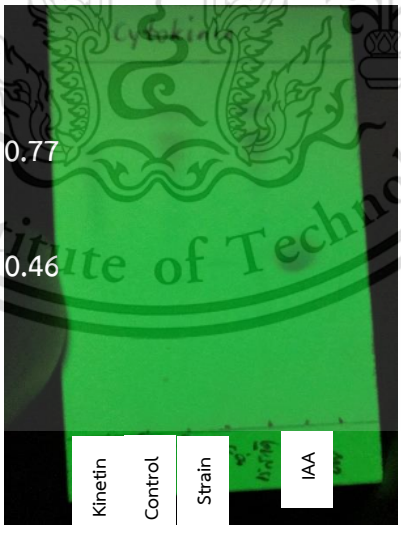


Figure 4.12 Cytokinin (kinetin), control and isolates spots observed under UV_{254nm}.

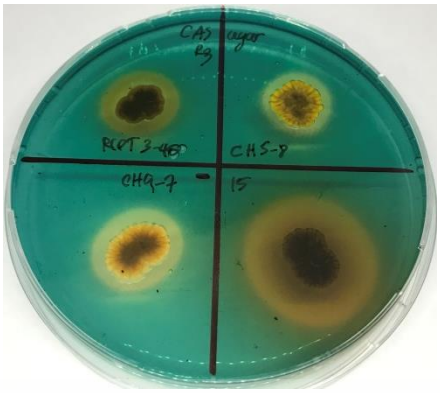


Figure 4.13 Halo formations around colonies of actinomycete isolates on CAS agar.

Table 4.6 Indole acetic acid (IAA), gibberellic acid (GA), cytokinin, and siderophore production of the selected actinomycetes.

Isolates	IAA production (µg/ml)	GA production (µg/ml)	Cytokinin production	Siderophore production index
RBST1–4	11.54±0.26	42.99±1.13	+	1.43±0.02
RBST2–5	32.63±1.83	31.18±0.78	+	1.20±0.12
RBST2–9	34.79±0.42	48.45±1.82	+	1.92±0.08
RBST2–11	25.52±1.09	92.81±5.06	+	1.81±0.24
RBST2–20	31.25±0.37	61.99±6.12	+	1.62±0.15
RBST2–21	21.25±0.11	50.98±1.36	+	1.62±0.24
RBST2–41	36.60±1.95	80.09±0.45	+	1.59±0.15
RBST2–53	32.78±1.75	30.59±0.25	+	1.33±0.23
RBST2–54	29.83±0.09	57.40±2.07	+	1.58±0.12
RBST2–65	26.39±3.39	36.86±1.17	+	1.42±0.13
SBST1–1	25.71±1.35	58.98±1.72	+	1.48±0.21
SBST1–23	22.83±0.27	226.60±3.19	+	1.56±0.09
SBST1–27	17.89±0.62	222.93±1.31	+	1.10±0.04
SBST2–5	40.11±1.88	31.53±0.36	+	1.94±0.10
RCPT1–4	12.57±1.89	36.57±1.11	+	1.14±0.06
RCPT3–25	12.80±0.22	52.69±2.17	+	1.27±0.05
RCPT3–26	17.76±0.16	48.61±0.65	+	1.41±0.07
RCPT3–27	6.98±1.49	47.81±1.05	+	1.28±0.08
RCPT3–28	6.40±0.42	40.46±2.20	+	1.19±0.11
RCPT3–35	13.26±1.19	47.01±1.01	+	1.66±0.16
RCPT3–40	18.62±1.55	35.87±0.37	+	1.69±0.17
CH5–8	37.21±0.49	56.90±1.58	+	1.36±0.03
CH9–7	19.75±0.83	39.19±2.50	+	1.53±0.12
NKS15	19.48±0.79	40.13±1.67	+	2.28±0.15

Note: Mean ± Standard deviation, + = positive, N = 3

4.5 Measurement of extracellular enzyme production of the selected actinomycetes

From 24 actinomycete isolates, twenty of which isolates (70.83%) were able to grow and sporulate on DF agar medium amended with ACC. This result showed that these actinomycetes have a positive effect on ACC deaminase production. Eighteen isolates (75.00%) were able to produce urease (Table 4.7).

For extracellular enzyme production, 22 (91.67%), 24 (100.00%), 24 (100.00%), and 22 (91.67%) isolated produced enzyme amylase, gelatinase, caseinase, and lipase, respectively. The levels of enzymatic activity (LEA) of each isolate and enzyme are shown in Table 4.7.

Table 4.7 Qualitative ACC deaminase and urease production, and levels of enzymatic activity (LEA) of the selected actinomycetes.

Isolates	ACC deaminase	Urease	Amylase	Gelatinase	Caseinase	Lipase	
						Tributyrin	Tween 80
RBST1–4	+	+	0.00±0.00	4.26±0.48	3.58±0.31	1.23±0.04	3.71±0.19
RBST2–5	+	+	4.18±0.20	4.08±0.12	4.10±0.01	1.66±0.31	3.81±0.09
RBST2–9	+	-	2.66±0.17	2.40±0.19	5.68±0.45	1.47±0.15	2.55±0.17
RBST2–11	+	-	4.85±0.08	7.51±1.57	6.77±1.09	2.39±0.16	4.01±0.45
RBST2–20	-	+	4.42±0.26	3.50±0.60	5.59±0.00	1.82±0.04	3.67±0.03
RBST2–21	-	+	4.84±0.11	3.82±1.34	4.39±0.01	1.78±0.16	3.64±0.29
RBST2–41	+	-	1.62±0.09	2.04±0.05	2.86±0.03	1.52±0.11	1.83±0.33
RBST2–53	-	+	4.77±0.05	4.20±0.48	4.64±0.51	1.75±0.07	3.31±0.04
RBST2–54	w	+	4.58±0.23	4.43±0.20	5.15±0.21	2.05±0.07	3.27±0.30
RBST2–65	+	+	4.32±0.12	4.45±0.31	4.78±0.31	1.68±0.17	3.33±0.47
SBST1–1	+	+	4.06±0.17	4.29±0.40	3.98±0.15	1.89±0.31	4.14±0.55
SBST1–23	-	+	1.70±0.22	2.38±1.01	3.26±0.45	1.82±0.26	1.49±0.12
SBST1–27	+	-	2.58±0.11	2.88±0.72	1.24±0.04	1.60±0.10	1.65±0.35
SBST2–5	w	-	1.67±0.05	1.67±0.16	2.29±0.05	1.89±0.15	1.22±0.13
RCPT1–4	+	+	1.55±0.32	2.20±0.28	2.43±0.23	2.05±0.06	5.51±0.25
RCPT3–25	+	+	4.27±0.10	4.13±0.29	3.46±0.06	2.00±0.15	4.31±0.09
RCPT3–26	+	+	4.04±0.10	4.35±0.28	3.69±0.05	1.81±0.01	5.79±0.51
RCPT3–27	+	+	3.02±0.05	5.28±0.67	4.68±0.18	1.46±0.00	2.63±0.05
RCPT3–28	+	+	2.82±0.15	4.80±0.28	4.00±0.28	1.64±0.26	2.97±0.21
RCPT3–35	w	+	5.29±0.10	4.83±0.24	5.31±0.09	1.53±0.17	4.97±0.05
RCPT3–40	+	+	3.79±0.28	4.50±0.00	3.91±0.26	2.03±0.04	4.36±0.10
CH5–8	+	-	0.00±0.00	1.71±0.40	3.10±0.01	0.00±0.00	0.00±0.00
CH9–7	w	+	5.63±0.03	1.77±0.08	4.35±0.49	0.00±0.00	0.00±0.00
NKS15	+	+	1.77±0.01	1.71±0.29	3.68±0.45	1.31±0.02	2.50±0.04

Note: Mean ± Standard deviation, + = positive, w = weakly positive, - = negative, N = 3

ACC deaminase–producing bacteria have been known to promote plant growth by decreasing ethylene inhibition in various plant processes (Husen *et al*., 2011). Several actinomycetes genera, such as *Streptomyces* (El–Tarabily, 2008 ; Jaemsaeng *et al*., 2018 ; Shan *et al*., 2018 ; Djebaili *et al*., 2021), *Nocardiopsis* (Shan *et al*., 2018 ; Djebaili *et al*., 2021), *Micrococcus* (Siddikee *et al*., 2010) and *Arthrobacter* (Krishnan *et al*., 2016) can produce ACC deaminase.

Extracellular hydrolytic enzymes from microorganisms including actinobacteria play an important role in the breakdown complex molecules into micromolecules for microbial activity, growth, and survival in their habitat (Kamalanathan *et al*., 2018). Ureases can breakdown urea into ammonia and carbon dioxide. Amylases can breakdown starch into simple sugar. Gelatinase can breakdown gelatin into smaller peptides, peptides, and amino acids. Caseinases or proteinases can breakdown protein into amino acids. Lipases can breakdown lipid into fatty acids. Various genera of actinomycetes have been reported to produce a wide array of potential extracellular enzymes (Nawani *et al*., 2013).

4.6 Measurement of stress, chemical fertilizer, and fungicide tolerance properties of the selected actinomycetes

For pH range for growth, twenty–four actinomycetes grew in different pH. The minimum and maximum pH for growth was 4 and 11. Actinomycete isolates tolerated maximum NaCl concentration varied from 4 to 10 and tolerated maximum temperature varied from 45 to 70 °C. For drought tolerance using polyethylene glycol 6000, actinomycete isolates tolerated maximum PEG 6000 varied from 30 to 35% or water potentials from -10.27 to -13.68 mPa (Table 4.8).

The samples were collected from different soil habitats and types of compost. So, actinomycete isolates were found to have varying levels of tolerance.

For chemical fertilizer and fungicide tolerance, all twenty–four actinomycetes were able to grow in ISP 2 media supplemented with the chemical fertilizer formula of 46–0–0, 0–0–60, 13–13–21, 25–7–7, 8–8–24 and 16–16–16, and fungicide 2.0% benzimidazole (carbendazim).

Considering the importance of soil microorganisms, they were grown in chemical fertilizer and fungicides for their deciding application as bio–inoculants to control phytopathogens or increase crop production in chemical fertilizer or fungicide stressed conditions.

Table 4.8 Tolerance property of the selected actinomycetes.

Isolates	pH range for growth	Maximum for growth		
		NaCl (% w/v)	Temperature (°C)	PEG 6000 (% w/v)
RBST1–4	5–10	9	70	35
RBST2–5	6–10	9	60	35
RBST2–9	6–10	4	55	34
RBST2–11	6–10	4	45	35
RBST2–20	6–11	10	70	34
RBST2–21	6–10	10	70	35
RBST2–41	6–10	5	65	34
RBST2–53	5–11	10	70	35
RBST2–54	4–11	10	70	35
RBST2–65	6–10	9	70	35
SBST1–1	6–11	10	70	34
SBST1–23	6–10	9	70	30
SBST1–27	6–10	9	65	35
SBST2–5	6–10	6	65	35
RCPT1–4	6–10	6	60	35
RCPT3–25	6–10	9	70	34
RCPT3–26	5–10	9	70	34
RCPT3–27	6–10	9	65	35
RCPT3–28	6–10	9	65	35
RCPT3–35	4–10	10	70	35
RCPT3–40	6–11	9	65	35
CH5–8	6–9	7	60	32
CH9–7	6–10	7	60	34
NKS15	6–10	6	45	35

Note: N = 3

4.7 Growth measurement of the selected actinomycetes

In the liquid medium or submerge fermentation using ISP 2 media, the growth of 24 actinomycetes in CFU number varied from 1.60×10^6 to 5.21×10^8 CFU/ml (Table 4.9). Strain SBST1–23 showed the lowest CFU number in the medium, while RBST2–54 showed the highest CFU number in the medium.

The solid substrates which results from the growth of all actinomycetes varied from 1.67×10^7 to 5.08×10^9 CFU/g sample (Table 4.9). Strain RBST2–5 showed the lowest CFU number in the medium, while SBST2–5 showed the highest CFU number in the medium.

Table 4.9 The colony forming units (CFU) of the selected actinomycetes from submerge fermentation and solid–state fermentation.

Isolates	Submerge fermentation	Solid–state fermentation
	CFU/ml	CFU/g
RBST1–4	2.84×10^7	4.44×10^9
RBST2–5	1.00×10^7	1.67×10^7
RBST2–9	8.70×10^6	3.99×10^7
RBST2–11	3.66×10^7	5.35×10^8
RBST2–20	3.11×10^7	8.00×10^7
RBST2–21	1.23×10^7	8.00×10^7
RBST2–41	1.10×10^7	9.10×10^7
RBST2–53	2.72×10^8	1.36×10^8
RBST2–54	5.21×10^8	1.11×10^9
RBST2–65	4.39×10^8	2.91×10^9
SBST1–1	3.90×10^7	1.30×10^8
SBST1–23	1.60×10^6	5.20×10^6
SBST1–27	9.30×10^6	2.26×10^7
SBST2–5	2.04×10^8	5.08×10^9
RCPT1–4	1.18×10^7	9.35×10^7
RCPT3–25	5.18×10^7	5.98×10^8
RCPT3–26	4.67×10^7	2.23×10^8
RCPT3–27	8.33×10^7	2.20×10^8
RCPT3–28	1.47×10^8	8.35×10^8
RCPT3–35	2.89×10^8	3.25×10^9
RCPT3–40	4.26×10^7	2.23×10^8
CH5–8	1.87×10^8	1.07×10^9
CH9–7	2.32×10^8	2.31×10^8
NKS15	1.16×10^7	1.97×10^8

Note: N = 3

Most actinomycetes were able to grow in solid–state fermentation better than in submerge fermentation. The results suggest the growth depending on actinomycetes species and the nutritional composition of the culture medium. Glucose, yeast extract, malt extract, and $MgSO_4.7H_2O$ in the solid–state medium reduced the lag phase of actinobacterial growth and enhanced cell mass. Extracellular enzymes from actinomycetes were rapidly produced to degrade biomass in different sources medium (red sorghum, fine rice bran, and bat guano) for their growth. Moreover, it was observed that after two to three days of submerge

fermentation, many spherical–shaped actinobacterial pellets were formed. Conversely, the actinobacterial spores were formed in solid–state fermentation. The hydric stress in solid–state fermentation could favor the sporulation, which increases of sporulation index and minimize the time production. It is necessary to optimize the processes to allow maximum production of the spores. A successful biological control product needs a long shelf–life (Cruz–Quiroz *et al*., 2019).

4.8 Banana toxicity of the selected actinomycetes

The selected actinomycetes with no cytotoxic on banana plants with the DSI for leaf symptom index (LSI) and rhizome discoloration index (RDI) values of 1.00 were selected (Table 4.10). This result showed that all the selected actinomycetes had potential use for product formulation.

Table 4.10 Disease severity index (DSI) for leaf symptoms and rhizome discoloration of the selected actinomycetes.

Isolates	Disease severity index (DSI)	
	Leaf symptom index (LSI)	Rhizome discoloration index (RDI)
RBST1–4	1.00 ± 0.00	1.00 ± 0.00
RBST2–5	1.00 ± 0.00	1.00 ± 0.00
RBST2–9	1.00 ± 0.00	1.00 ± 0.00
RBST2–11	1.00 ± 0.00	1.00 ± 0.00
RBST2–20	1.00 ± 0.00	1.00 ± 0.00
RBST2–21	1.00 ± 0.00	1.00 ± 0.00
RBST2–41	1.00 ± 0.00	1.00 ± 0.00
RBST2–53	1.00 ± 0.00	1.00 ± 0.00
RBST2–54	1.00 ± 0.00	1.00 ± 0.00
RBST2–65	1.00 ± 0.00	1.00 ± 0.00
SBST1–1	1.00 ± 0.00	1.00 ± 0.00
SBST1–23	1.00 ± 0.00	1.00 ± 0.00
SBST1–27	1.00 ± 0.00	1.00 ± 0.00
SBST2–5	1.00 ± 0.00	1.00 ± 0.00
RCPT1–4	1.00 ± 0.00	1.00 ± 0.00
RCPT3–25	1.00 ± 0.00	1.00 ± 0.00
RCPT3–26	1.00 ± 0.00	1.00 ± 0.00
RCPT3–27	1.00 ± 0.00	1.00 ± 0.00
RCPT3–28	1.00 ± 0.00	1.00 ± 0.00
RCPT3–35	1.00 ± 0.00	1.00 ± 0.00
RCPT3–40	1.00 ± 0.00	1.00 ± 0.00
CH5–8	1.00 ± 0.00	1.00 ± 0.00
CH9–7	1.00 ± 0.00	1.00 ± 0.00
NKS15	1.00 ± 0.00	1.00 ± 0.00

Note: Mean ± Standard deviation, N = 3

4.9 Antagonistic activity among the selected actinomycetes

Table 4.11 showed the inhibitory activity among the selected actinomycetes. No antagonistic activity was observed of eleven isolates (45.8%), such as RBST1–4, RBST2–20, RBST2–21, RBST2–53, RBST2–54, SBST1–1, RCPT1–4, RCPT3–25, RCPT3–26, RCPT3–40, and CH9–7, against other selected actinomycete isolates.

Table 4.11 Antagonistic activity among the selected actinomycetes.

Isolates	Antagonistic actinomycetes
RBST2–5	RBST2–9, RBST2–11, RBST2–41, CH5–8
RBST2–9	RCPT3–27, RCPT3–28, SBST2–5, CH5–8, NKS15
RBST2–11	RBST2–5, RCPT3–27, RCPT3–28, SBST2–5, CH5–8, NKS15
RBST2–41	RBST2–5, RBST2–65, RCPT3–27, RCPT3–28, SBST2–5, CH5–8, NKS15
RBST2–65	RBST2–41
SBST1–23	SBST2–5
SBST1–27	SBST2–5
SBST2–5	RBST2–9, RBST2–11, RBST2–41, SBST1–23, SBST1–27
RCPT3–27	RBST2–9, RBST2–11, RBST2–41, RCPT3–35
RCPT3–28	RBST2–9, RBST2–11, RBST2–41, RCPT3–35
RCPT3–35	RCPT3–27, RCPT3–28
CH5–8	RBST2–5, RBST2–9, RBST2–11, RBST2–41
NKS15	RBST2–9, RBST2–11, RBST2–41

Note: N = 3

4.10 Taxonomic characterization of the selected actinomycetes

4.10.1 Morphological characteristics

Morphology is an important characteristic for the description of taxa, it is not adequate in itself to differentiate between many genera and it was the only character used in many early descriptions. Most of twenty–four actinomycete isolates grew well on ISP 2 agar. The colors of the substrate mycelium ranged from yellow series to very deep red. White to grey series aerial spore masses are formed on ISP 2 agar, except RBST2–9, RBST2–11, and NKS15. Most produced diffusible or soluble pigments ranged from yellow series to deep red, except for RBST1–4, RBST2–9, RBST2–11, and RBST2–41. The cultural characteristics of the selected actinomycetes are shown in Table 4.12. Spiral and retinaculum–apertum spore chains were observed under a light microscope with total 400X magnifications (Figures 4.14–4.37).

Table 4.12 Cultural characteristics of the selected twenty–four selected actinomycete strains on ISP 2 agar.

Isolates	Growth	Color of substrate mycelium	Color of aerial spore mass	Soluble pigment
RBST1–4	Abundant	Light yellow	Light grey	-
RBST2–5	Good	Light greenish yellow	Greenish white	Light greenish yellow
RBST2–9	Moderate	Light yellow	Pinkish white	-
RBST2–11	Moderate	Light yellow	-	-
RBST2–20	Abundant	Brilliant yellow	Yellowish white	Light yellow
RBST2–21	Abundant	Brilliant yellow	Yellowish white	Light yellow
RBST2–41	Poor	Light yellow	-	-
RBST2–53	Abundant	Brilliant yellow	Yellowish white	Light yellow
RBST2–54	Abundant	Brilliant yellow	Yellowish white	Light yellow
RBST2–65	Abundant	Strong yellow	Yellowish white	Brilliant yellow
SBST1–1	Abundant	Light greenish yellow	Yellowish white	Light greenish yellow
SBST1–23	Good	Moderate olive	Light grey	Pale greenish yellow
SBST1–27	Abundant	Strong greenish yellow	Grey	Light yellowish green
SBST2–5	Good	Dark orange yellow	Light grey	Dark yellow
RCPT1–4	Good	Light yellowish brown	Light grey	Light yellow
RCPT3–25	Abundant	Light greenish yellow	Yellowish white	Light yellow
RCPT3–26	Abundant	Strong yellow	Yellowish white	Light yellow
RCPT3–27	Good	Yellowish brown	Grey	Light yellow
RCPT3–28	Good	Pale greenish yellow	Light grey	Light yellow
RCPT3–35	Abundant	Strong yellow	Yellowish white	Light yellow
RCPT3–40	Abundant	Strong yellow	Yellowish white	Light yellow
CH5–8	Good	Vivid greenish yellow	Medium grey	Brilliant yellow
CH9–7	Good	Light yellow	Light grey	Pale yellow
NKS15	Poor	Very deep red	-	Deep red

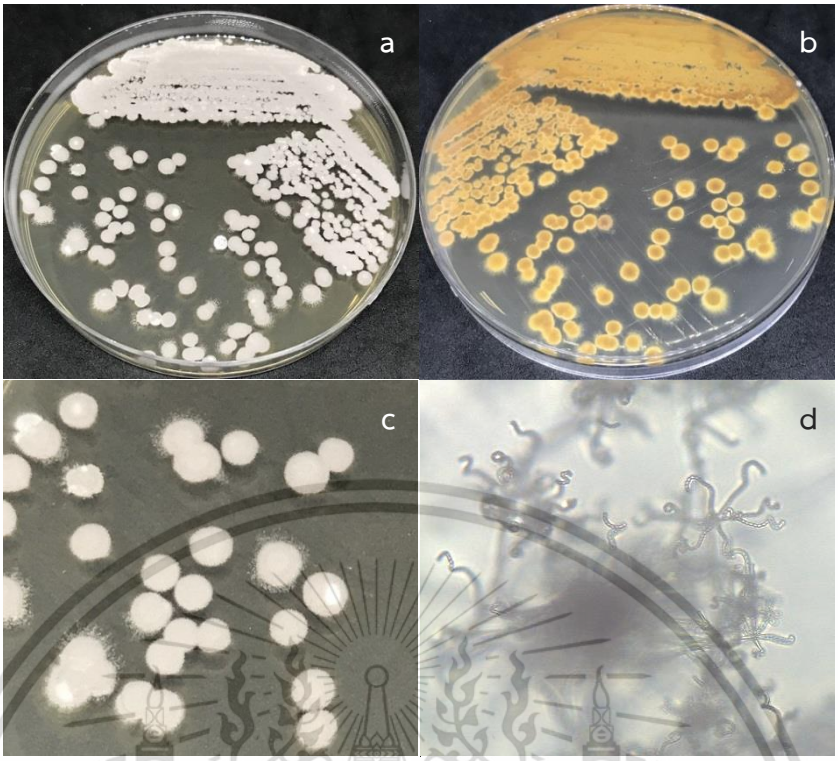


Figure 4.14 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST1–4.

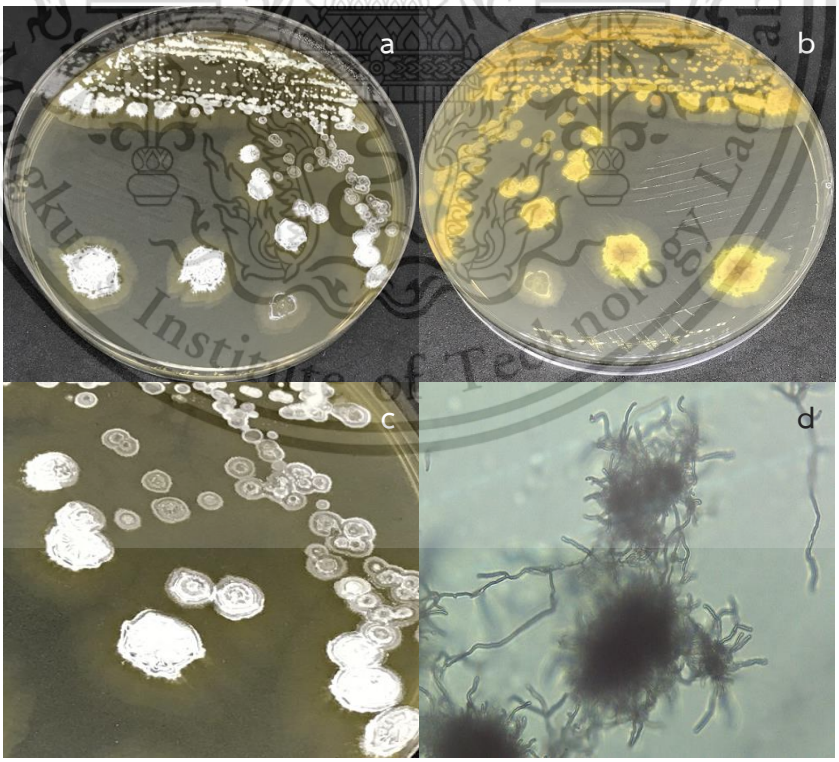


Figure 4.15 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–5.

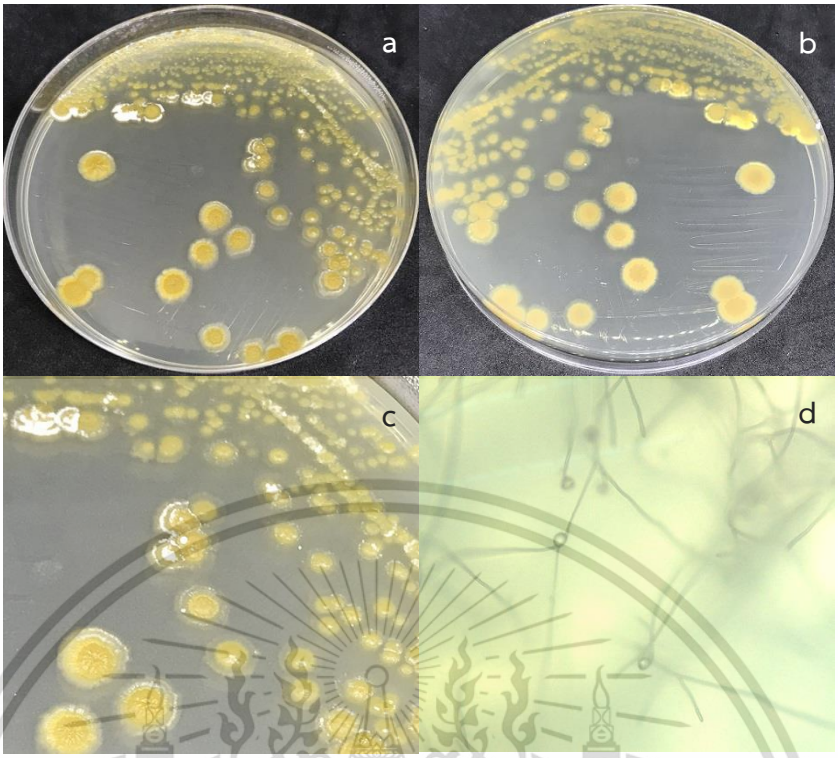


Figure 4.16 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–9.

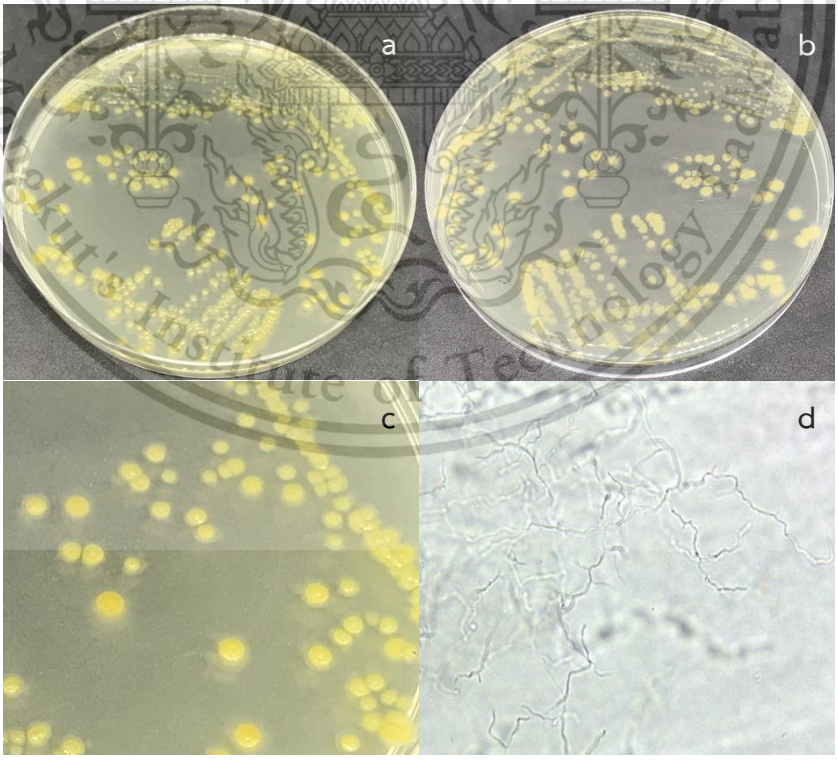


Figure 4.17 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–11.

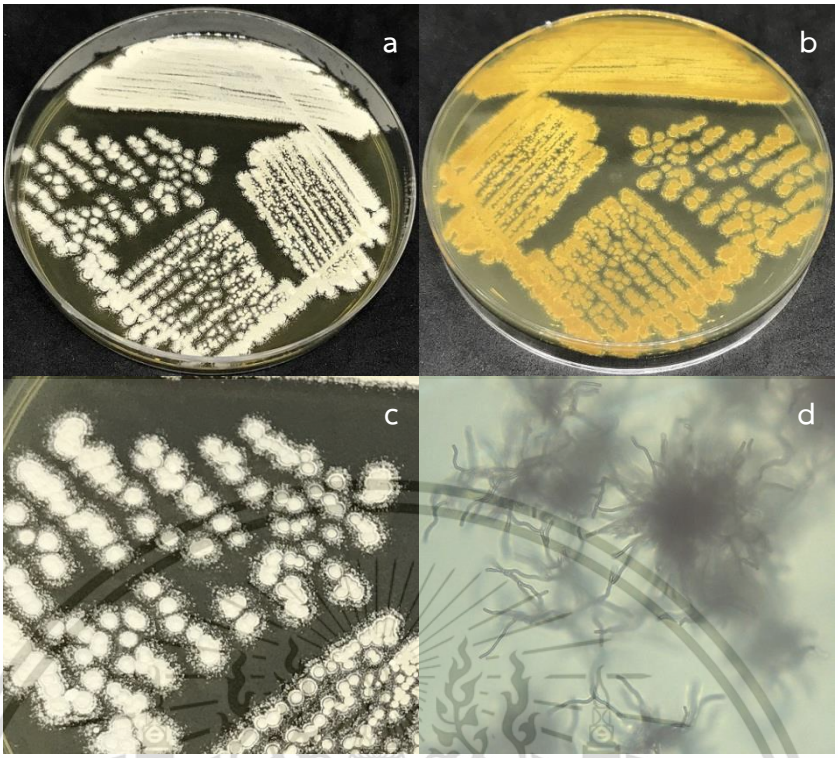


Figure 4.18 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–20.

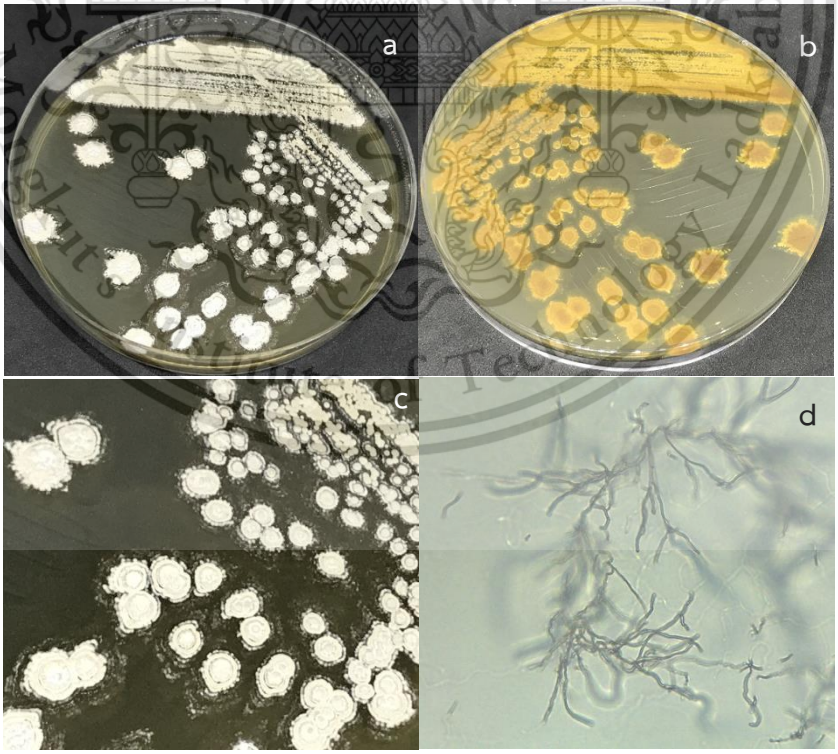


Figure 4.19 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–21.

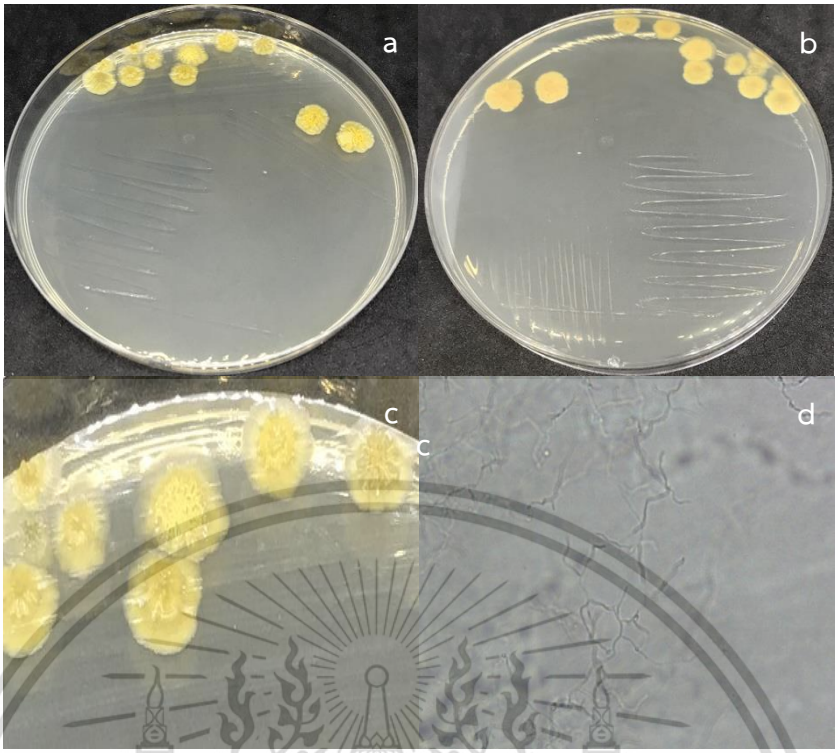


Figure 4.20 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–41.

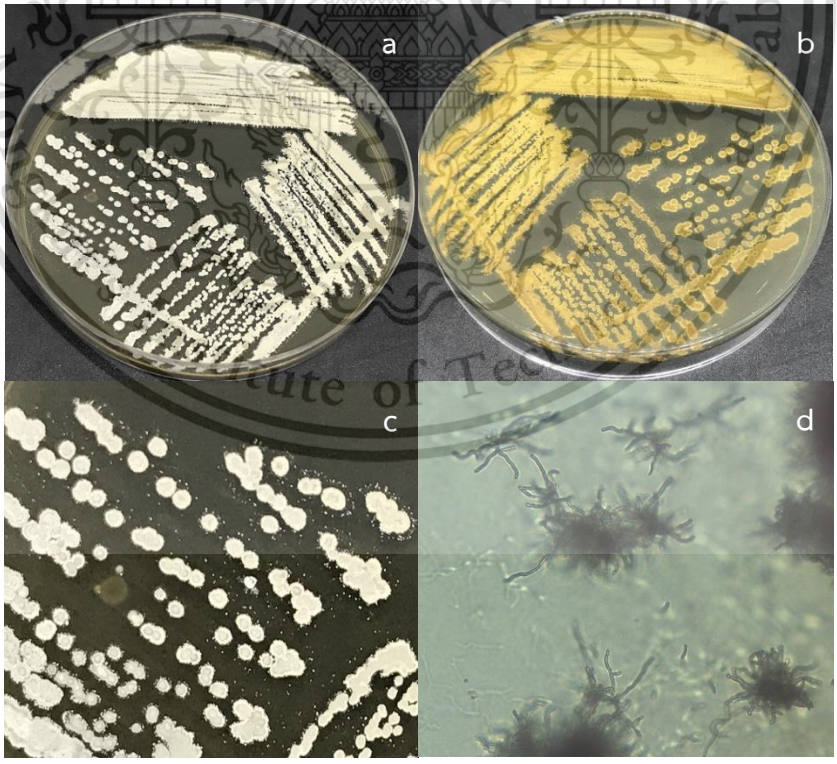


Figure 4.21 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–53.

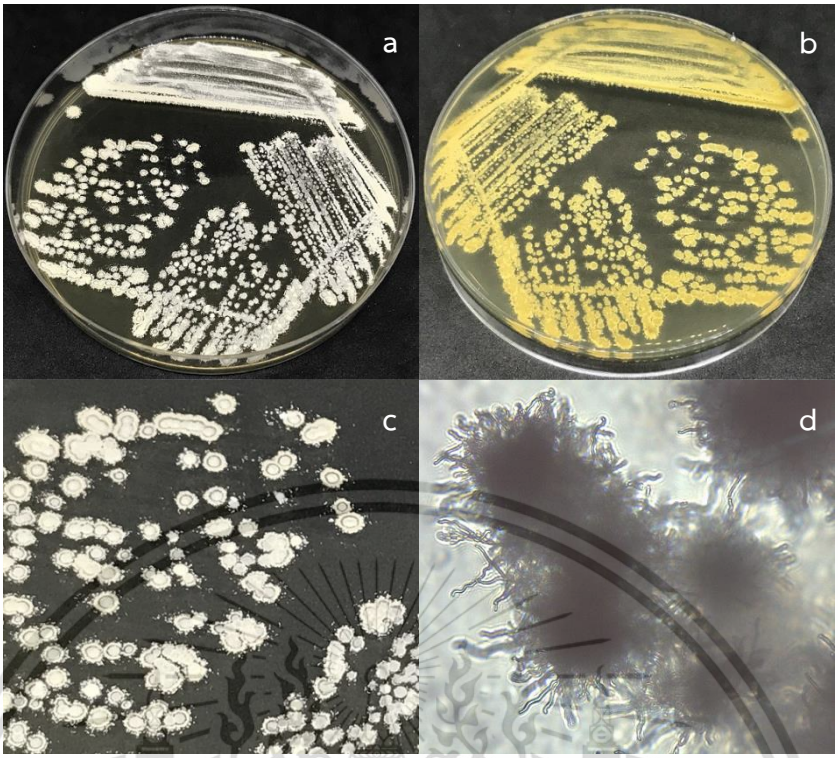


Figure 4.22 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–54.

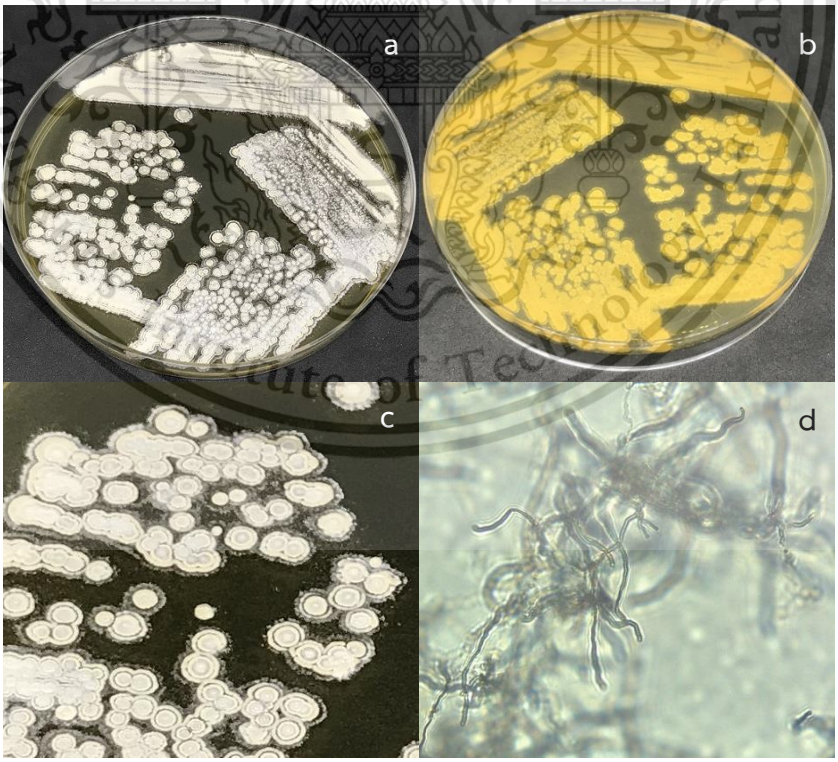


Figure 4.23 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RBST2–65.

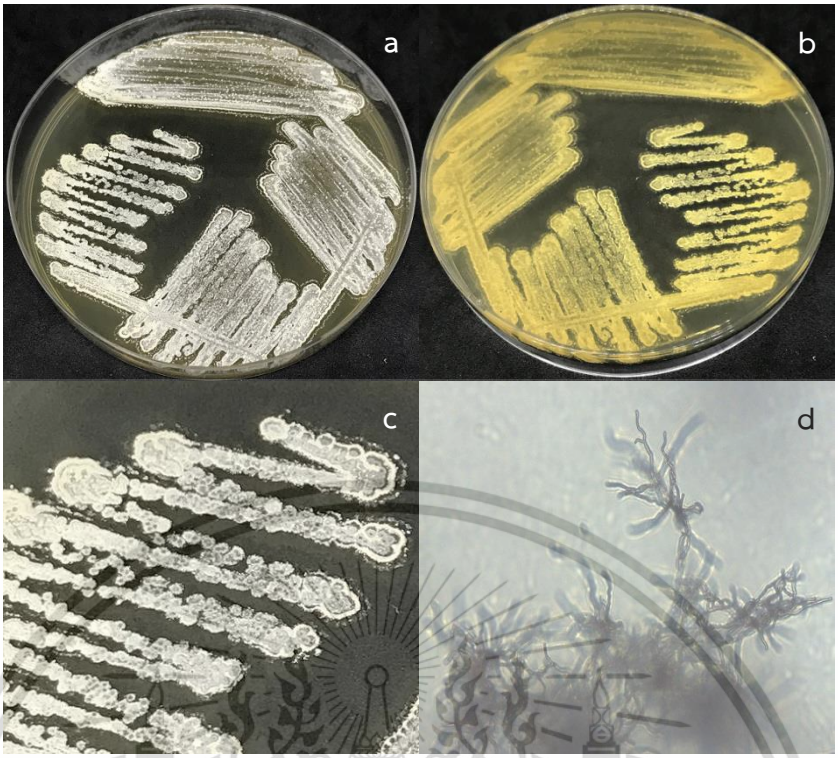


Figure 4.24 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain SBST1–1.

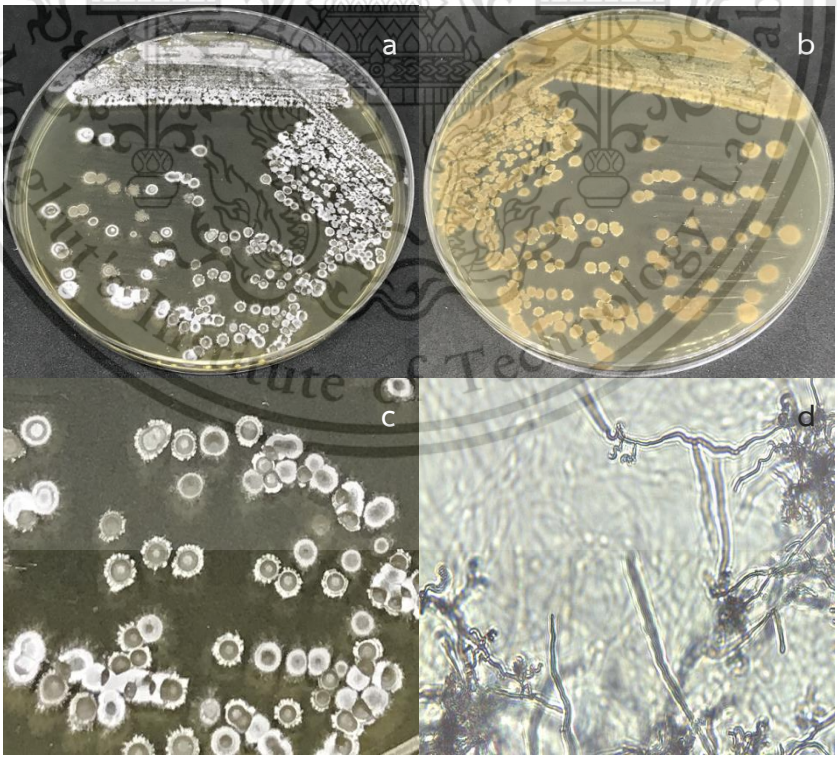


Figure 4.25 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain SBST1–23.

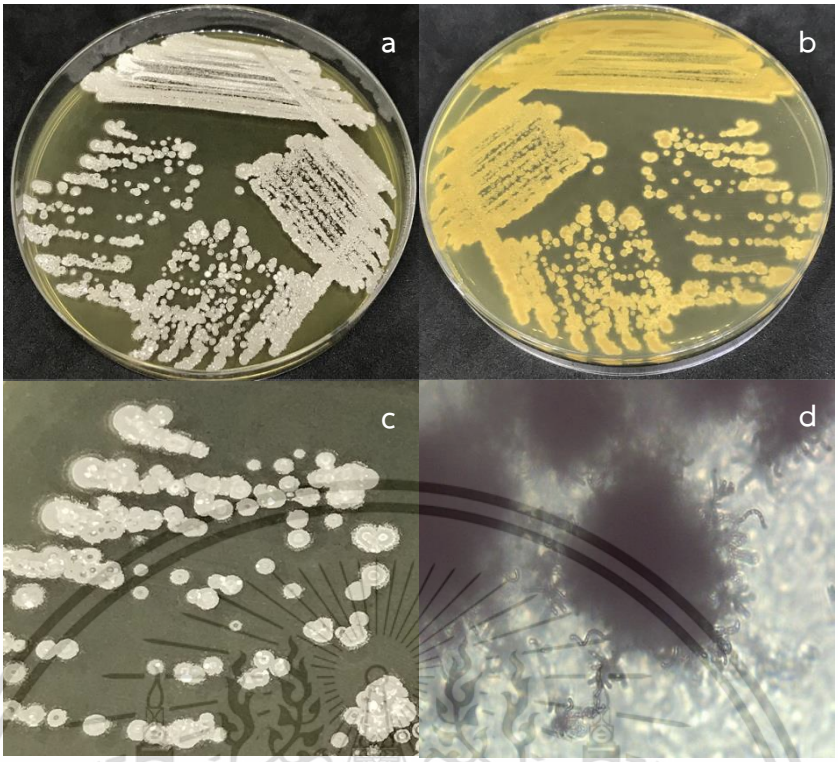


Figure 4.26 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain SBST1–27.

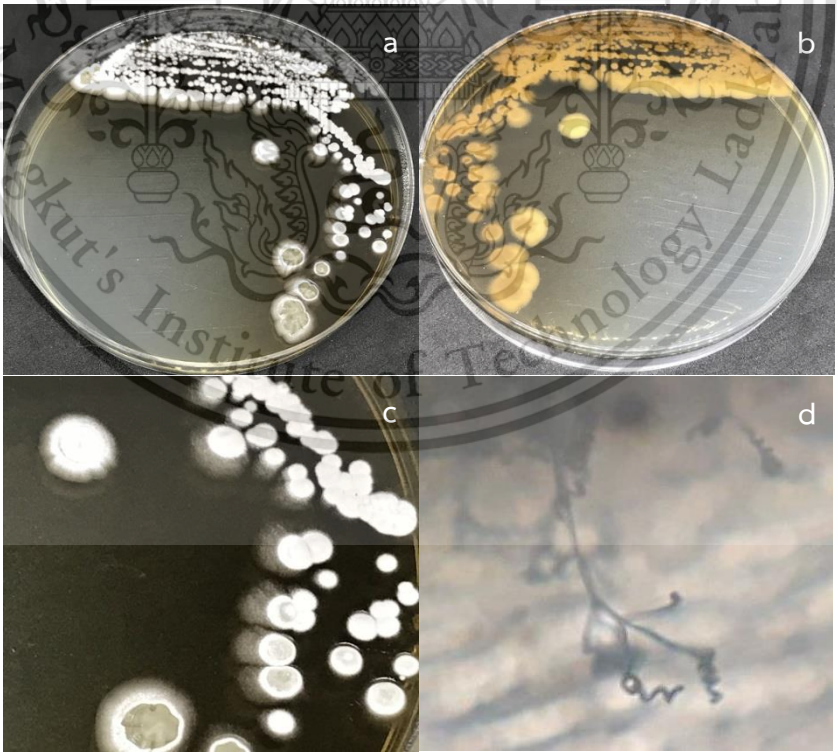


Figure 4.27 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain SBST2–5.

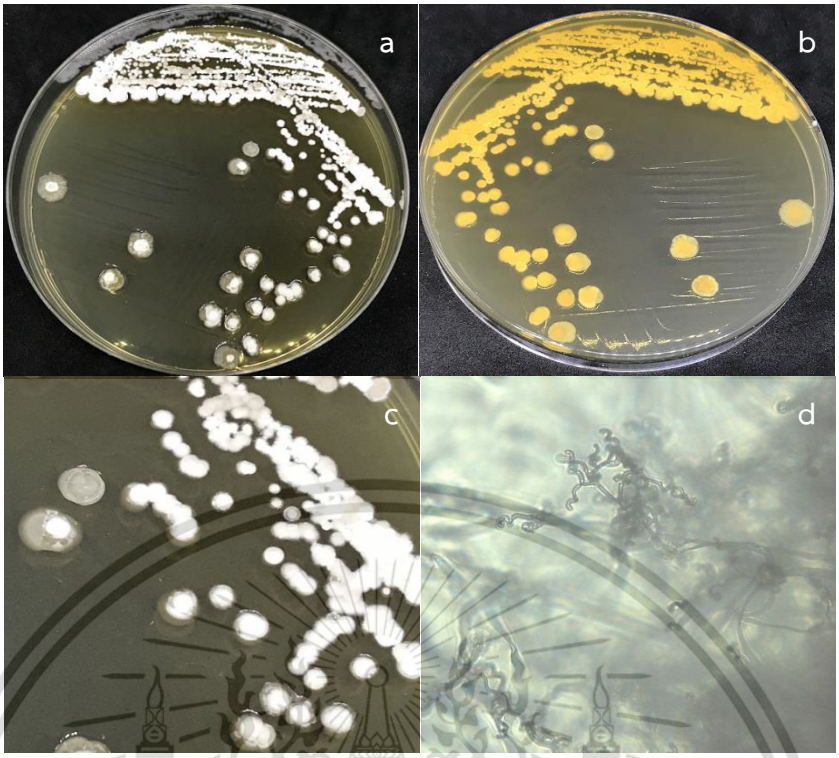


Figure 4.28 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RCPT1–4.

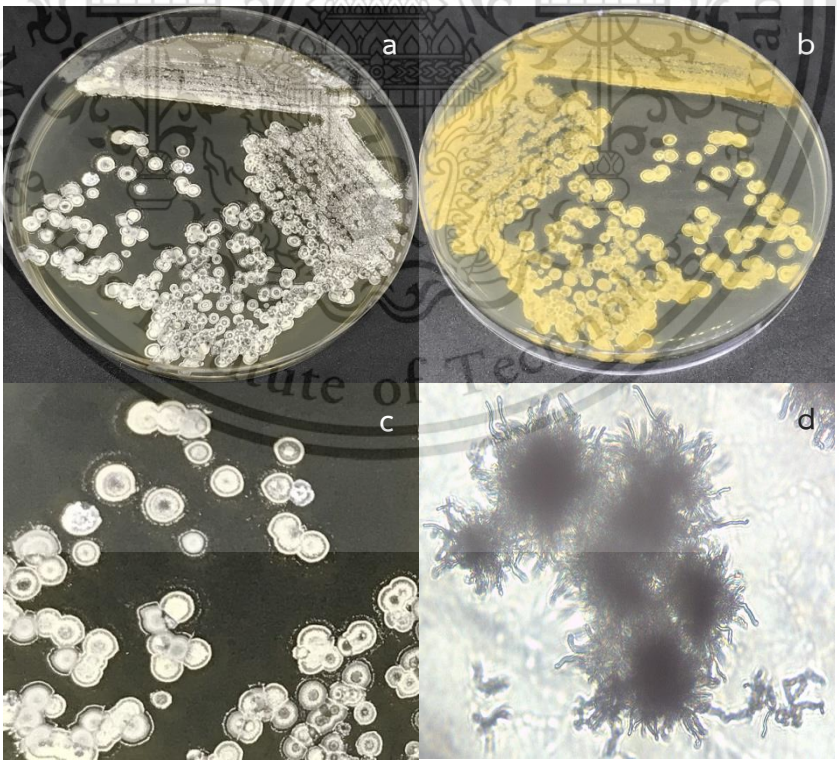


Figure 4.29 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RCPT3–25.

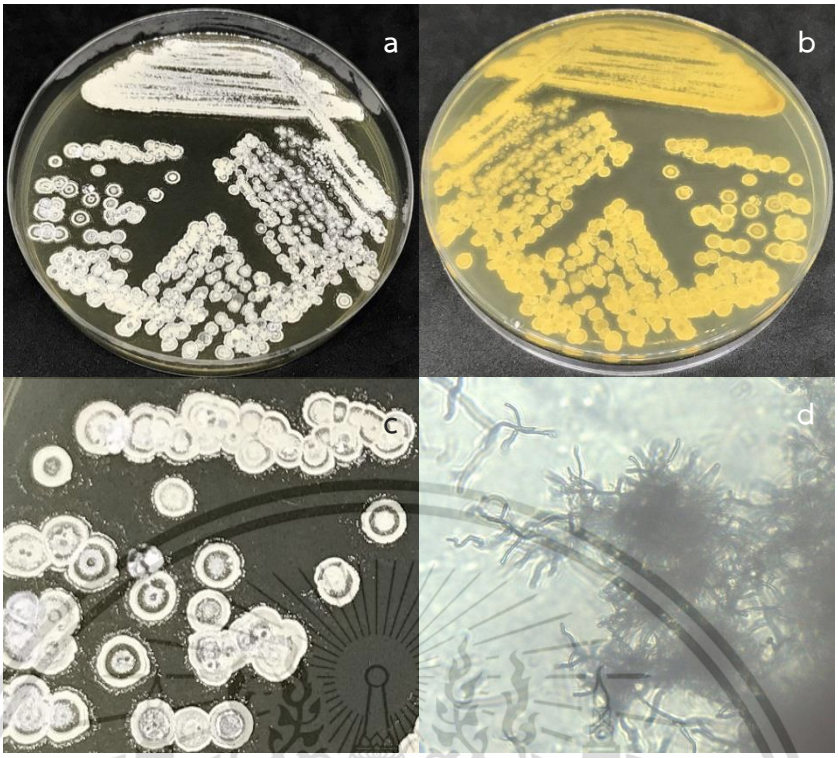


Figure 4.30 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RCPT3–26.

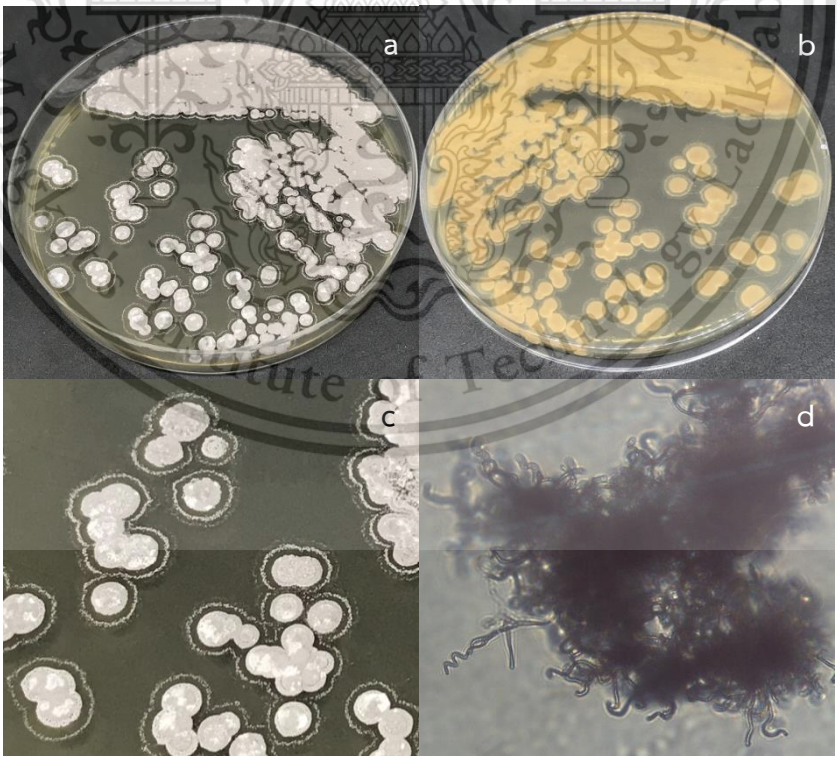


Figure 4.31 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RCPT3–27.

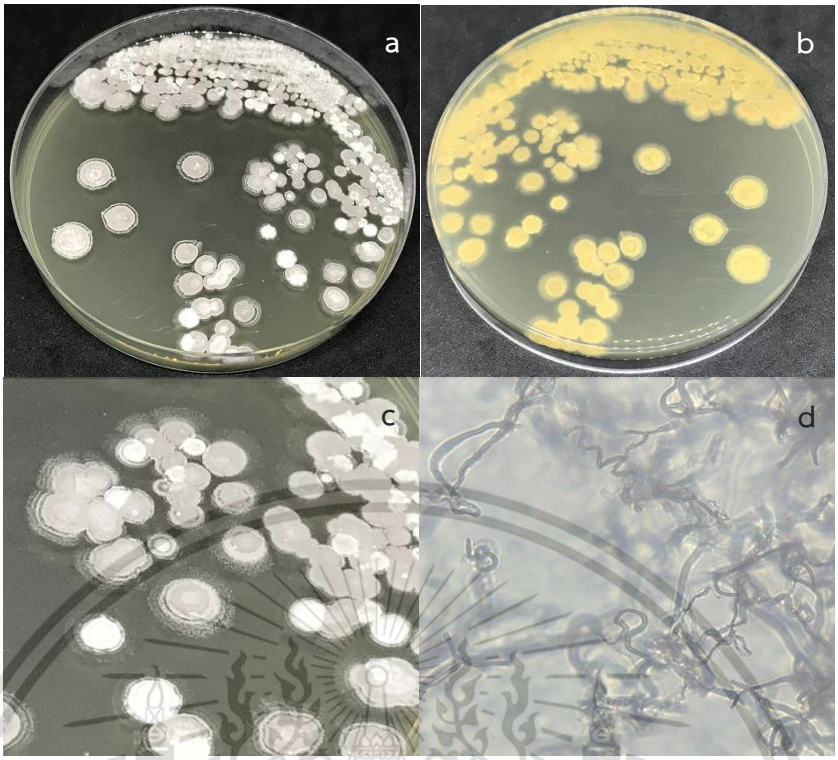


Figure 4.32 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RCPT3–28.

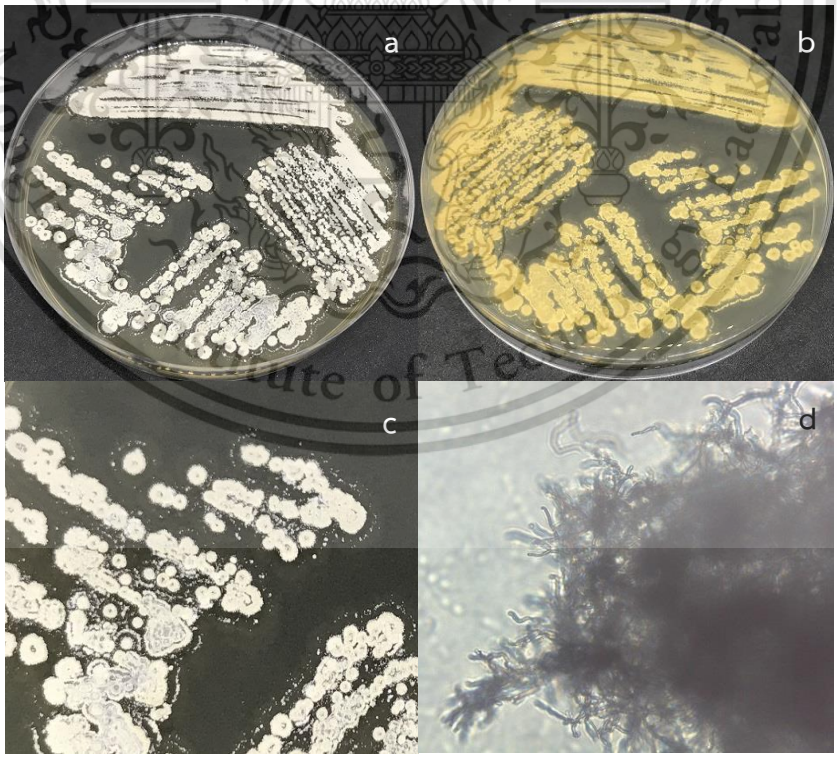


Figure 4.33 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RCPT3–35.

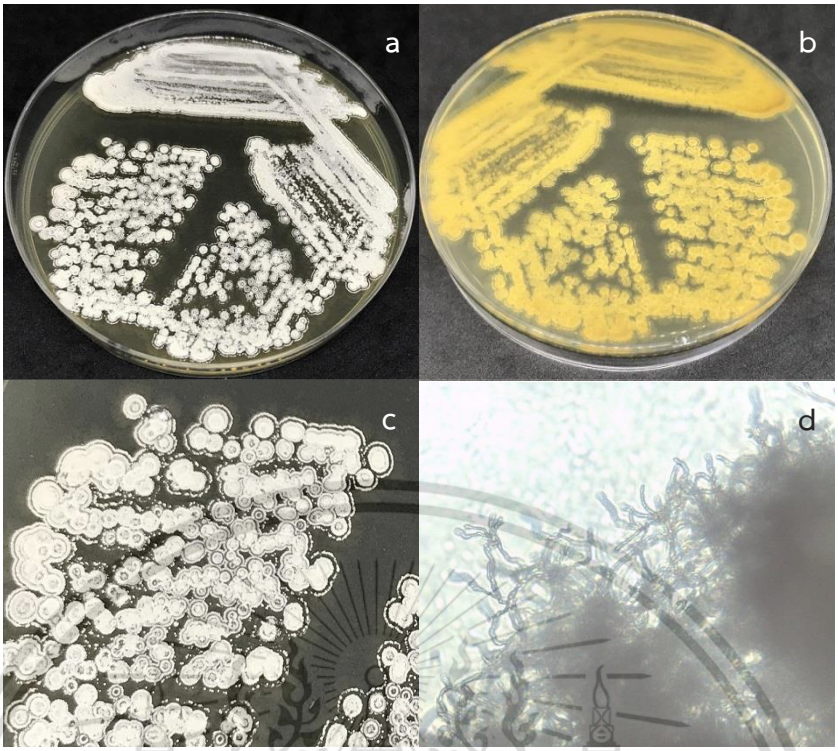


Figure 4.34 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain RCPT3–40.

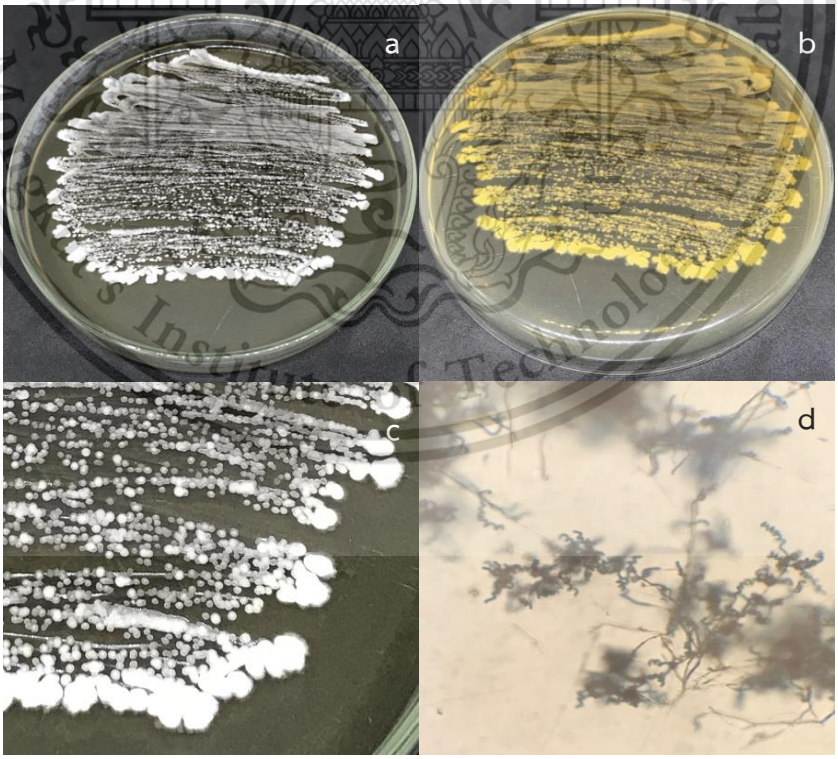


Figure 4.35 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain CH5–8.

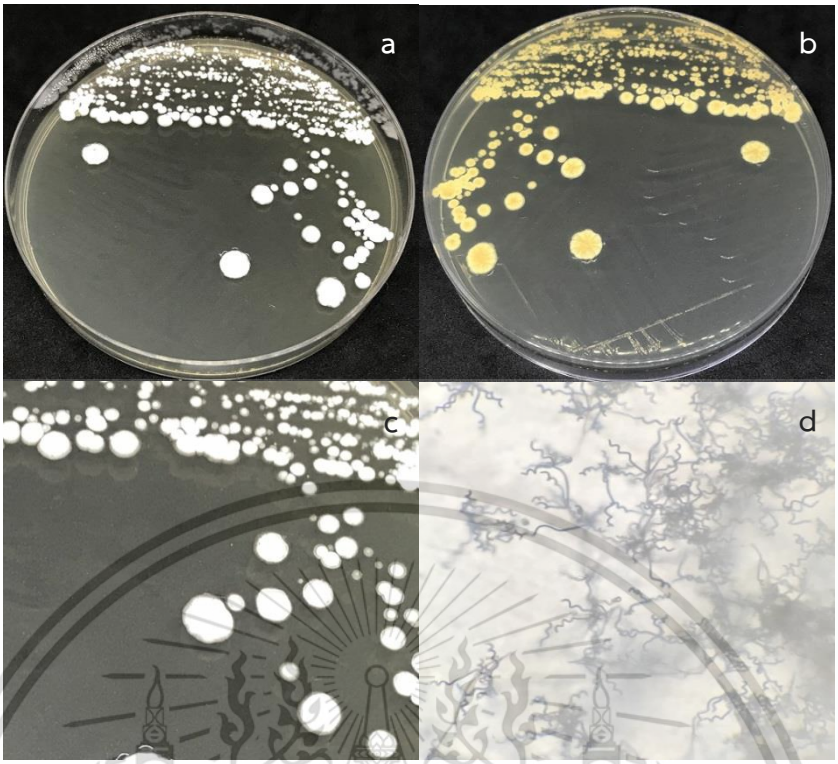


Figure 4.36 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain CH9–7.

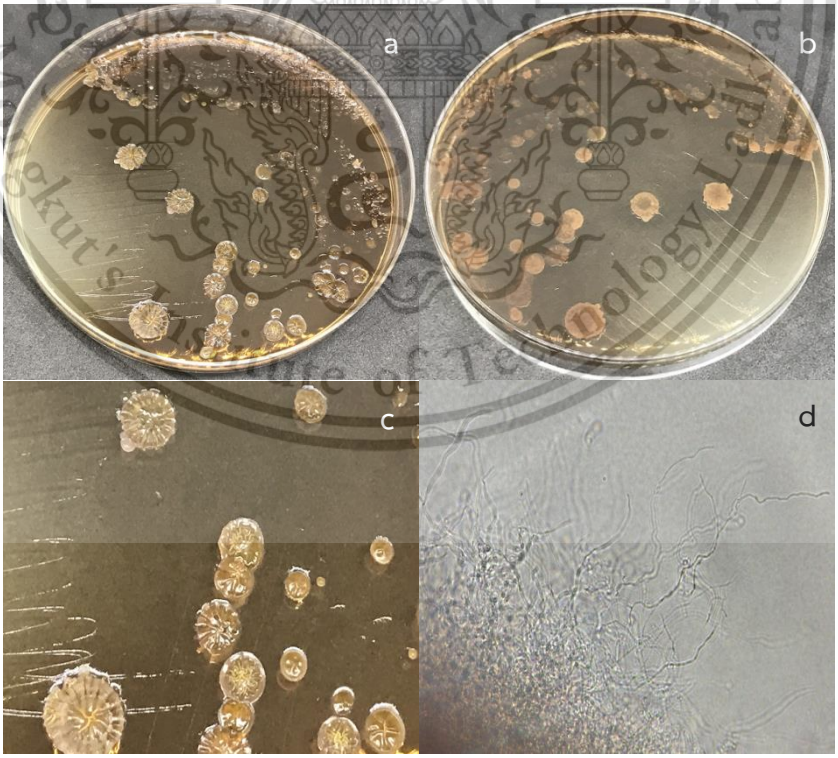


Figure 4.37 Colonial appearance on ISP 2 agar (a, b and c) and light micrograph (d) of *Streptomyces* sp. strain NKS15.

4.10.2 Identification of the selected actinomycetes by 16S rRNA gene sequencing

Almost complete 16S rRNA gene sequences (1,405–1,520 nt) of each strain were used for phylogenetic analysis and compared with 16S rRNA gene sequences of members of the family *Streptomycetaceae*. Phylogenetic analysis revealed that all strains were placed within the cluster of the genus *Streptomyces* (Figures 4.38–4.49). All strains shared the highest 16S rRNA gene sequence similarities with the genus *Streptomyces* ranging from 98.85 to 100.00% (Table 4.13).

Table 4.13 16S rRNA gene sequences of the selected actinomycete strains.

Isolates/Accession number	Identification	%Similarity	Total base pairs
RBST1–4 (LC489245)	*Streptomyces rochei* NRRL B-2410^T	100.00	1,519
RBST2–5 (LC489248)	*Streptomyces daghestanicus* NRRL B-5418^T *Streptomyces albidoflavus* DSM 40455^T *Streptomyces violascens* ISP 5183^T	99.93	1,490
RBST2–9 (LC489255)	*Streptomyces fradiae* DSM 40063^T	100.00	1,456
RBST2–11 (LC489256)	*Streptomyces fradiae* DSM 40063^T	100.00	1,448
RBST2–20 (LC489250)	*Streptomyces daghestanicus* NRRL B-5418^T *Streptomyces albidoflavus* DSM 40455^T *Streptomyces violascens* ISP 5183^T	99.86	1,435
RBST2–21 (LC489251)	*Streptomyces daghestanicus* NRRL B-5418^T *Streptomyces albidoflavus* DSM 40455^T *Streptomyces violascens* ISP 5183^T	100.00	1,471
RBST2–41 (LC489257)	*Streptomyces fradiae* DSM 40063^T	100.00	1,444
RBST2–53 (LC489258)	*Streptomyces daghestanicus* NRRL B-5418^T *Streptomyces albidoflavus* DSM 40455^T *Streptomyces violascens* ISP 5183^T	100.00	1,405
RBST2–54 (LC489259)	*Streptomyces daghestanicus* NRRL B-5418^T *Streptomyces albidoflavus* DSM 40455^T *Streptomyces violascens* ISP 5183^T	100.00	1,520
RBST2–65 (LC489260)	*Streptomyces daghestanicus* NRRL B-5418^T *Streptomyces albidoflavus* DSM 40455^T *Streptomyces violascens* ISP 5183^T	99.93	1,469
SBST1–1 (LC489249)	*Streptomyces daghestanicus* NRRL B-5418^T *Streptomyces albidoflavus* DSM 40455^T *Streptomyces violascens* ISP 5183^T	100.00	1,457
SBST1–23 (LC489246)	*Streptomyces speibonae* NRRL B-24240^T	99.72	1,486
SBST1–27 (LC489247)	*Streptomyces albogriseolus* NRRL B-1305^T	100.00	1,451
SBST2–5 (LC430996)	*Streptomyces thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T	98.94	1,430
RCPT1–4 (OM661191)	*Streptomyces fumigatiscleroticus* NBRC 12999^T	99.22	1,413

Table 4.13 16S rRNA gene sequences of the selected actinomycete strains (continue).

Isolates/Accession number	Identification	%Similarity	Total base pair
RCPT3–25 (LC489243)	*Streptomyces daghestanicus* NRRL B-5418^T	99.93	1,445
	Streptomyces albidoflavus DSM 40455^T		
	Streptomyces violascens ISP 5183^T		
RCPT3–26 (LC489244)	*Streptomyces daghestanicus* NRRL B-5418^T	99.93	1,481
	Streptomyces albidoflavus DSM 40455^T		
	Streptomyces violascens ISP 5183^T		
RCPT3–27 (LC489252)	*Streptomyces diastaticus* subsp. *ardesiacus* NRRL B-1773^T	99.72	1,430
RCPT3–28 (LC489253)	*Streptomyces diastaticus* subsp. *ardesiacus* NRRL B-1773^T	99.72	1,494
RCPT3–35 (LC553842)	*Streptomyces albidoflavus* DSM 40455^T	99.51	1,464
RCPT3–40 (LC489254)	*Streptomyces daghestanicus* NRRL B-5418^T	100.00	1,488
	Streptomyces albidoflavus DSM 40455^T		
	Streptomyces violascens ISP 5183^T		
CH5–8 (LC489239)	*Streptomyces echinatus* NBRC 12763^T	98.86	1,425
	Streptomyces actinomycinicus RCU-197^T	98.85	
CH9–7 (LC489240)	*Streptomyces lydicus* ATCC 25470^T	99.86	1,414
	Streptomyces chattanoogensis NRRL ISP-5002^T		
NKS15 (LC489241)	*Streptomyces tuirus* NBRC 15617^T	99.79	1,485

Phylogenetic relationship of strain RBST1–4 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. rochei* NRRL B–2410^{T} and *S. mutabilis* NBRC 12800^{T} with the bootstrap values of 58% (Figure 4.38).

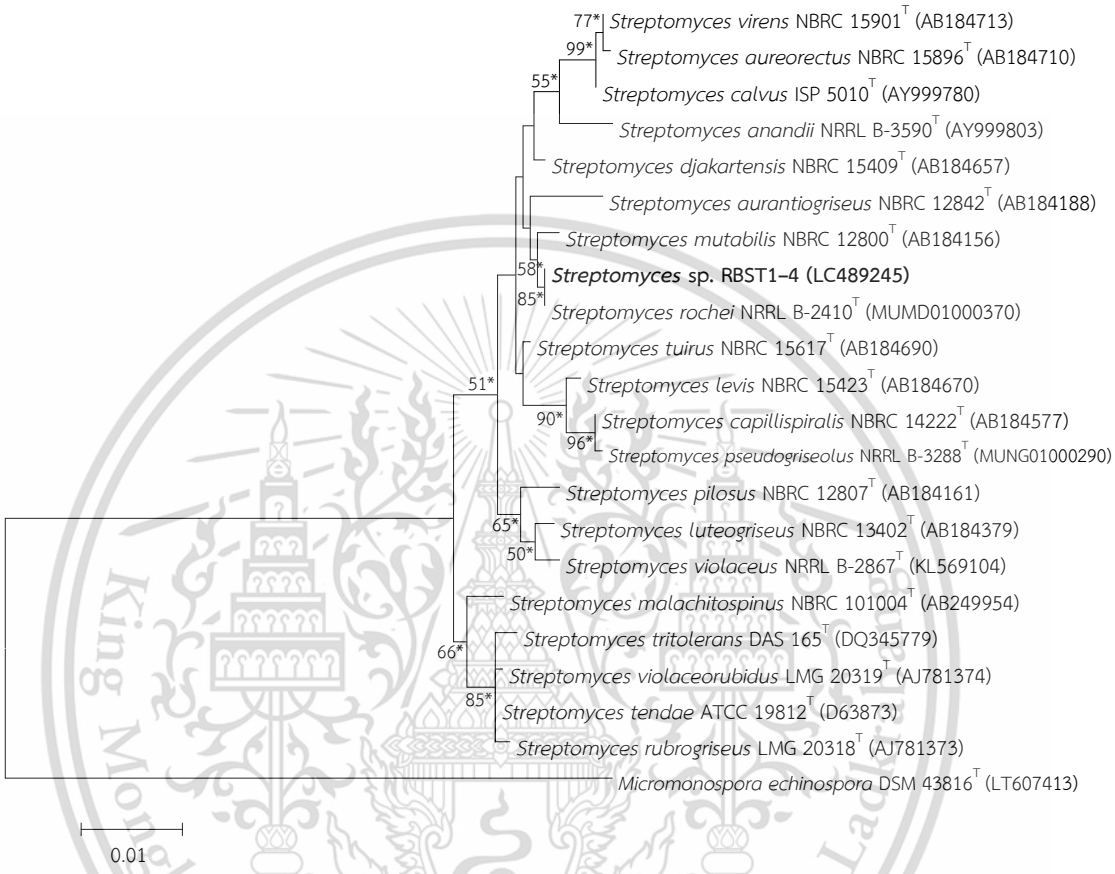


Figure 4.38 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain RBST1–4 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of actinomycetes group I strains, including RBST2–5, RBST2–20, RBST2–21, RBST2–53, RBST2–54, RBST2–65, SBST1–1, RCPT3–25, RCPT3–26, and RCPT3–40 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. daghestanicus* NRRL B–5418^{T}, *S. albidoflavus* DSM 40455^{T} and *S. violascens* ISP 5183^{T} with the bootstrap values of 63% (Figure 4.39). Pairwise comparisons of these actinomycetes are shown in Table 4.14.

Table 4.14 Similarity values (%) of actinomycetes group I.

Taxa	1	2	3	4	5	6	7	8	9	10
1.RBST2–5	100.00	99.93	99.93	99.93	99.86	99.86	99.93	99.79	99.86	99.93
2.RBST2–20	99.93	100.00	99.93	99.86	99.86	99.79	99.86	99.79	99.79	99.86
3.RBST2–21	99.93	99.93	100.00	100.00	100.00	99.86	100.00	99.93	99.93	100.00
4.RBST2–53	99.93	99.86	100.00	100.00	100.00	99.93	100.00	99.93	99.93	100.00
5.RBST2–54	99.86	99.86	100.00	100.00	100.00	99.93	100.00	99.93	99.93	100.00
6.RBST2–65	99.86	99.79	99.86	99.93	99.93	100.00	99.93	99.86	99.86	99.93
7.SBST1–1	99.93	99.86	100.00	100.00	100.00	99.93	100.00	99.93	99.93	100.00
8.RCPT3–25	99.79	99.79	99.93	99.93	99.93	99.86	99.93	100.00	100.00	99.93
9.RCPT3–26	99.86	99.79	99.93	99.93	99.93	99.86	99.93	100.00	100.00	99.93
10.RCPT3–40	99.93	99.86	100.00	100.00	100.00	99.93	100.00	99.93	99.93	100.00

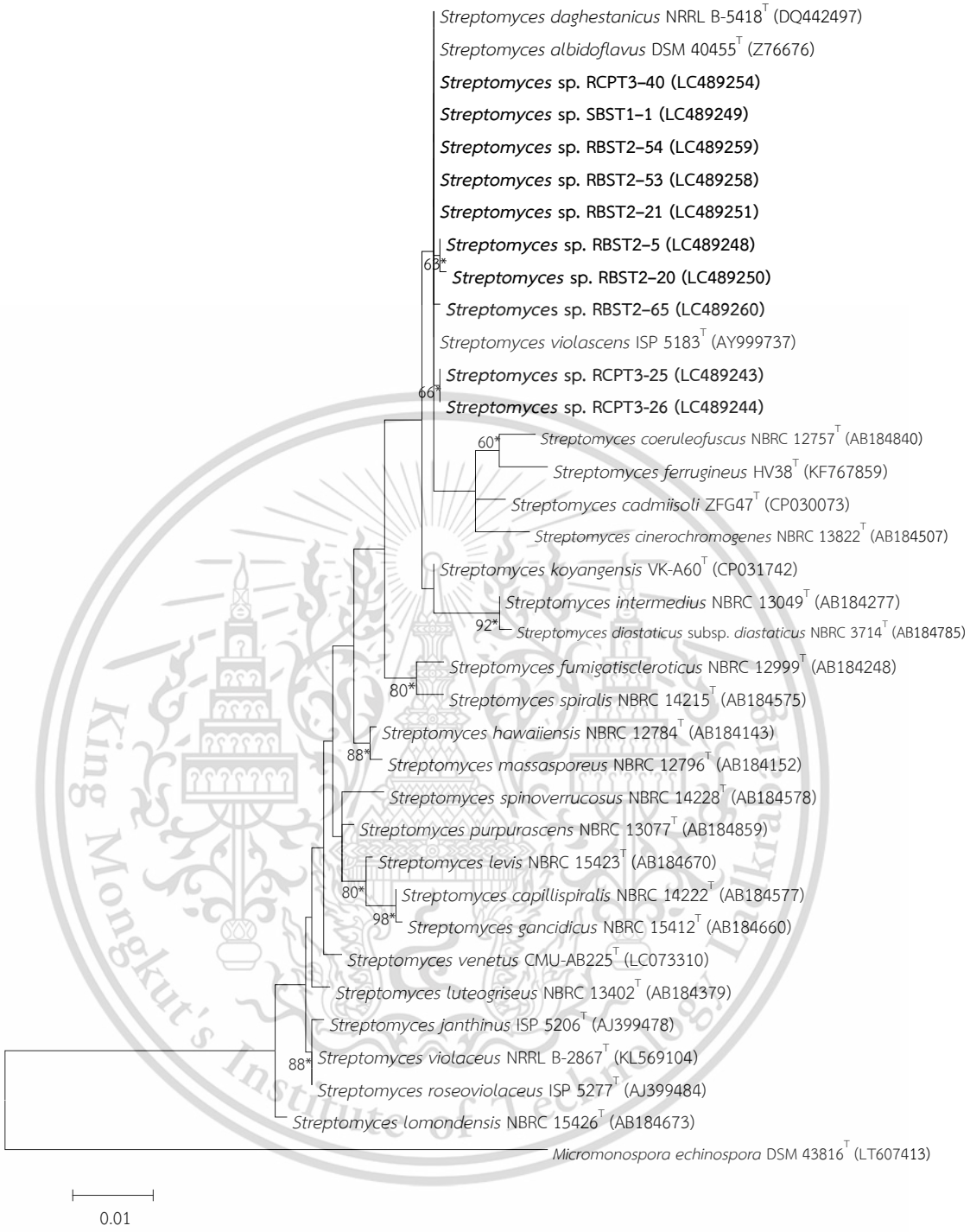


Figure 4.39 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain RBST2–5, RBST2–20, RBST2–21, RBST2–53, RBST2–54, RBST2–65, SBST1–1, RCPT3–25, RCPT3–26, RCPT3–40, and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of actinomycetes group II strains, including RBST2–9, RBST2–11, and RBST2–41 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. fradiae* DSM 40063^{T} with the bootstrap values of 99% (Figure 4.40). Pairwise comparisons of these actinomycetes were 100%.

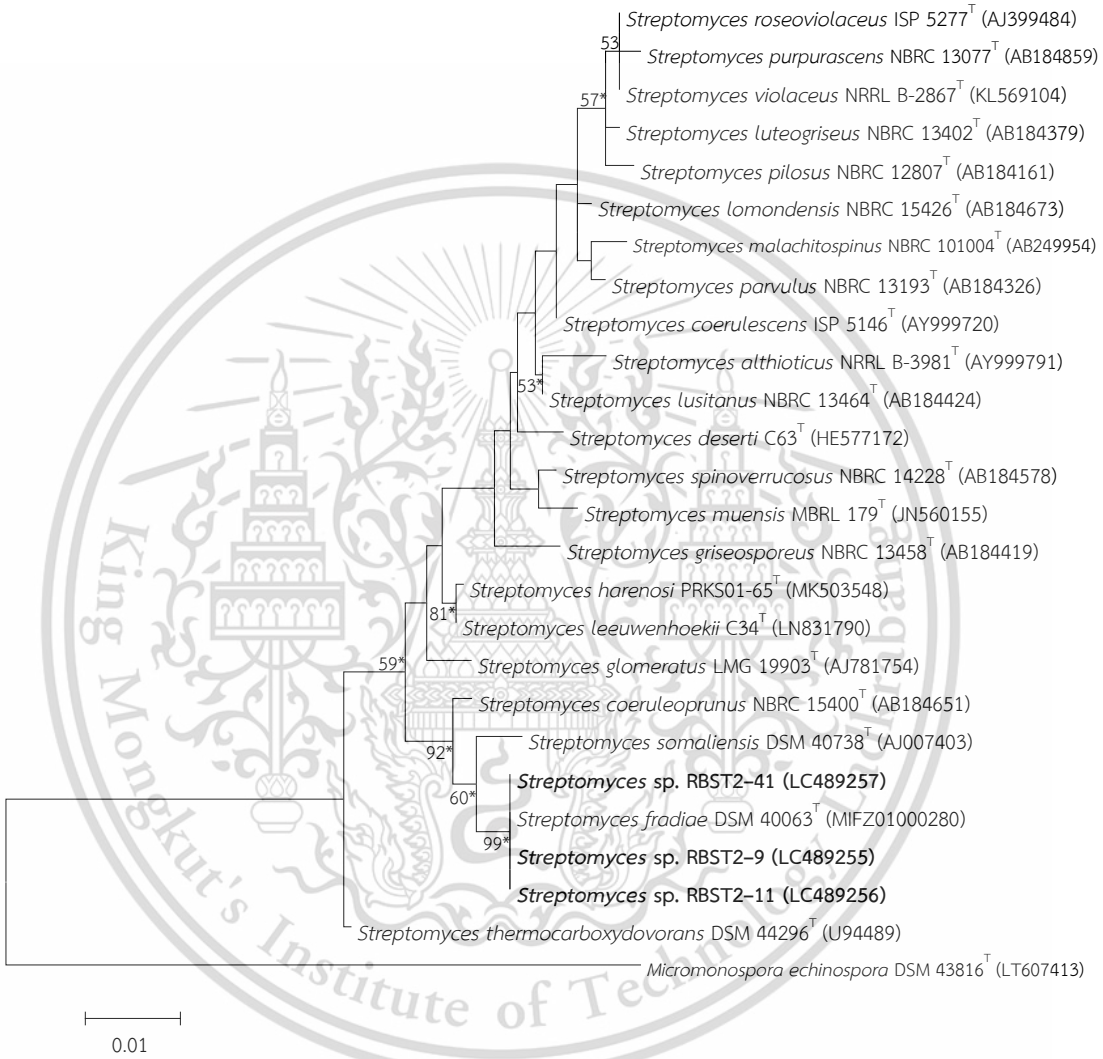


Figure 4.40 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain RBST2–9, RBST2–11, RBST2–41, and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of strain SBST1–23 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. speibonae* NRRL B–24240^{T} with the bootstrap values of 93% (Figure 4.41).

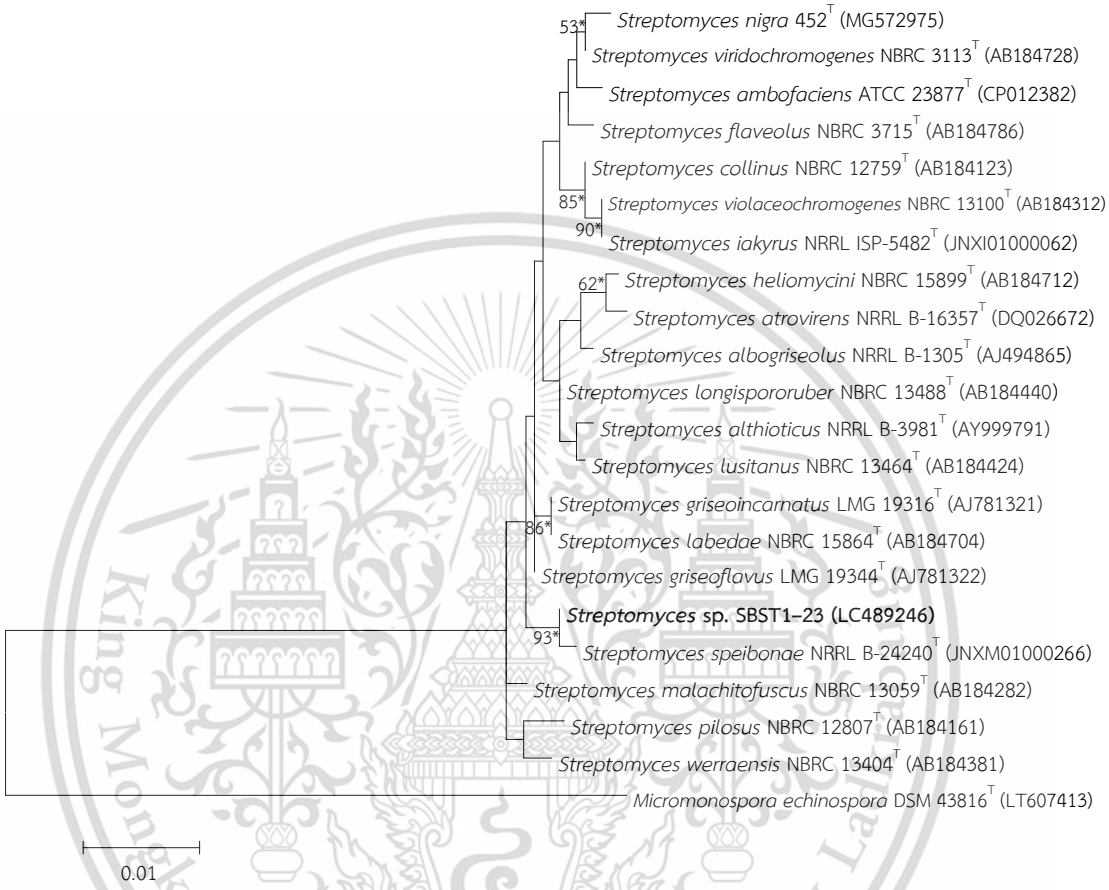


Figure 4.41 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain SBST1–23 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of strain SBST1–27 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. albogriseolus* NRRL B–1305^{T}, *S. speibonae* NRRL B–24240^{T}, *S. atrovirens* NRRL B–16357^{T} and *S. heliomycini* NBRC 15899^{T} with low bootstrap values (<50%) (Figure 4.42).

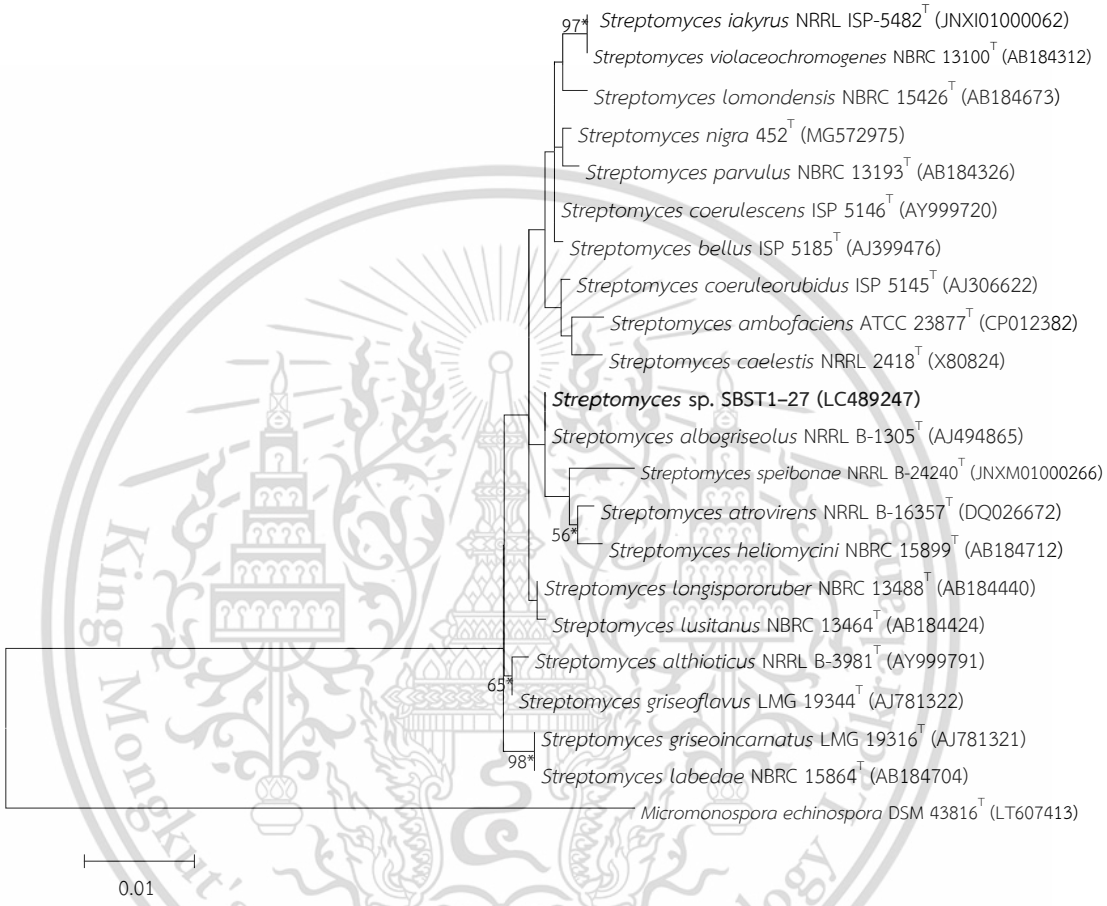


Figure 4.42 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain SBST1–27 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of strain SBST2–5 based on maximum–likelihood and neighbor–joining method showed that the strain formed a distinct monophyletic line within the genus *Streptomyces* (Figure 4.43). It was found to be most closely related to the type strains of *S. thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^{T} (98.94%).

Figure 4.43 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain SBST2–5 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of strain RCPT1–4 based on maximum–likelihood and neighbor–joining method showed that the branches for this strain were separated from *S. fumigatiscleroticus* NBRC 12999^{T} and *S. spiralis* NBRC 14215^{T} with low bootstrap values (<50%) (Figure 4.44).

Figure 4.44 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain RCPT1–4 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of actinomycetes group III strains, including RCPT3–27 and RCPT3–28 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. diastaticus* subsp. *diastaticus* NRRL B-1773^{T} with the bootstrap values of 92% (Figure 4.45). Pairwise comparisons of two actinomycetes were 100%.

Figure 4.45 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain RCPT3–27, RCPT3–28, and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of strain RCPT3–35 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. violascens* ISP 5183^{T}, *S. albidoflavus* DSM 40455^{T} and *S. daghestanicus* NRRL B–5418^{T} with low bootstrap values (<50%) (Figure 4.46).

Figure 4.46 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain RCPT3–35, and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of strain CH5–8 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. actinomycinicus* RCU–197^{T} and *S. echinatus* NBRC 12763^{T} with the bootstrap values of 62% (Figure 4.47).

Figure 4.47 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain CH5–8 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of strain CH9–7 based on maximum–likelihood and neighbor–joining method showed that the branches for this strain were separated from *S. lydicus* ATCC 25470^{T} and *S. chattanoogensis* NRRL ISP–5002^{T} with low bootstrap values (<50%) (Figure 4.48).

Figure 4.48 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain CH9–7 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

Phylogenetic relationship of strain NKS15 based on maximum–likelihood and neighbor–joining method showed that the strain formed a monophyletic line with *S. tuirus* NBRC 15617^{T} with low bootstrap values (<50%) (Figure 4.49).

Figure 4.49 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain NKS15 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

4.11 Product formation from the selected actinomycetes

From the above experiment data, three actinomycete products (Table 4.15) based on all complete properties were formulated (Tables 4.16–4.17). ATU–Bio 1 consisted of the strain RBST1–4, RCPT3–28, and CH9–7, ATU–Bio 2 consisted of the strain CH5–8, RCPT1–4, and RBST2–54, and ATU–Bio 3 consisted of the strain RCPT1–4, RCPT3–40, and RBST2–21, respectively.

Table 4.15 Actinomycete products of this study.

Products	Isolates
ATU–Bio 1	RBST1–4, RCPT3–28, CH9–7
ATU–Bio 2	CH5–8, RCPT1–4, RBST2–54
ATU–Bio 3	RCPT1–4, RCPT3–40, RBST2–21

Table 4.16 Anti–*Foc* activity, macronutrients, phytohormones and siderophore production of the selected actinomycetes in the products.

Products/ Isolates	Anti–*Foc* activity[a]	Macronutrients			Phytohormones			Siderophore[f]
		N_2–fixation[b]	Phosphate[c]	Potassium[d]	IAA[e]	GA[e]	Cytokinin[b]	
ATU–Bio 1								
RBST1–4	33.30	+	0.00	119.00	11.54	42.99	+	1.43
RCPT3–28	26.62	+	0.00	0.00	6.40	40.46	+	1.19
CH9–7	57.60	+	108.16	131.32	19.75	39.19	+	1.53
ATU–Bio 2								
CH5–8	50.34	+	102.55	113.93	37.21	56.90	+	1.36
RCPT1–4	39.15	+	130.43	166.30	12.57	36.57	+	1.14
RBST2–54	43.75	+	0.00	117.97	29.83	57.40	+	1.58
ATU–Bio 3								
RCPT1–4	39.15	+	130.43	166.30	12.57	36.57	+	1.14
RCPT3–40	41.92	+	0.00	0.00	18.62	35.87	+	1.69
RBST2–21	21.04	+	0.00	116.42	21.25	50.98	+	1.62

Note: Negative results for H_2S and HCN production

[a] Mean of the percentage inhibition of radial growth of *Foc*

[b] Qualitative analysis: + = positive

[c] Mean of the phosphate solubilization efficiency (PE)

[d] Mean of the potassium solubilization efficiency (PSE)

[e] Mean of the phytohormone production (µg/ml)

[f] Mean of the siderophore production index (SPI)

Table 4.17 Extracellular enzyme production, stress tolerance, and growth of the selected actinomycetes in the products.

Products/ Isolates	Extracellular enzyme production						Stress tolerance[c]				Growth[d] (CFU/g)
	Acc[a]	Ure[a]	Ge[b]	Am[b]	Ca[b]	Lp[b]	pH	NaCl	Temp.	PEG	
ATU–Bio 1											
RBST1–4	+	+	4.26	0.00	3.58	1.23	5–10	9	70	35	$4.44x10^9$
RCPT3–28	+	+	4.80	2.82	4.00	1.64	6–10	9	65	35	$8.35x10^8$
CH9–7	w	+	1.77	5.63	4.35	0.00	6–10	7	60	34	$2.31x10^8$
ATU–Bio 2											
CH5–8	+	-	1.71	0.00	3.10	0.00	6–9	7	60	32	$1.07x10^9$
RCPT1–4	+	+	2.20	1.55	2.43	2.05	6–10	6	60	35	$9.35x10^7$
RBST2–54	w	+	4.43	4.58	5.15	2.05	4–11	10	70	35	$1.11x10^9$
ATU–Bio 3											
RCPT1–4	+	+	2.20	1.55	2.43	2.05	6–10	6	60	35	$9.35x10^7$
RCPT3–40	+	+	4.50	3.79	3.91	2.03	6–11	9	65	35	$2.23x10^8$
RBST2–21	-	+	3.82	4.84	4.39	1.78	6–10	10	70	35	$8.00x10^7$

Note: Acc = 1–aminocyclopropane–1–carboxylate (ACC) deaminase, Ure = Urease, Ge = Gelatinase, Am = Amylase, Ca = Caseinase, Lp = Lipase.
All strains were able to grow in ISP 2 media supplemented with chemical fertilizer formula of 46–0–0, 0–0–60, 13–13–21, 25–7–7, 8–8–24 and 16–16–16, and fungicide 2.0% benzimidazole.

[a] Qualitative analysis: + = positive, w = weakly positive, - = negative

[b] Mean of the levels of enzymatic activity (LEA)

[c] pH range for growth and maximum growth in NaCl (% w/v), Temperature (°C), PEG 6000 (% w/v)

[d] Mean of the actinomycetes growth in solid–state fermentation

For shelf–life determination, at 0 months of actinomycete populations of all products were detected ranging from 2.36×10^9 to 4.47×10^9 CFU/g sample. At 4 months of preservation, the loads in ATU–Bio 1 rapidly decreased to 2.20×10^7 CFU/g samples, the loads in ATU–Bio 2 slightly decreased to 3.48×10^8 CFU/g samples, while the loads in ATU–Bio 3 stabled at 1.43×10^9 CFU/g samples. At 7 months, all actinomycete loads in all products were detected at 10^7 CFU/g samples. At 9 and 12 months, the actinomycete loads in all products decreased to 10^6 CFU/g samples. At the last time at 16 months, all actinomycete loads in all products remained 10^5 CFU/g samples (Table 4.18).

Survival actinomycetes in the product were affected by several variables such as actinomycete species, the physiological state of actinomycetes when harvested from the medium, and the use of protective materials (Date, 2001).

Land Development Department (LDD), Ministry of Agriculture and Cooperatives, Thailand set the standard of microbial population in Microbial super LDD (product of LDD) that shall not be less than 10^7 CFU/g samples (LDD, n.d.). In this study, the optimum time for product preservation was 7 months.

Table 4.18 The colony forming units (CFU) of the actinomycete products during preservation time (month).

Products	Colony forming units (CFU)/g sample					
	0 months	4 months	7 months	9 months	12 months	16 months
ATU–Bio 1	$2.36x10^{9}$±0.15	$2.20x10^{7}$±0.46	$1.62x10^{7}$±0.48	$1.93x10^{6}$±0.24	$1.83x10^{6}$±0.07	$3.24x10^{5}$±0.26
ATU–Bio 2	$3.92x10^{9}$±0.39	$3.48x10^{8}$±0.42	$2.01x10^{7}$±0.43	$1.47x10^{6}$±0.06	$1.15x10^{6}$±0.91	$2.16x10^{5}$±0.24
ATU–Bio 3	$4.47x10^{9}$±1.10	$1.43x10^{9}$±0.18	$3.05x10^{7}$±0.18	$2.69x10^{6}$±0.45	$2.56x10^{6}$±0.33	$2.92x10^{5}$±0.50

Not: Mean ± Standard deviation, N = 3

4.12 Field experiment from actinomycete products

4.12.1 Plant growth promotion experiment

The banana seedlings possessed approximately the same properties at the beginning of the experiment. After twelve weeks after being transplanted to the pots, the plant height, root length, stem diameter, and fresh and dry weight of banana seedlings treated with the actinomycete products group was remarkably higher than that of the control group (no supplement) and commercial PGPR–2 ($P < 0.05$) (Table 4.19).

Plant height after being treated with ATU–Bio 1 showed the highest by 20.53 cm compared with other treatments but was not significantly different with ATU–Bio 2 and commercial fertilizer (16–16–16). Banana root length after being treated with actinomycete products group ranging from 25.40 to 26.54 cm, which was significantly higher than the carrier and commercial fertilizer (16–16–16) ($P < 0.05$). Banana stem diameter using the actinomycete products group was ranging from 1.22 to 1.47 cm, which tended to have higher than the carrier and commercial fertilizer (16–16–16). Fresh and dry weights after being treated with ATU–Bio 1 and ATU–Bio 2 were 14.47, 14.62, and 1.58, 1.62 g, respectively, which were significantly higher than ATU Bio–3 and other treatments ($P < 0.05$). All experimental data were shown in Table 4.19 and Figure 4.50.

From the above experiments, all actinomycetes in products have investigated for the ability to produce macronutrients (nitrogen, phosphate, and potassium solubilization), phytohormone (IAA, gibberellins, and cytokinin), and siderophores, which act as inducers of plant metabolic processes (Moncaleán *et al*., 2002). Actinomycetes such as *S. olivaceoviridis*, *S. rimosus*, *S. rochei*, *S. griseoviridis*, and *S. lydicus* could improve plant growth by increasing seed germination, root elongation, and root dry weight by producing phytohormones and siderophores (Mahadevan and Crawford, 1997 ; Uphoff *et al*., 2009 ; Rungin *et al*., 2012).

Table 4.19 Effect of actinomycete products and tested materials on plant height, root length, stem diameter, fresh and dry weight of banana plantlets.

Treatments	Plant height (cm)	Root length (cm)	Stem diameter (cm)	Fresh weight (g)	Dry weight (g)
No supplement (control)	6.27 ± 1.25^{d}	9.37 ± 0.76^{c}	0.60 ± 0.20^{d}	3.77 ± 0.15^{d}	0.36 ± 0.01^{e}
Carrier (well compost)	13.40 ± 1.79^{bc}	19.14 ± 1.64^{ab}	1.11 ± 0.21^{abc}	10.74 ± 0.80^{b}	1.05 ± 0.01^{c}
Chemical fertilizer (16–16–16)	16.60 ± 1.64^{ab}	16.17 ± 1.61^{bc}	1.07 ± 0.25^{bc}	8.83 ± 0.47^{c}	0.87 ± 0.05^{d}
Commercial PGPR–2	10.10 ± 2.51^{cd}	16.26 ± 2.9^{bc}	0.78 ± 0.28^{cd}	7.90 ± 0.45^{c}	0.85 ± 0.02^{d}
ATU–Bio 1	20.53 ± 4.91^{a}	25.40 ± 1.83^{a}	1.47 ± 0.15^{a}	14.47 ± 0.46^{a}	1.58 ± 0.07^{a}
ATU–Bio 2	16.12 ± 3.94^{ab}	26.24 ± 5.93^{a}	1.24 ± 0.20^{ab}	14.62 ± 2.26^{a}	1.62 ± 0.09^{a}
ATU–Bio 3	14.76 ± 2.30^{b}	26.54 ± 3.93^{a}	1.22 ± 0.25^{ab}	12.04 ± 1.01^{b}	1.34 ± 0.07^{b}

Note: Mean ± Standard deviation, N = 5

Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT)

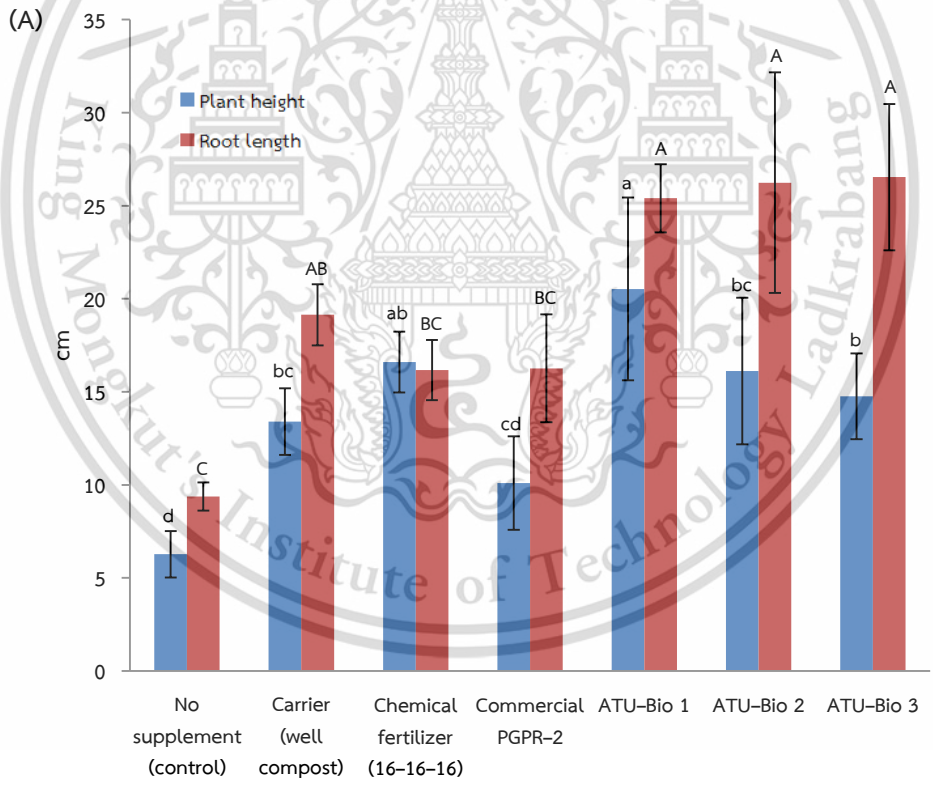


Figure 4.50 (A) Plant height and root length, (B) stem diameter and (C) fresh and dry weight of banana plantlets treated with different treatments. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation of five replications.

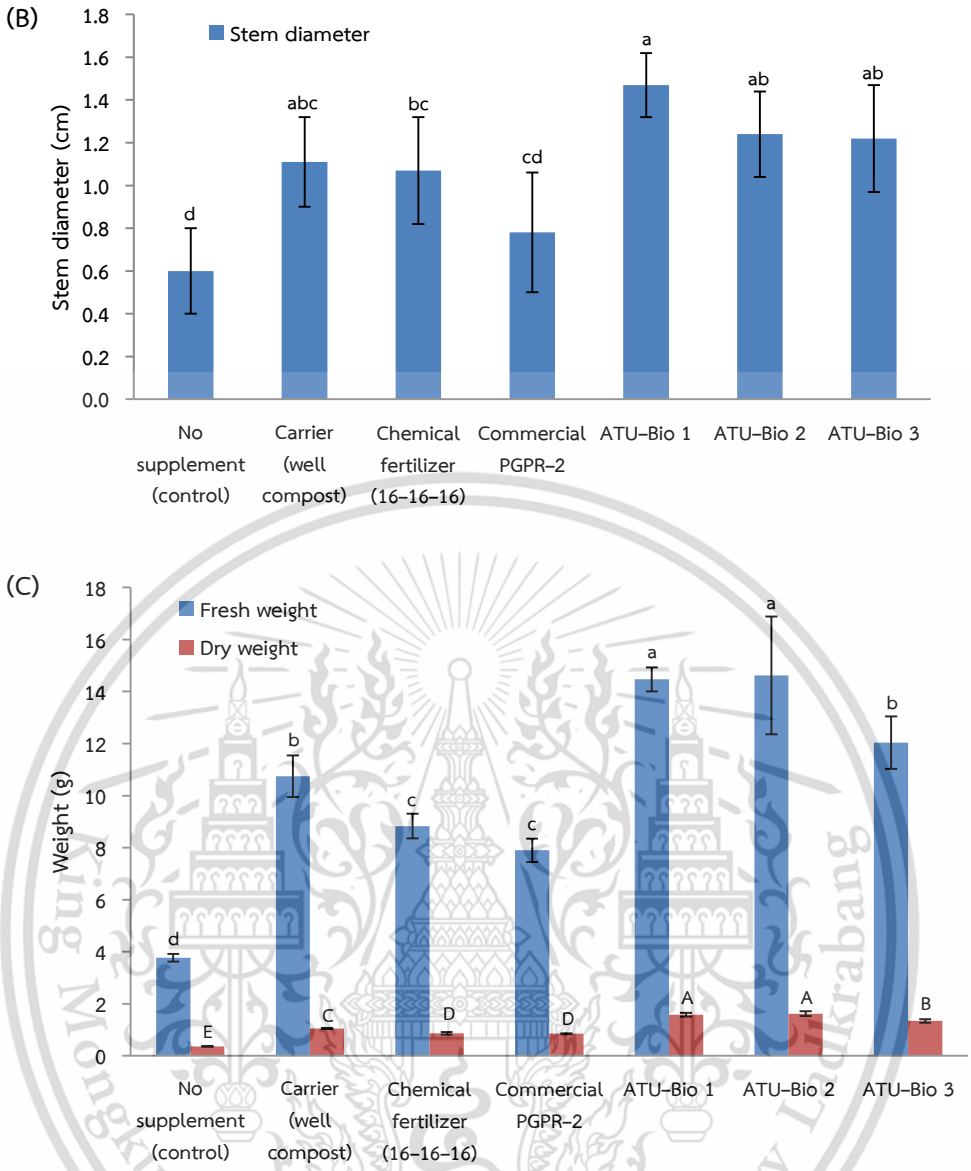


Figure 4.50 (A) Plant height and root length, (B) stem diameter and (C) fresh and dry weight of banana plantlets treated with different treatments. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) ($P<0.05$) are marked with different letters. Error bars indicate the standard deviation of five replications (continued).

Figure 4.51 Growth of banana plantlets treated with different treatments after 12 weeks, T1: No supplement (control), T2: Carrier (well compost), T3: Chemical fertilizer (16–16–16), T4: Commercial PGPR–2, T5: ATU–Bio 1, T6: ATU–Bio 2, T7: ATU–Bio 3.

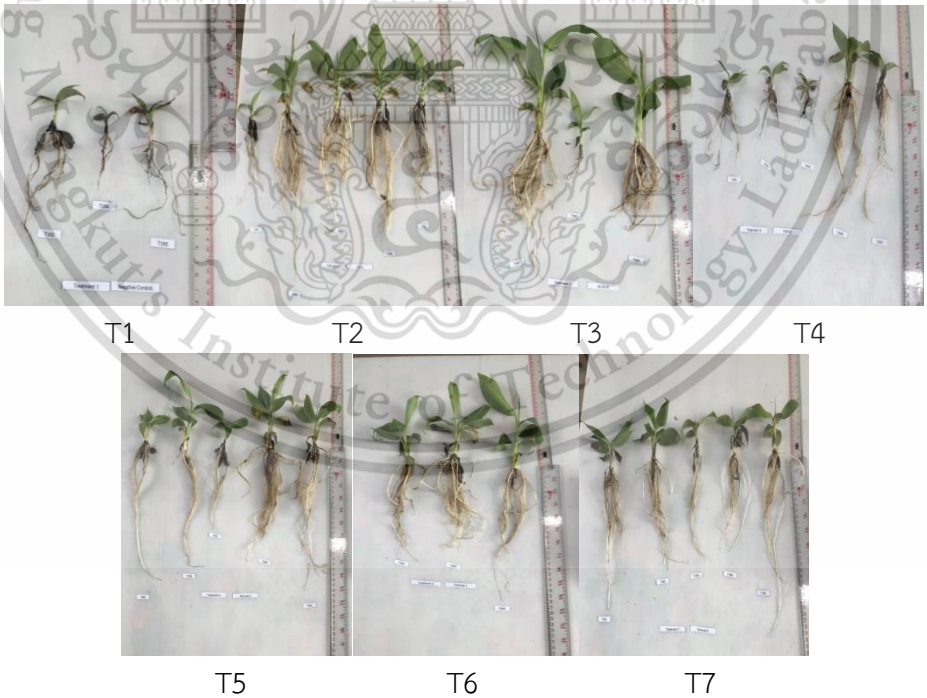


Figure 4.52 Plant height and root length of banana plantlets treated with different treatments after 12 weeks, T1: No supplement (control), T2: Carrier (well compost), T3: Chemical fertilizer (16–16–16), T4: Commercial PGPR–2, T5: ATU–Bio 1, T6: ATU–Bio 2, T7: ATU–Bio 3.

4.12.2 Plant growth promotion and disease control experiment

4.12.2.1 Protective experiment

At the last week, the number of survivors and dead banana plantlets after being inoculated with *Foc* were shown in Table 4.20. The results showed that more than 50% of banana plantlets were able to survive in all treatments. It was observed that the severity index (DSI) for LSI and RDI were significantly (*P < 0.05*) reduced in plantlets treated with the actinomycete products group compared to control (no supplement) and carrier, and tended to have better than other control agents (2% carbendazim and commercial *Trichoderma harzianum*) (Table 4.21, Figures 4.53–4.54, and Appendix E). For LSI, ATU–Bio 1 reduced the LSI on all *Fusarium*–inoculated plantlets ranging from 38.46 to 62.50%, ATU–Bio 2 ranging from 38.46 to 50.00%, and ATU–Bio 3 ranging from 30.77 to 50.00% compared to untreated plantlets (no supplement). For RDI, ATU–Bio 1 reduced the LSI on all *Fusarium*–inoculated plantlets ranging from 40.74 to 57.14%, ATU–Bio 2 ranging from 37.04 to 50.00%, and ATU–Bio 3 ranging from 34.62 to 46.15% compared to untreated plantlets (no supplement).

After twelve weeks after transplanting the banana plantlets to the pot filled with treatment–contained soils (factor A) and nine weeks after *Foc* inoculation (factor B), almost plant height, root length, stem diameter, fresh and dry weight of banana seedlings treated with actinomycete products group were remarkably higher than that of the control group (no supplement) and 2% carbendazim (*P < 0.05*) (Tables 4.22–4.23, Figures 4.55–4.59, and Appendix E).

Plant height with ATU–Bio 1 showed the highest ranging from 11.23 to 14.88 cm (except with *Foc* 8) compared with other treatments. In the actinomycete products group, ATU–Bio 1 was a significant difference in plant height from ATU–Bio 2 and 3 (Table 4.22, Figure 4.55, and Appendix E). Almost banana root length with actinomycete products group was higher than using the carrier and commercial *T. harzianum* (*P < 0.05*) (Table 4.22, Figure 4.56, and Appendix E). ATU–Bio 1 tended to have a root length higher than other actinomycete products.

Banana stem diameter using the actinomycete products group ranging from 0.87 to 1.25 cm, which was significantly higher than other treatments (*P < 0.05*) (Table 4.22, Figure 4.57, and Appendix E). Fresh and dry weight with actinomycete products group was higher than other treatments (*P < 0.05*) (Table 4.23, Figures 4.58–4.59, and Appendix E).

Overall data indicated that actinomycete products had the potential to protect *Foc* infection–causing Fusarium wilt disease in banana. For plant

growth promoters under *Foc* infection, most of the plant growth parameters were significantly lower than control (no *Foc*) ($P < 0.05$).

Table 4.20 Number of survival and dead banana plantlets after inoculation with *Foc* at 12 weeks in field protective experiment.

Treatments	*Foc* strains									
	Foc 1		*Foc* 6		*Foc* 7		*Foc* 8		No *Foc* (control)	
	Suvivor	Dead	Suvivor	Dead	Suvivor	Dead	Suvivor	Dead	Suvivor	Dead
No supplement (control)	3	1	3	1	2	2	3	1	4	0
Carrier (well compost)	3	1	3	1	3	1	3	1	3	0
2% Carbendazim	3	1	3	1	3	1	3	1	4	0
Commercial *Trichoderma harzianum*	2	2	2	2	3	1	3	1	4	0
ATU–Bio 1	3	1	3	1	3	1	3	1	4	0
ATU–Bio 2	2	2	3	1	2	2	3	1	4	0
ATU–Bio 3	3	1	3	1	3	1	2	2	4	0

Table 4.21 Leaf symptom index (LSI) and rhizome discoloration index (RDI) from the protective effect of actinomycete products with *Foc* on banana plantlets.

Treatments	LSI		RDI	
	Foc 8	No *Foc* (control)	*Foc* 8	No *Foc* (control)
No supplement (control)	3.00 ± 1.41^{b}	1.00 ± 0.50^{a}	6.50 ± 1.00^{D}	1.00 ± 0.00^{A}
Carrier (well compost)	3.00 ± 1.41^{b}	1.00 ± 0.00^{a}	5.50 ± 1.73^{CD}	1.00 ± 0.00^{A}
2% Carbendazim	3.00 ± 1.41^{b}	1.00 ± 0.00^{a}	4.75 ± 2.22^{BCD}	1.00 ± 0.00^{A}
Commercial *Trichoderma harzianum*	3.00 ± 0.82^{b}	1.00 ± 0.00^{a}	4.75 ± 1.71^{BCD}	1.00 ± 0.00^{A}
ATU–Bio 1	1.25 ± 0.50^{a}	1.00 ± 0.00^{a}	3.00 ± 1.41^{B}	1.00 ± 0.00^{A}
ATU–Bio 2	1.50 ± 0.58^{a}	1.00 ± 0.00^{a}	3.75 ± 1.71^{BC}	1.00 ± 0.00^{A}
ATU–Bio 3	1.75 ± 0.96^{a}	1.00 ± 0.00^{a}	4.25 ± 1.50^{BC}	1.00 ± 0.00^{A}

Note: Mean ± Standard deviation. Different letters indicated a significant difference at the $P<0.05$ levels by Duncan's new multiple range test (DMRT).

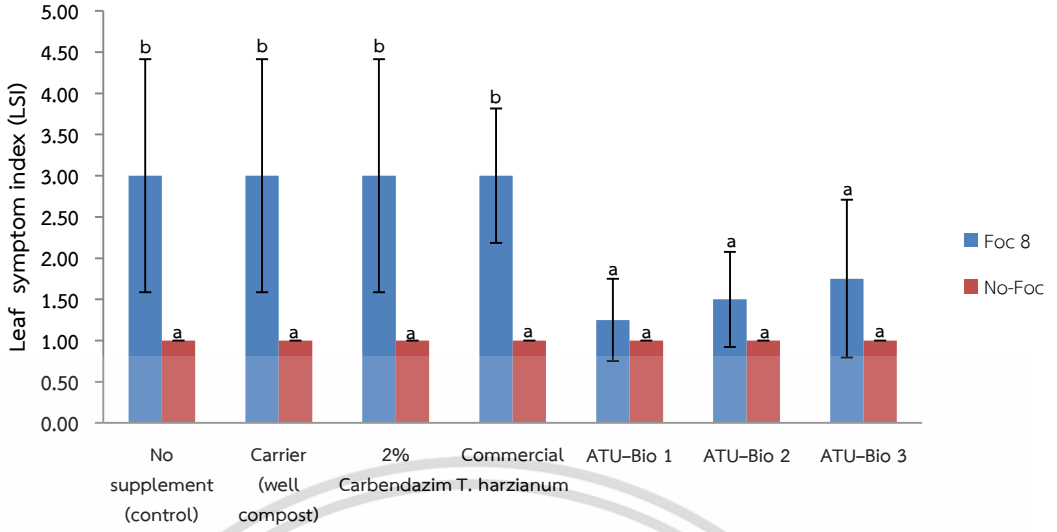


Figure 4.53 Leaf symptom index (LSI) from the protective effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation of four replications.

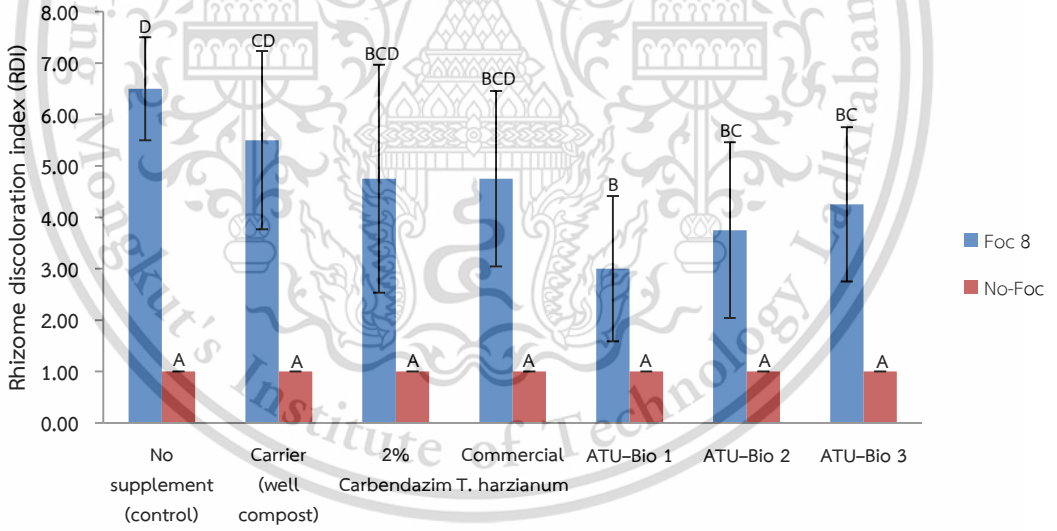


Figure 4.54 Rhizome discoloration index (RDI) from the protective effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation of four replications.

Table 4.22 Protective effect of actinomycete products with *Foc* on plant height, root length, and stem diameter of banana plantlets.

Treatments	Plant height (cm)		Root length (cm)		Stem diameter (cm)	
	Foc 8	No *Foc* (control)	*Foc* 8	No *Foc* (control)	*Foc* 8	No *Foc* (control)
No supplement (control)	4.30 ± 0.79^{f}	6.98 ± 0.21^{e}	4.90 ± 0.46^{f}	8.88 ± 0.44^{e}	0.47 ± 0.12^{f}	0.80 ± 0.12^{de}
Carrier (well compost)	9.43 ± 0.59^{d}	12.70 ± 1.31^{b}	11.60 ± 0.53^{d}	19.15 ± 2.22^{b}	0.67 ± 0.58^{ef}	1.03 ± 0.21$^{a–d}$
2% Carbendazim	7.20 ± 0.27^{e}	7.18 ± 0.26^{e}	11.30 ± 0.63^{c}	12.08 ± 0.94^{d}	0.90 ± 0.17$^{b–e}$	0.80 ± 0.10^{de}
Commercial *Trichoderma harzianum*	7.43 ± 0.15^{e}	9.80 ± 1.10^{d}	11.97 ± 0.25^{d}	13.55 ± 1.06^{d}	0.83 ± 0.12$^{c–e}$	0.85 ± 0.13$^{b–e}$
ATU–Bio 1	11.23 ± 0.90^{c}	14.88 ± 0.46^{a}	19.50 ± 1.80^{b}	22.58 ± 1.10^{a}	0.87 ± 0.21$^{b–e}$	1.25 ± 0.19^{a}
ATU–Bio 2	10.73 ± 1.36^{cd}	13.18 ± 1.08^{b}	16.30 ± 0.79^{c}	22.58 ± 1.31^{a}	1.03 ± 0.06$^{a–d}$	1.10 ± 0.18^{ab}
ATU–Bio 3	12.00 ± 0.71^{bc}	12.75 ± 0.44^{b}	17.95 ± 0.35^{bc}	22.60 ± 2.05^{a}	1.05 ± 0.07$^{a–d}$	1.08 ± 0.10^{abc}

Note: Mean ± Standard deviation. Different letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

Table 4.23 Protective effect of actinomycete products with *Foc* on fresh and dry weight of banana plantlets.

Treatments	Fresh weight (g)		Dry weight (g)	
	Foc 8	No *Foc* (control)	*Foc* 8	No *Foc* (control)
No supplement (control)	1.17 ± 0.15^{h}	2.85 ± 0.34^{g}	0.12 ± 0.01^{e}	0.31 ± 0.04^{d}
Carrier (well compost)	3.27 ± 0.25^{fg}	4.95 ± 0.74^{cd}	0.34 ± 0.34^{cd}	0.51 ± 0.10^{b}
2% Carbendazim	3.87 ± 0.42^{ef}	4.38 ± 0.21^{de}	0.39 ± 0.03^{c}	0.41 ± 0.01^{c}
Commercial *Trichoderma harzianum*	3.50 ± 0.30^{f}	4.80 ± 0.41^{cd}	0.35 ± 0.01^{cd}	0.50 ± 0.06^{b}
ATU–Bio 1	4.80 ± 0.35^{cd}	7.05 ± 0.42^{a}	0.49 ± 0.04^{b}	0.66 ± 0.02^{a}
ATU–Bio 2	4.83 ± 0.21^{cd}	6.53 ± 0.46^{ab}	0.48 ± 0.01^{b}	0.62 ± 0.02^{a}
ATU–Bio 3	5.10 ± 0.14^{c}	6.13 ± 0.22^{b}	0.51 ± 0.01^{b}	0.60 ± 0.02^{a}

Note: Mean ± Standard deviation. Different letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

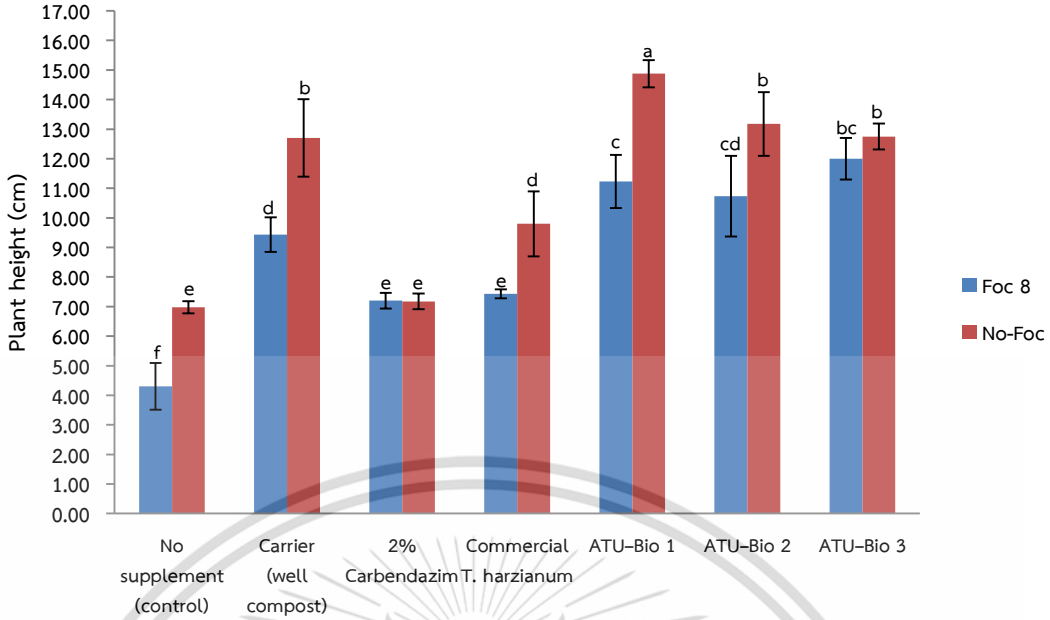


Figure 4.55 Plant height (cm) of banana plantlets from the protective effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

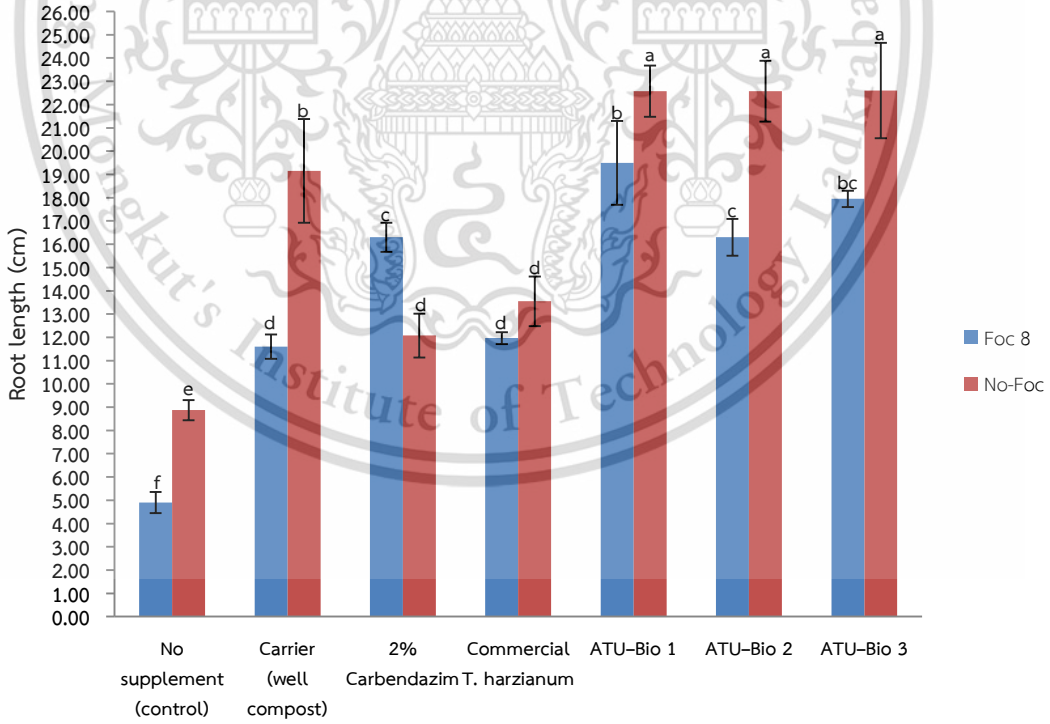


Figure 4.56 Root length (cm) of banana plantlets from the protective effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

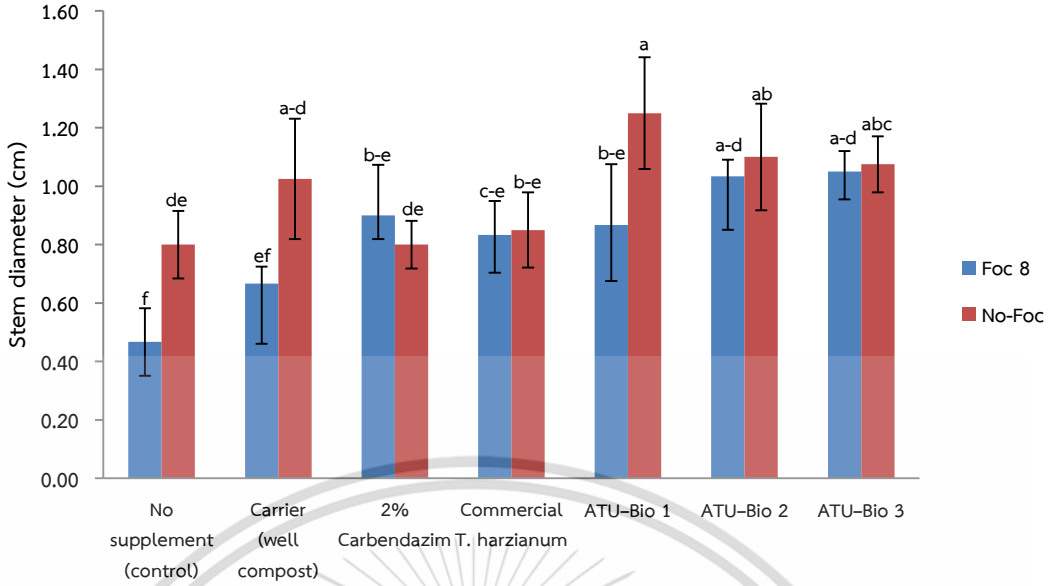


Figure 4.57 Stem diameter (cm) of banana plantlets from the protective effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

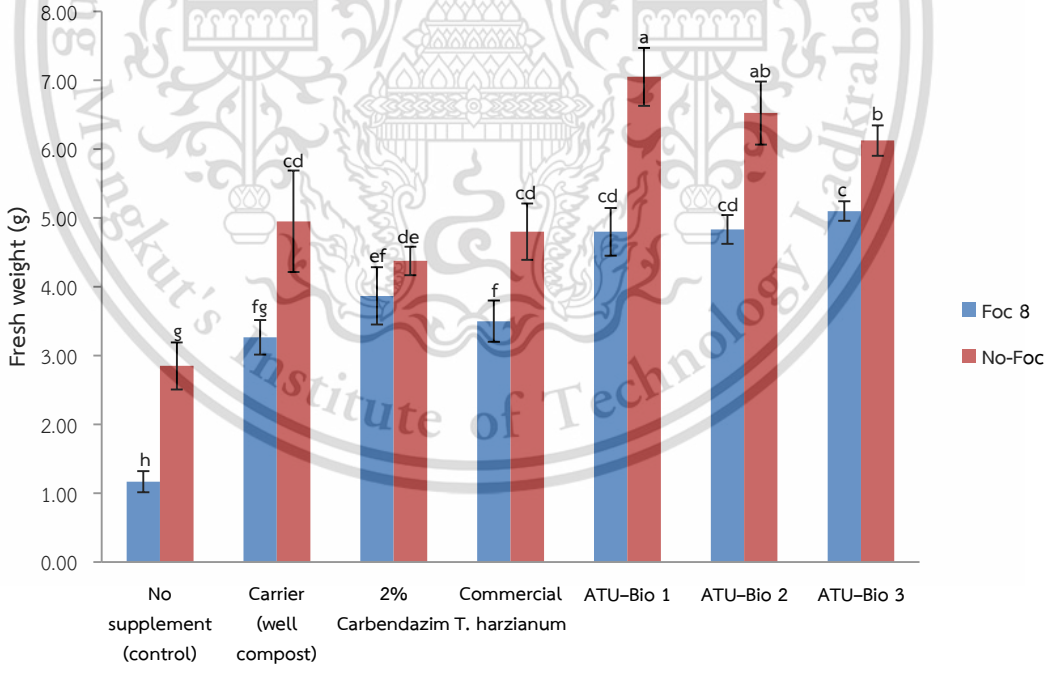


Figure 4.58 Fresh weight (g) of banana plantlets from the protective effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

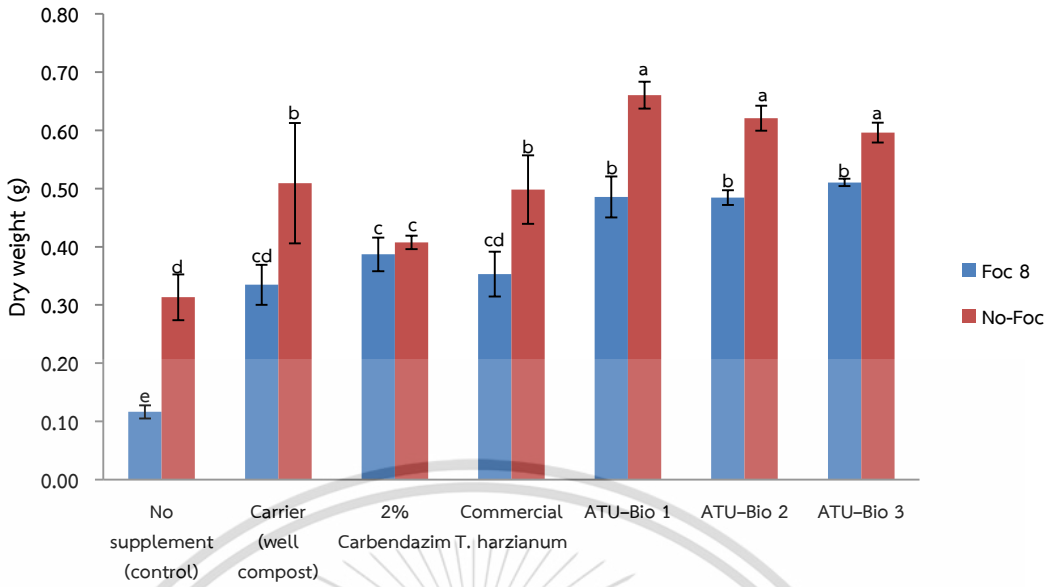


Figure 4.59 Dry weight (g) of banana plantlets from the protective effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

Control (no supplement) Healthy plantlet Infected plantlet

Figure 4.60 Leaf symptoms of banana plantlets in the protective experiment.

Healthy plantlet Infected plantlet

Figure 4.61 Rhizome symptoms of banana plantlets in the protective experiment.

Healthy plantlet Infected plantlet

Figure 4.62 Stem and root of banana plantlets in the protective experiment.

4.12.2.2 Curative experiment

In the last week, the number of survival and dead banana plantlets after being inoculated with *Foc* was shown in Table 4.24. The results showed that 46.43% (65 of 140) of banana plantlets were able to survive in all treatments. It was observed that the severity index (DSI) for LSI and RDI were not significantly different between treatments (Table 4.25, Figures 4.64–4.65, and Appendix E). In details of LSI, ATU–Bio 1 reduced the LSI on *Fusarium*–inoculated plantlets ranging from 0.00 to 21.05%, ATU–Bio 2 ranging from 0.00 to 15.00%, and ATU–Bio 3 ranging from 0.00 to 10.53% compared to untreated plantlets (no supplement). For RDI, ATU–Bio 1 reduced the LSI on all *Fusarium*–inoculated plantlets ranging from 0.00 to 25.00%, ATU–Bio 2 ranging from 0.00 to 9.38%, and ATU–Bio 3 ranging from 0.00 to 6.45% compared to untreated plantlets (no supplement).

After immersion of *Foc* strains (factor B) to banana root plantlets, treatments (factor A) were added into a pot nearly plantlets root and soil after it showed the light yellow coloring of the lower leaves or wilting. Almost plant height, root length, stem diameter, and fresh and dry weight of remaining banana seedlings after being treated with actinomycete products group tended to have higher than other treatments (Tables 4.26–4.27, Figures 4.66–4.70, and Appendix E). Overall data indicated that actinomycete products had a poor–efficiency capacity to cure *Foc*–infection causing Fusarium wilt disease in banana. For plant growth promoters under *Foc*infection, all of the plant growth parameters were lower than the control (no *Foc*).

Infected plantlet Dead plantlet

Figure 4.63 Leaf symptoms of banana plantlets in the curative experiment.

Table 4.24 Number of survival and dead banana plantlets after inoculation with *Foc* at 12 weeks in field curative experiments.

Treatments	*Foc* strains									
	Foc 1		*Foc* 6		*Foc* 7		*Foc* 8		No *Foc* (control)	
	Suvivor	Dead	Suvivor	Dead	Suvivor	Dead	Suvivor	Dead	Suvivor	Dead
No supplement (control)	1	3	0	4	1	3	1	3	4	0
Carrier (well compost)	1	3	1	3	2	2	0	4	4	0
2% Carbendazim	3	1	1	3	2	2	2	2	4	0
Commercial *Trichoderma harzianum*	2	2	1	3	0	4	2	2	4	0
ATU–Bio 1	1	3	3	1	3	1	0	4	4	0
ATU–Bio 2	0	4	2	2	1	3	2	2	4	0
ATU–Bio 3	0	4	1	3	2	2	2	2	4	0

Table 4.25 Leaf symptom index (LSI) and rhizome discoloration index (RDI) from the curative effect of actinomycete products with *Foc* on banana plantlets.

Treatments	LSI		RDI	
	Foc 8	No *Foc* (control)	*Foc* 8	No *Foc* (control)
No supplement (control)	4.75 ± 0.50^{b}	1.00 ± 0.00^{a}	7.75 ± 0.50^{B}	1.00 ± 0.00^{A}
Carrier (well compost)	5.00 ± 0.00^{b}	1.00 ± 0.00^{a}	8.00 ± 0.00^{B}	1.00 ± 0.00^{A}
2% Carbendazim	4.25 ± 0.96^{b}	1.00 ± 0.00^{a}	7.25 ± 0.96^{B}	1.00 ± 0.00^{A}
Commercial *Trichoderma harzianum*	4.25 ± 1.00^{b}	1.00 ± 0.00^{a}	7.25 ± 0.96^{B}	1.00 ± 0.00^{A}
ATU–Bio 1	5.00 ± 0.00^{b}	1.00 ± 0.00^{a}	8.00 ± 0.00^{B}	1.00 ± 0.00^{A}
ATU–Bio 2	4.25 ± 0.96^{b}	1.00 ± 0.00^{a}	7.25 ± 0.96^{B}	1.00 ± 0.00^{A}
ATU–Bio 3	4.25 ± 0.96^{b}	1.00 ± 0.00^{a}	7.25 ± 0.96^{B}	1.00 ± 0.00^{A}

Note: Mean ± Standard deviation. Different letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

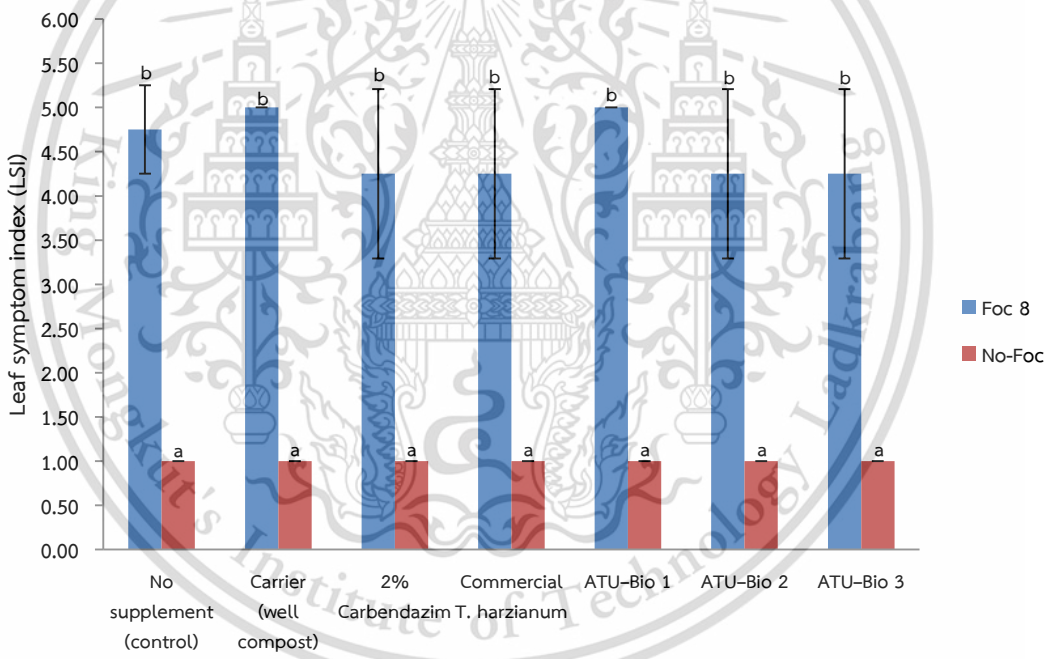


Figure 4.64 Leaf symptom index (LSI) from the curative effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation of four replications.

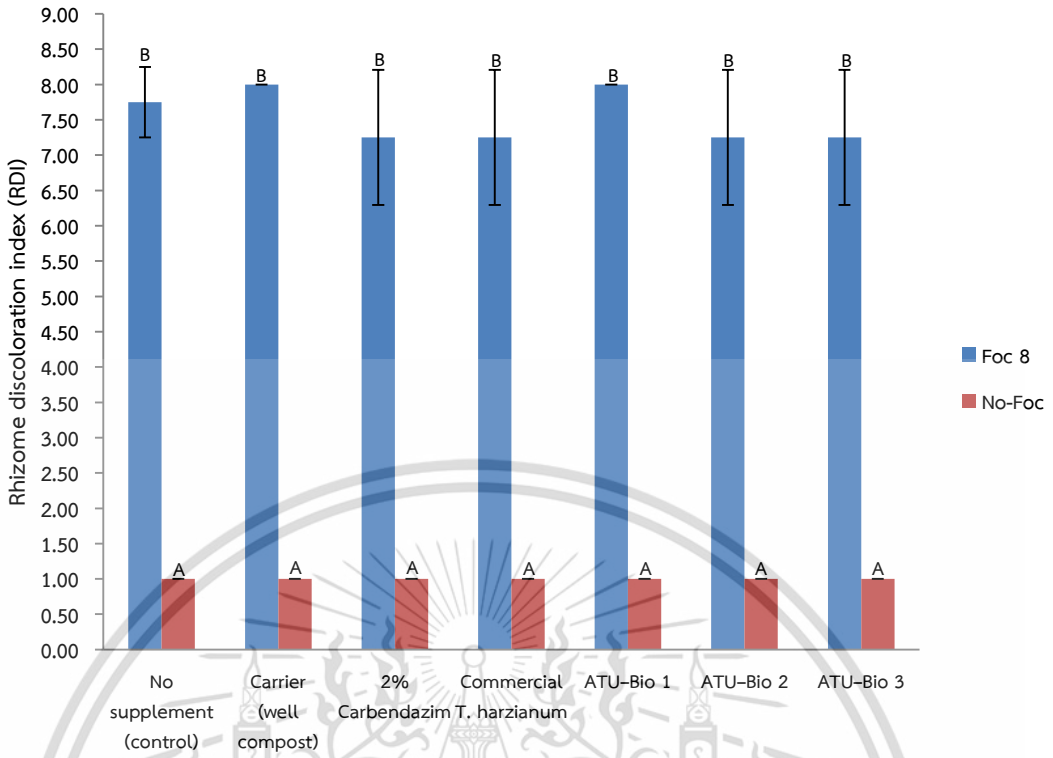


Figure 4.65 Rhizome discoloration index (RDI) from the curative effect of actinomycete products with *Foc* on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) ($P<0.05$) are marked with different letters. Error bars indicate the standard deviation of four replications.

Table 4.26 Curative effect of actinomycete products with *Foc* on plant height, root length, and stem diameter of remaining banana plantlets.

Treatments	Plant height (cm)		Root length (cm)		Stem diameter (cm)	
	Foc 8	No *Foc* (control)	*Foc* 8	No *Foc* (control)	*Foc* 8	No *Foc* (control)
No supplement (control)	2.50 ± 0.00	6.65 ± 0.39	2.90 ± 0.00	8.73 ± 0.49	0.30 ± 0.00	0.80 ± 0.08
Carrier (well compost)	0.00 ± 0.00	12.43 ± 0.94	0.00 ± 0.00	18.38 ± 1.60	0.00 ± 0.00	0.90 ± 0.18
2% Carbendazim	4.00 ± 0.71	7.08 ± 0.18	4.95 ± 0.50	11.98 ± 0.87	0.40 ± 0.14	0.88 ± 0.10
Commercial *Trichoderma harzianum*	4.00 ± 0.14	9.65 ± 0.94	5.00 ± 0.28	13.60 ± 0.91	0.45 ± 0.07	0.88 ± 0.05
ATU–Bio 1	0.00 ± 0.00	14.43 ± 0.56	0.00 ± 0.00	22.20 ± 0.48	0.00 ± 0.00	1.15 ± 0.13
ATU–Bio 2	3.85 ± 0.21	13.05 ± 0.81	5.35 ± 0.50	22.48 ± 1.21	0.55 ± 0.07	1.05 ± 0.17
ATU–Bio 3	3.80 ± 0.14	12.78 ± 0.53	5.10 ± 0.14	22.55 ± 2.14	0.50 ± 0.00	1.10 ± 0.08

Note: Mean ± Standard deviation.

Table 4.27 Curative effect of actinomycete products with *Foc* on fresh and dry weight of remaining banana plantlets.

Treatments	Fresh weight (g)		Dry weight (g)	
	Foc 8	No *Foc* (control)	*Foc* 8	No *Foc* (control)
No supplement (control)	1.10 ± 0.00	2.78 ± 0.34	0.09 ± 0.00	0.31 ± 0.04
Carrier (well compost)	0.00 ± 0.00	4.90 ± 0.59	0.00 ± 0.00	0.50 ± 0.09
2% Carbendazim	1.25 ± 0.21	4.28 ± 0.28	0.13 ± 0.03	0.40 ± 0.02
Commercial *Trichoderma harzianum*	1.35 ± 0.07	4.65 ± 0.33	0.14 ± 0.01	0.48 ± 0.05
ATU–Bio 1	0.00 ± 0.00	6.98 ± 0.33	0.00 ± 0.00	0.65 ± 0.01
ATU–Bio 2	1.45 ± 0.85	6.70 ± 0.85	0.15 ± 0.06	0.64 ± 0.06
ATU–Bio 3	1.45 ± 0.07	6.13 ± 0.15	0.15 ± 0.01	0.60 ± 0.02

Note: Mean ± Standard deviation.

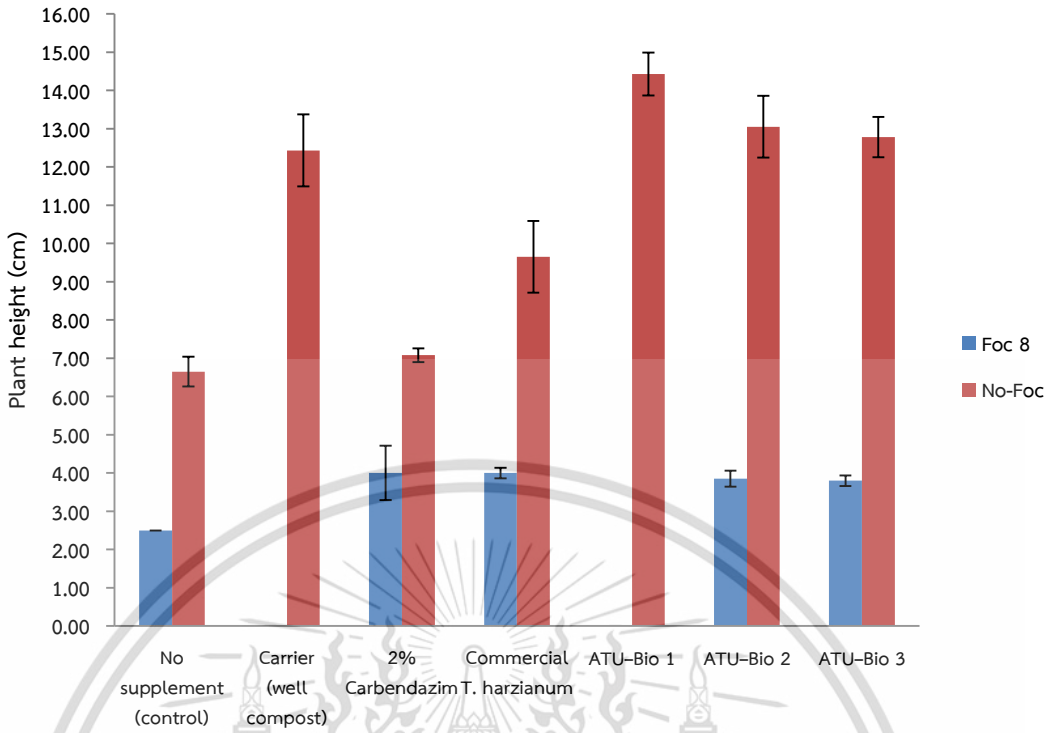


Figure 4.66 Plant height (cm) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* on banana plantlets. Error bars indicate the standard deviation.

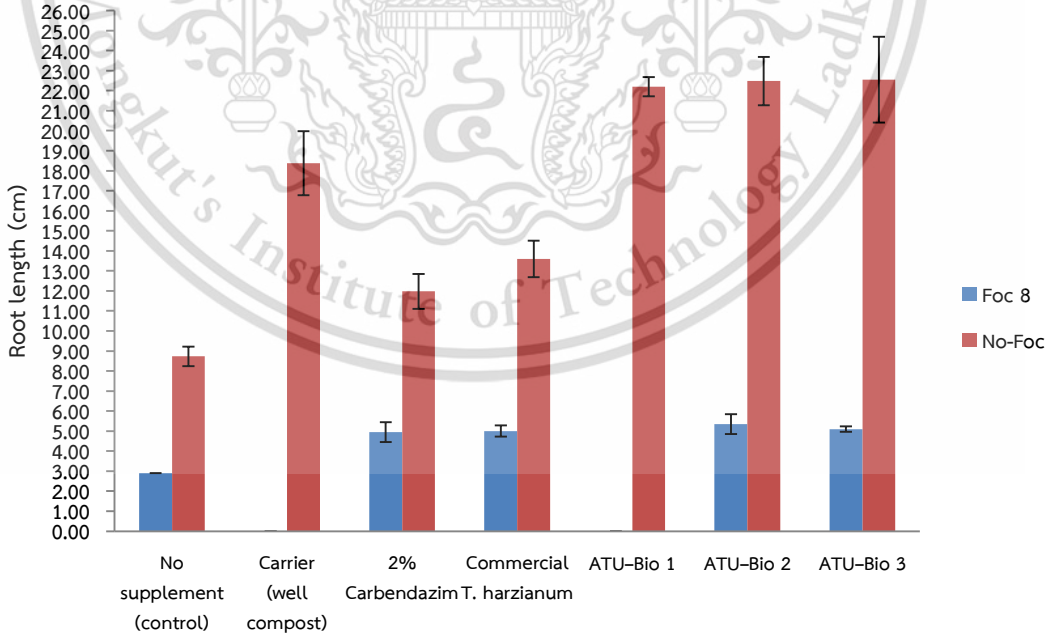


Figure 4.67 Root length (cm) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* on banana plantlets. Error bars indicate the standard deviation.

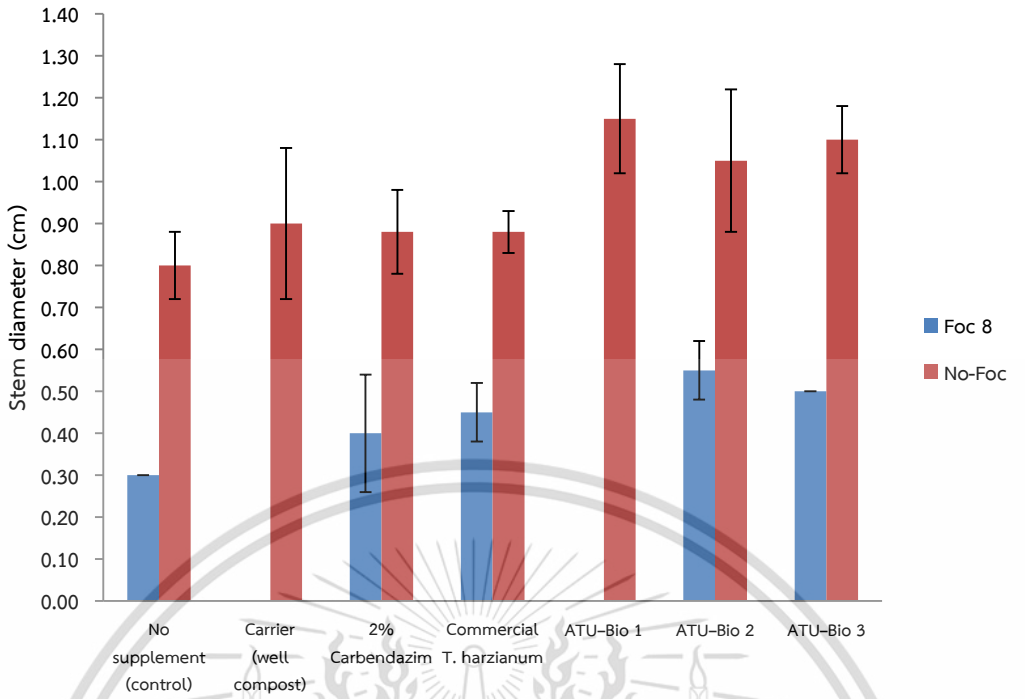


Figure 4.68 Stem diameter (cm) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* on banana plantlets. Error bars indicate the standard deviation.

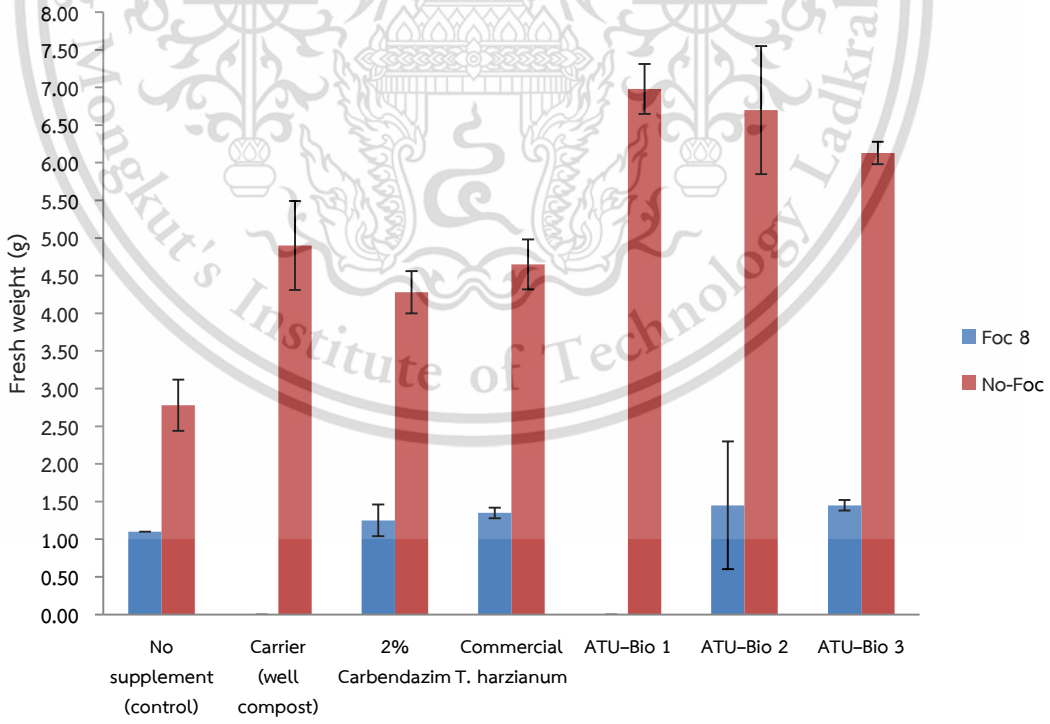


Figure 4.69 Fresh weight (g) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* on banana plantlets. Error bars indicate the standard deviation.

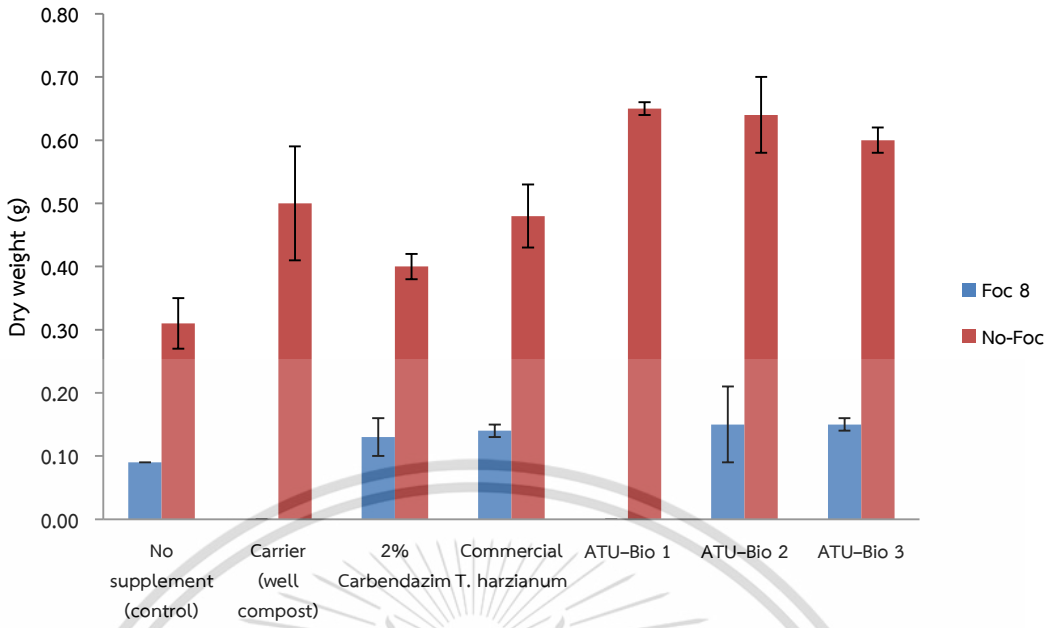


Figure 4.70 Dry weight (g) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* on banana plantlets. Error bars indicate the standard deviation.

From the protective and curative experiments, the protective evidence presented here suggests a possible biological control for Fusarium wilt of banana. According to Marois (1990), it may be possible to establish antagonistic microbial communities that would increase the buffering capacity of soils against subsequent *Foc* aggression in areas where the pathogen has not yet occurred. However, this way may not be suitable in soils where the pathogen is already established. In such cases, it would be better to protect the infection sites rather than alter the entire soil microbial community. The host plant provides the habitat and nutrients necessary for the control agent to become established at the infection site. Since actinomycetes colonize soil or rhizosphere externally to plant tissues, the critical step in achieving active disease control would be to target the pathogen before it gains entry into the corm or root tissues. Many researchers have shown that actinomycetes can control *Foc* in banana *in planta* experiment. Cao *et al*. (2005) showed that the disease severity index (DSI) was significantly ($P = 0.05$) reduced, while mean fresh weight was increased ($P = 0.001$) in banana plantlets treated with *S. griseorubiginosus* S96 compared with those grown in the absence of the biocontrol strain. Getha *et al*. (2005) reported that the final disease severity index for leaf symptom (LSI) and rhizome discoloration (RDI) was reduced by about 47 and 53%, respectively after being treated with *Streptomyces* sp. strain g10 in banana plantlets

compared with untreated plantlets. According to the experiment of Chen *et al*. (2018b), the incidence of banana seedlings was reduced after using *Streptomyces* sp. CB–75 treatment with disease index of 10.23. The prevention and control effect was 83.12% and it also had a growth–promoting effect on banana plants. The fermentation broth of *Streptomyces* sp. strain JBS5–6 significantly reduced the disease index of banana seedlings by inhibiting the infection of *Foc* TR4 with a 64.94% inhibition percentage in a pot experiment (Jing *et al*., 2020). The fermentation broth of *Streptomyces* sp. H3–2 also strong significantly controlled Fusarium wilt disease and promoted the growth of banana seedlings including stem diameter, plant height, leaf area, chlorophyll content, and dry weight in comparison to the *Foc* TR4 treatment group (Zou *et al*., 2021) that similar to the experiment of Duan *et al*. (2020). Li *et al*. (2021) reported that the crude extract of *Streptomyces* sp. H4 inhibited mycelial growth, spore germination, and tube elongation of *Foc* TR4. The fermentation broth of H4 significantly decreased the disease index of *Foc* TR4 and improved the growth of banana seedlings in pot test.

4.13 Evaluation of the mechanism against *Foc*

4.13.1 Extracellular enzyme production

Three selected isolates were able to produce enzyme chitinase with the highest activity in RCPT1–4. Only two strains, RCPT1–4 and CH5–8, produced the enzyme CMCase. None of the isolates was able to produce avicelase (Table 4.28).

Table 4.28 Levels of enzymatic activity (LEA) related to the fungal cell wall of the selected actinomycetes.

Isolates	Levels of enzymatic activity (LEA)		
	Chitinase	CMCase	Avicelase
RCPT1–4	2.00±0.18	3.23±0.02	0.00±0.00
CH5–8	1.15±0.09	3.18±0.00	0.00±0.00
CH9–7	1.30±0.05	0.00±0.00	0.00±0.00

Note: Mean ± Standard deviation, N = 3

One of antifungal mechanism has been attributed to the action of hydrolytic extracellular enzymes such as chitinase, β–1, 3–glucanase, β–1, 4–glucanase, and protease (Macagnan *et al*., 2008). Production of these enzymes by microbes was related to fungal growth inhibition and biocontrol of fungal phytopathogens due to the cell wall degrading enzyme activity. Fungal cell wall lysis by microbe leads to leakage of cell contents and collapse of the fungal cell wall

(Bressan, 2003). Interestingly, strain RCPT1–4, CH5–8, and CH9–7 were able to produce chitinase and proteinase, while only RCPT1–4 and CH5–8 were able to produce β–1, 4–endoglucanase in form of CMCase. Zacky and Ting (2013) reported *Streptomyces griseus* (St 4) was able to produce chitinase and β–1, 3–glucanase to lysis of *Foc* cell wall and inhibit sporulation. *Streptomyces* spp. from rhizospheric soil showed enzyme activity of cellulase, chitinase, protease, and β–1, 3–glucanase against *Fusarium oxysporum* causing Fusarium wilt disease (Chaiharn *et al*., 2018). Chitinase and β–1, 3–glucanase from *Streptomyces* BITDG-11 lead to the destruction of cell wall and inhibit the growth of *Foc* (Zhang *et al*., 2021).

4.13.2 Secondary metabolite production

4.13.2.1 Isolation, purification, structure elucidation, and anti–*Foc* activity of the secondary metabolite from strain CH5–8

The EtOAc crude extract from strain CH5–8 was isolated using column chromatography by Sephadex LH20, eluted with 100% MeOH. The crude extract was separated into five fractions (F1–F5) based on thin layer chromatography technique (stationary phase: TLC silica gel 60 F_{254} plates and mobile phase: methanol : chloroform, 1 : 9.

Only the fraction 2 (F2) exhibited antifungal activity against *Foc* (*Foc* 2–*Foc* 8) with an inhibition zones at the concentration of 100 µg/disc ranging from 11.83 to 20.10 mm (Table 4.30). This fraction was purified by using Sephadex LH20 column eluted with 100% MeOH to give three fractions (F2F1–F2F3). All three fractions were then tested for anti–*Foc* activity. The results showed that only F2H2 displayed anti-*Foc* activity with the inhibition zones at the concentration of 100 µg/disc ranging from 9.00 to 23.33 mm (Table 4.30).

The fraction F2F2 composed of one interesting peak that was determined by HPLC at RT 6.742 (peak A). The UV profiles of all peak was compared with BIOTEC's in–house database. The chemical profiles were shown in Figures 4.71–4.72.

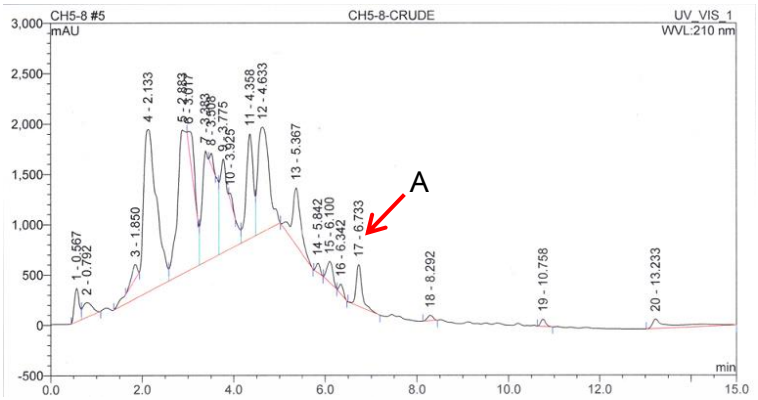


Figure 4.71 Chemical profiles of EtOAc crude extract from the strain CH5–8.

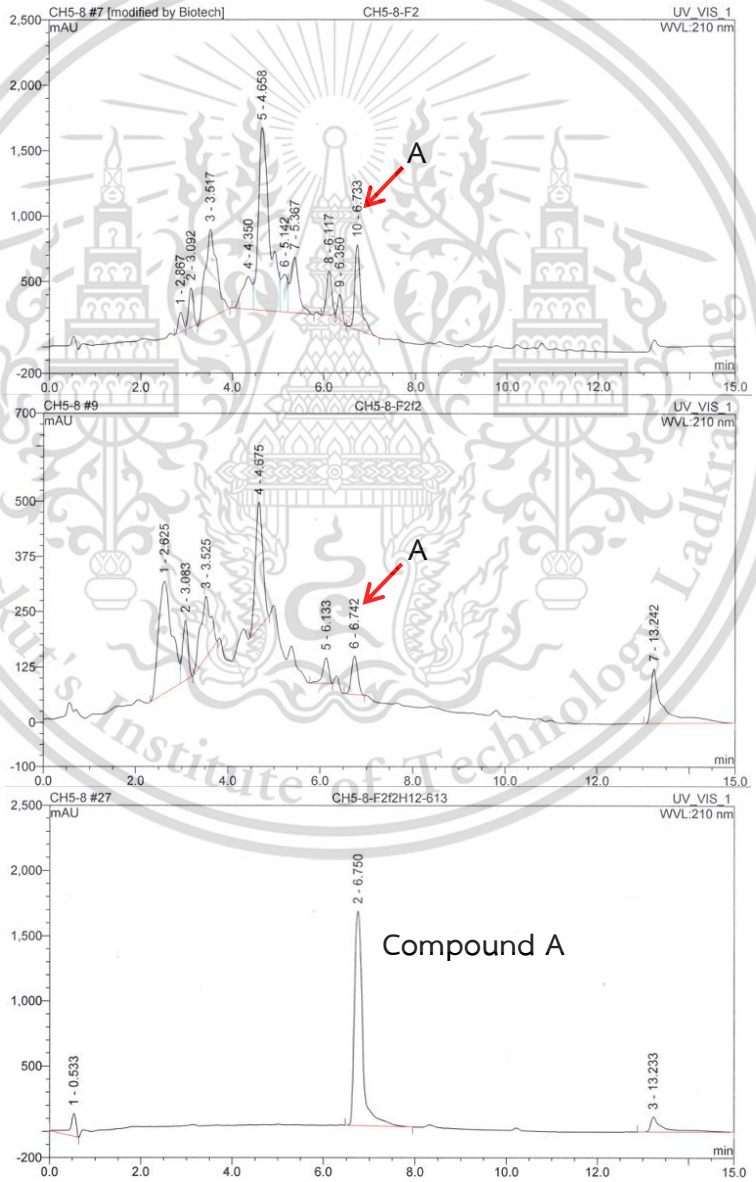


Figure 4.72 Chemical profiles of fraction F2, F2F2 crude extract, and compound A (F2F2H12) from the strain CH5–8.

The fraction F2F2 was purified by semi–preparative HPLC to give one interesting compound (compound A, CH5–8 F2F2H12). The chemical structure of compound A (CH5–8 F2F2H12) was elucidated by NMR spectral analysis.

Compound A (CH5–8 F2F2H12)	:	Yellow viscous liquid
^{1}H NMR (DMSO–d_6, 400 MHz) (Appendix F, Figure 1)	:	δ_H 0.84 (12–CH_3), 0.97-1.07 (H–11), 1.14 (13–CH_3), 1.43–1.53 (H–12), 1.39 (4–CH_3), 1.55 (8–CH_3), 1.74–1.85 and 2.02–2.10 (H–10), 3.16 (7–OCH_3), 4.05 (H–7), 4.46 (H–13), 5.21 (H–9), 5.62 (H–6), 5.69 (H–5), 5.72 (H–2), 6.86 (H–3)
^{13}C NMR (DMSO–d_6, 100 MHz) (Appendix F, Figure 2)	:	δ_C 13.5 (8–CH_3), 15.0 (12–CH_3), 17.4 (13–CH_3), 26.8 (C–10), 26.5 (4–CH_3), 33.6 (C–11), 38.1 (C–12), 56.1 (7–OCH_3), 71.9 (C–4), 74.7 (C–13), 84.3 (C–7), 114.3 (C–2), 127.6 (C–9), 129.5 (C–6), 136.5 (C–5), 136.8 (C–8), 156.7 (C–3), 165.7 (C–1)
HRESIMS spectrum (Appendix F, Figure 3)	:	$C_{18}H_{28}O_4$, *m/z* 331.19 $[M+Na]^+$

The spectroscopic data were identical to those reported for albocycline in the literature (Nagahama *et al*., 1971 ; Thomas and Chidester, 1982 ; Harada *et al*., 1984 ; Gu *et al*., 2009). Thus compound A was identified as albocycline (Table 4.29).

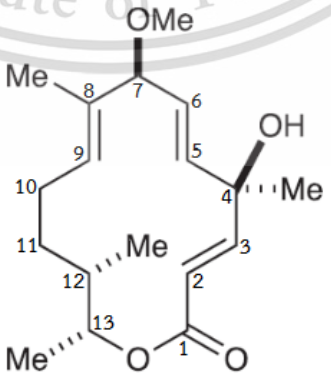


Figure 4.73 Chemical structure of compound A (CH5–8 F2F2H12).

Table 4.29 ^{1}H and ^{13}C NMR data of compound A (CH5–8 F2F2H12) in DMSO–d_6 and albocycline in chloroform–*d* ($CDCl_3$).

Position	Compound A (CH5–8 F2F2H12)		Albocycline (Ohike *et al.*, 2018)	
	δ_C (ppm)[a]	δ_H(ppm)[b], multiplicity, (*J* in Hz)	δ_C (ppm)[c]	δ_H(ppm)[d], multiplicity, (*J* in Hz)
1	165.7	-	166.3	-
2	114.3	5.72, *d* (15.4)	115.3	5.83, *d* (15.4)
3	156.7	6.86, *d* (15.4)	154.8	6.84, *d* (15.5)
4	71.9	-	73.0	-
5	136.5	5.69, *d* (16.1)	135.9	5.73, *dd* (0.5, 16.2)
6	129.5	5.62, *dd* (5.7, 16.1)	130.6	5.61, dd (6.2, 16.2)
7	84.3	4.05, *d* (5.7)	84.8	4.03, *d* (6.2)
8	136.8	-	136.5	-
9	127.6	5.21, *t* (6.3)	129.1	5.24, *t* (6.3)
10	26.8	1.74–1.85, *m* 2.02–2.10, *m*	24.6	1.80, *m*
11	33.6	0.97–1.07, *m*	34.2	1.13, *m*
12	38.1	1.43–1.53, *m*	39.0	1.40, *m*
13	74.7	4.46, *dq* (6.3, 8.3)	75.5	4.51, *dq* (6.4, 8.5)
4–CH_3	26.5	1.39, *s*	26.9	1.49, *s*
7–OCH_3	56.1	3.16, *s*	56.9	3.26, *s*
8–CH_3	13.5	1.55, *s*	13.9	1.60, *s*
12–CH_3	15.0	0.84, *d* (6.6)	15.6	0.84, *d* (6.9)
13–CH_3	17.4	1.14, *d* (6.4)	17.8	1.17, *d* (6.4)

[a]100 MHz, [b]400 MHz, [c]150 MHz, [d]600 MHz

Anti–*Foc* activity against *Foc* 1 – *Foc* 8 of albocycline (compound A, CH5–8 F2F2H12) was measured and compared to the references of antifungal agents, which were cycloheximide, nystatin, and benzimidazole (carbendazim). The results showed that all *Foc* strains were inhibited by albocycline (compound A, CH5–8 F2F2H12) with the inhibition zone ranging from 13.83 to 34.00 mm (Table 4.30). Compared with the control, albocycline (compound A, CH5–8 F2F2H12) displayed a larger inhibition zone than control, except *Foc* 7 and *Foc* 1 with cycloheximide and benzimidazole (carbendazim) (Table 4.30). The result indicated that albocycline (compound A, CH5–8 F2F2H12) had good anti–*Foc* activity.

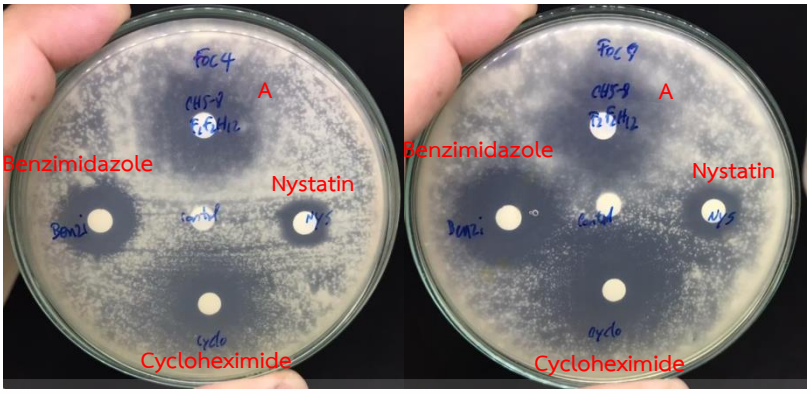


Figure 4.74 Anti–*Foc* activity of compound A (F2F2H12) from strain CH5–8.

Albocycline is a macrocyclic lactone antibiotic with a 14–membered macrocyclic ring without any carbohydrate substituent (Thomas and Chidester, 1982). It was previously isolated from various actinomycetes such as *Streptomyces bruneogriseus* (Nagahama *et al*., 1967), *S. roseocinereus* (Furumai *et al*., 1968), *S. roseochromogenes* (Furumai *et al*., 1968), *S. maizeus* (Reusser, 1969 ; Liang *et al*., 2018), *Propionicimonas* sp. ENT–18 (Zucchi *et al*., 2014), *Streptomyces* strain AR10 (Ohike *et al*., 2018), and *Streptomyces* strain 4205 (Gu *et al*., 2019) with potent antimicrobial activity against methicillin–resistant *Staphylococcus aureus* (MRSA), vancomycin–intermediate *S. aureus* (VISA), and vancomycin–resistant *S. aureus* (VRSA) strains (Daher *et al*., 2020), *Candida albicans* ATCC 90028 (Gu *et al*., 2019), *Sclerotinia sclerotiorum* (Zucchi *et al*., 2014) and *Rhizoctonia solani* (Ohike *et al*., 2018). Moreover, albocycline was able to block nicotinate biosynthesis in *Bacillus subtilis* cells (Reusser, 1969). Many studies explained the antifungal mechanisms of albcycline. François *et al*. (2005) assumed that this macrolide has affect glucan biosynthesis or other ways of macrolides mechanisms (Mazzei *et al*., 1993 ; Gaynor and Mankin, 2003).

Table 4.30 Anti–*Foc* activity of ethyl acetate crude extract and compound from strain CH5–8.

Crude extracts/ compounds	Inhibition zone (mm)							
	Foc 1	*Foc* 2	*Foc* 3	*Foc* 4	*Foc* 5	*Foc* 6	*Foc* 7	*Foc* 8
F1 (100 µg/disc)	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
F2 (100 µg/disc)	Inactive	19.00 ± 0.50	20.10 ± 0.30	15.67 ± 0.29	11.83 ± 0.29	19.00 ± 0.50	12.83 ± 0.30	14.50 ± 0.50
F3 (100 µg/disc)	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
F4 (100 µg/disc)	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
F5 (100 µg/disc)	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
F2F1 (100 µg/disc)	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
F2F2 (100 µg/disc)	9.67 ± 0.29	16.17 ± 0.29	23.33 ± 1.04	13.00 ± 0.50	11.50 ± 0.50	22.33 ± 0.29	9.00 ± 0.50	14.67 ± 0.29
F2F3 (100 µg/disc)	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
A (F2F2H12) (60 µg/disc)	22.67 ± 0.58	29.67 ± 0.58	34.00 ± 0.00	23.83 ± 0.29	24.67 ± 0.58	29.83 ± 0.76	13.83 ± 0.29	24.67 ± 0.29
Cycloheximide (50 µg/disc)	24.67 ± 0.29	24.83 ± 0.29	29.83 ± 0.76	18.00 ± 0.50	18.17 ± 0.29	23.83 ± 0.29	17.67 ± 0.29	20.17 ± 0.29
Nystatin (50 µg/disc)	20.83 ± 0.29	15.67 ± 0.29	20.33 ± 0.29	12.83 ± 0.29	12.31 ± 0.58	12.67 ± 1.04	14.67 ± 0.29	14.17 ± 0.29
Benzimidazole (100 µg/disc)	32.83 ± 0.76	24.83 ± 0.29	22.00 ± 0.00	17.67 ± 0.29	17.17 ± 0.29	22.50 ± 0.50	31.50 ± 0.50	22.50 ± 0.50

Note: Mean ± Standard deviation, N = 3

4.13.2.2 Isolation, purification, structure elucidation, and anti–*Foc* activity of the secondary metabolite from strain CH9–7

The EtOAc crude extract from strain CH9–7 was analyzed by HPLC. The chemical profile showed several peaks of major and minor components (Figures 4.75–4.76). The UV profiles of peaks were compared with BIOTEC's in–house database. The crude was isolated using a Sephadex LH20 column eluted with 100% MeOH to give five fractions (F1–F5). The HPLC profiles showed one interesting peak at RT 2.3717 (peak B) of fraction F1 (Figure 4.75). The F1 crude extract was purified by semi–preparative HPLC to give compound B (CH9–7 F1H2). The chemical structure of compound B (CH9–7 F1H2) was elucidated by NMR spectral analysis.

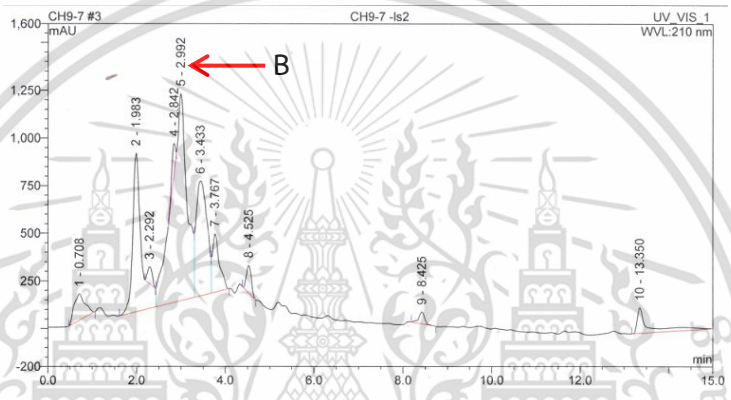


Figure 4.75 Chemical profiles of EtOAc crude extract from the strain CH9–7.

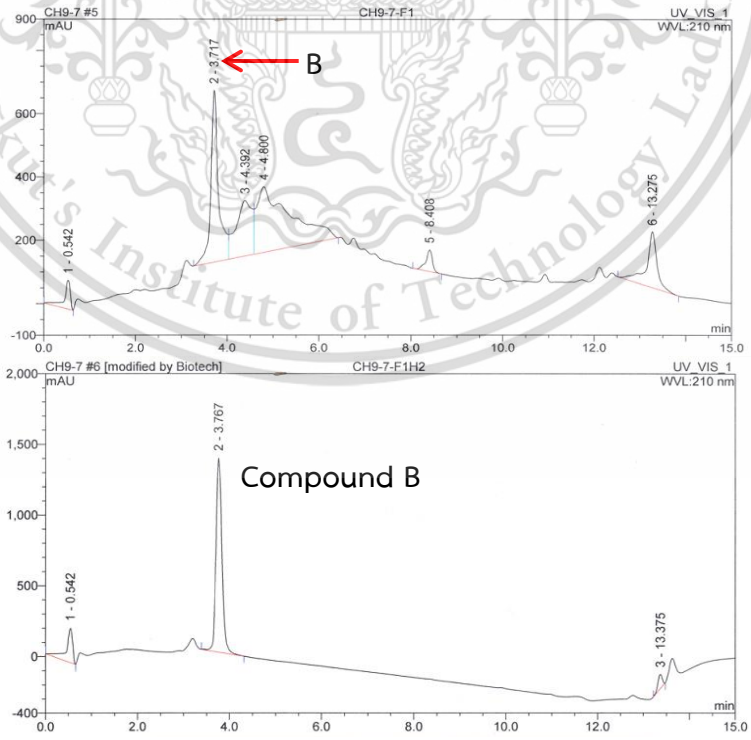


Figure 4.76 Chemical profiles of fraction F1 crude extract and compound B (F1H2) from the strain CH9–7.

Compound B (CH9–7 F1H2)	:	White solid
^{1}H NMR (DMSO–d_6, 500 MHz) (Appendix F, Figure 4)	:	δ_H 2.28 (6H, H–2, 11, 20), 2.58 (6H, H–3, 12, 21), 3.46 (6H, H–5, 14, 23), 1.49 (6H, H–6, 15, 24), 1.20 (6H, H–7, 16, 25), 1.37 (6H, H–8, 17, 26), 3.00 (6H, H–9, 18, 27), 9.62 (3H, N–OH), 7.37 (3H, NH)
^{13}C NMR (DMSO–d_6, 125 MHz) (Appendix F, Figure 5)	:	δ_C 171.4 (C–1, 10, 19), 29.9 (C–2, 11, 20), 27.4 (C–3, 12, 21), 172.0 (C–4, 13, 22), 46.8 (C–5, 14, 23), 25.8 (C–6, 15, 24), 23.1 (C–7, 16, 25), 28.5 (C–8, 17, 26), 38.8 (C–9, 18, 27)
HRESIMS spectrum (Appendix F, Figure 6)	:	$C_{27}H_{48}N_6$, *m/z* 623.34 $[M+Na]^+$

The spectroscopic data were identical to those reported for nocardamine in the literature (Meyer and Abdallah, 1980 ; Wang *et al*., 2000 ; Kalinovskaya *et al*., 2011 ; Li *et al*., 2018). Compound B was thus identified as nocardamine (Table 4.31).

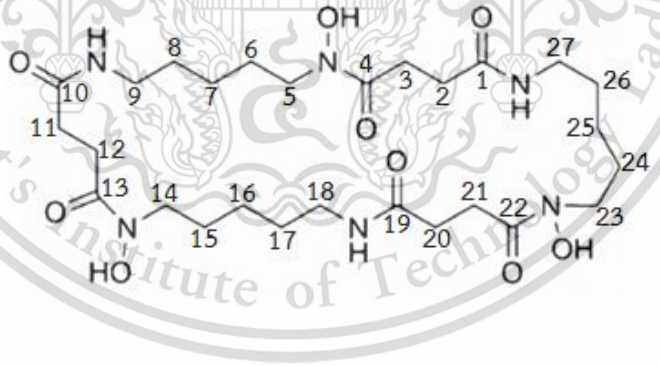


Figure 4.77 Chemical structure of compound B (CH9–7 F1H2).

Table 4.31 ^{1}H and ^{13}C NMR data of compound B (CH9–7 F1H2) in DMSO–d_6 and nocardamine in DMSO–d_6.

Position	Compound B (CH9–7 F1H2)		Nocardamine (Kalinovskaya *et al*., 2011)	
	δ_C (ppm)[a]	δ_H(ppm)[b], multiplicity, (*J* in Hz)	δ_C (ppm)[c]	δ_H(ppm)[d], multiplicity, (*J* in Hz)
1, 10, 19	171.4	-	171.4	-
2, 11, 20	29.9	2.28, *t* (6.8)	30.0	2.27, *t* (6.8)
3, 12, 21	27.4	2.58, *brt* (6.4)	27.4	2.57, *brt* (6.7)
4, 13, 22	172.0	-	172.0	-
5, 14, 23	46.8	3.46, *t* (6.7)	46.8	3.46, *t* (6.6)
6, 15, 24	25.8	1.49, *quint* (7.0)	25.8	1.49, *brquint* (7.1)
7, 16, 25	23.1	1.20, *quint* (7.0)	23.1	1.20, *m*
8, 17, 26	28.5	1.37, *quint* (7.0)	28.5	1.35, *m*
9, 18, 27	38.8	3.00, *q* (6.1)	38.3	2.30, *brq* (6.3)
N–OH	-	9.62, *s*	-	9.57, *brs*
NH	-	7.73, *brt* (4.9)	-	7.69, *brt* (5.3)

[a]125 MHz, [b]500 MHz, [c]75.48 MHz, [d]300.13 MHz

The EtOAc crude extract of the strain CH9–7 and compound B (CH9–7 F1H2) at the maximum concentration of 3 mg/ml (60 µg/disc) showed no activity against all *Foc* strains.

Nocardamine or desferrioxamine E is a cyclic hydroxamate siderophore that consisted of three units of 5–succinylated–1–amino–hydroxyamino–pentane belonging to the ferrioxamine group (Kalinovskaya *et al*., 2011). It was previously isolated from actinomycetes such as *Nocardia* sp. (Stoll *et al*., 1951), *Streptomyces* sp. strain Wak A–305 (Yang and Leong, 1982), a marined–derived *Streptomyces* sp. (Lee *et al*., 2005), *S. avermitilis* (Ueki *et al*., 2009), *S. griseus* (Yamanaka *et al*., 2005), a marine actinomycete *Citricoccus* sp. KMM 3890 (Kalinovskaya *et al*., 2011), *S. atratus* SCSIOZH16 (Li *et al*., 2018) and *Streptomyces* sp. H11890 (Mahmud *et al*., 2022) with potent activity against mycobacteria (Stoll *et al*., 1951), *Proteus vulgaris* (Deboer and Dietz, 1976), and *P. mirabilis* (Eiji *et al*., 1999). Few studies focused on antitumor activity aginst T–47D, SK–Mel–5, SK–Mel–28, and PRMI–7951 tumor cell line (Kameyama *et al*., 1987 ; Kalinovskaya *et al*., 2011). For antifungal activity, desferrioxamine E (nocardamine) from *Pseudomonas stutzeri* inhibited *Stemphylium botryosum* growth (Essén *et al*., 2007 ; Mokrani *et al*., 2019) and desferrioxamine E from *Streptomyces* sp. S29 starved *Botrytis cinerea* growth (Jarmusch *et al*., 2021). From this study, due to the limited concentration of

compound B (CH9–7 F1H2), anti–*Foc* activity was not observed at the concentration of 3 mg/ml. So, increasing the concentration may inhibit these fungi. Another reason, siderophores had affinity for iron which determines the growth of microbes under competitive conditions. So, siderophores produced by beneficial microorganisms were found to correlate with biocontrol of disease suppressive soils (Kleopper *et al*., 1980).

4.14 Polyphasic taxonomic characterization of the selected actinomycetes

4.14.1 Strain RCPT1–4

4.14.1.1 Phenotypic characteristics

Strain RCPT1–4 was aerobic, gram–stain–positive filamentous actinomycete that formed well–developed and nonfragmented branched substrate mycelia. Grew well on ISP 2, ISP 3, and ISP 7. Grew moderately on ISP 4, ISP 5, ISP 6, glucose–asparagine, and nutrient agar. Growth on Czapek's sucrose agar was poor. Yellowish–brown series substrate mycelium was observed on ISP 2 and ISP 3. White to grey series aerial spore masses were formed on ISP 2, ISP 3, ISP 4, ISP 6, ISP 7, and nutrient agar. Light yellow, pale greenish–yellow, and light greenish–yellow diffusible pigments were detected on ISP 2, ISP 5 and ISP 7 agar, respectively (Table 4.32). On ISP 2 medium, spore chains were spiral with hairy surfaces after 14 days of cultivation at 30 °C, and spores were non-motile (Figure 4.78). This spore morphology was consistent with the description of spore–chain morphology for the members of the genus *Streptomyces* (Waksman and Henrici, 1943 ; Pridham *et al*., 1958). In contrast, spiral spore chains with smooth spore surfaces have been reported in *Streptomyces fumigatiscleroticus* NBRC 12999^{T}, the closest relative according to the results of 16S rRNA gene analysis (Kämpfer, 2012).

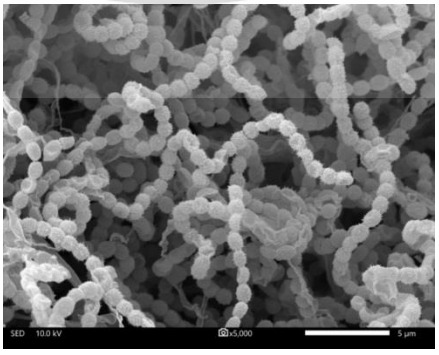


Figure 4.78 Scanning electron microscopy image of strain RCPT1–4 grown on ISP 2 medium at 30 °C for 14 days.

Table 4.32 Cultural characteristic of strain RCPT1–4 and the closest relatives.

Strains	Media	Growth	Substrate mycelium	Aerial spore mass	Soluble pigment
RCPT1–4	ISP 2	Good	Light yellowish-brown	Light grey	Light yellow
	ISP 3	Good	Yellowish-brown	Medium grey	-
	ISP 4	Moderate	Light greenish yellow	Yellowish grey	-
	ISP 5	Moderate	Greenish yellow	-	Pale greenish yellow
	ISP 6	Moderate	Pale greenish yellow	White	-
	ISP 7	Good	Brilliant orange yellow	Light grey	Light greenish yellow
	Glucose–asparagine	Moderate	Light greenish yellow	-	-
	Czapek's Sucrose	Poor	Pale greenish white	-	-
	NA	Moderate	Light orange yellow	Light grey	-
S. fumigatiscleroticus NBRC 12999^{T}	ISP 2	Good	Light orange yellow	Pale greenish yellow	-
	ISP 3	Good	Pale yellow	Moderate greenish yellow	-
	ISP 4	Moderate	Pale greenish yellow	-	-
	ISP 5	Good	Pale greenish yellow	-	-
	ISP 6	Moderate	Pale yellow	-	-
	ISP 7	Poor	Pale greenish yellow	-	-
	Glucose–asparagine	Poor	Pale greenish yellow	-	-
	Czapek's Sucrose	Good	Pale greenish white	-	-
	NA	Poor	Pale greenish yellow	-	-
S. spiralis NBRC 14215^{T}	ISP 2	Good	Strong yellowish orange	Greyish white	Strong yellowish brown
	ISP 3	Moderate	Pale orange yellow	Light grey	-
	ISP 4	Moderate	Pale yellowish brown	Greyish white	-
	ISP 5	Moderate	Pale yellow	Greyish white	Pale yellow
	ISP 6	Moderate	Pale yellow	-	-
	ISP 7	Good	Deep orange	Yellowish grey	-
	Glucose–asparagine	Moderate	Pale yellow	Greyish white	Pale yellow
	Czapek's Sucrose	Moderate	Deep orange	Greyish white	Light orange
	NA	Moderate	Pale yellow	-	-

Hydrolysis of starch and urea, liquefaction of gelatin, coagulation, and peptonization of milk, reduction of nitrate, and production of catalase and oxidase were positive. Negative results were observed for hydrogen sulfide and melanin production. Decomposed adenine, hypoxanthine, and L–tyrosine but not cellulose and xanthine. Utilized L–arabinose, cellobiose, dextran, D–fructose, D–galactose, D–glucose, glycerol, lactose, D–mannose, melibiose, D–ribose, trehalose, xylitol and D–xylose; weakly utilized *myo*–inositol, inulin, D–mannitol and sucrose; but does not utilized melezitose, raffinose or L–rhamnose, as sole carbon sources.

Utilized DL–2–aminobutyric acid, L–arginine, L–asparagine, L–cysteine, L–histidine, 4–hydroxyproline, L–methionine, L–phenylalanine, L–proline, L–serine, L–threonine, and L–valine as sole nitrogen sources. Produced acid from L–arabinose, cellobiose, dextran, D–fructose, D–galactose, D–glucose, glycerol, *myo*–inositol, inulin, lactose, D–mannose, D–ribose, trehalose, xylitol, and D–xylose. The growth temperature was between 15 and 47 °C, while the optimum range was 25–30 °C. Maximum NaCl concentration for growth was 6% (w/v). The pH range for growth was 5–10. According to the API ZYM system, cells can produce acid phosphatase, alkaline phosphatase, esterase lipase (C8), β–glucosidase, leucine arylamidase, α–mannosidase, N–acetyl–β–glucosaminidase, naphthol–AS–BI–phosphohydrolase, and valine arylamidase. Cells displayed weak activities of cystine arylamidase, esterase (C4), β–galactosidase, α–glucosidase, lipase (C14), and trypsin, and no activities of cells on α–chymotrypsin, α–fucosidase, α–galactosidase and β–glucuronidase were observed.

4.14.1.2 Chemotaxonomic characteristics

Cell wall peptidoglycan contained *LL*–diaminopimelic acid. Strain RCPT1–4 was found to have MK–9(H_6) (71.3%) and MK–9(H_4) (19.6%) as the major menaquinones, while MK–9(H_2) (6.7%) and MK–9(H_8) (2.4%) were also detected. Glucose, mannose, and ribose were detected as whole–cell sugars. The major fatty acids (>10%) were anteiso–$C_{15:0}$ (25.4%), anteiso–$C_{17:0}$ (19.5%) and iso–$C_{16:0}$ (18.7%). This major fatty acid pattern was also found in *S. fumigatiscleroticus* NBRC 12999^T and *S. spiralis* NBRC 14215^T with different proportions. In contrast, a minor fatty acid component, 3OH–$C_{18:0}$, was not detected in *S. fumigatiscleroticus* NBRC 12999^T (Table 4.33). Diphosphatidylglycerol (DPG), glycolic acid (GA), hydroxyl–phosphatidylethanolamine OH–PE), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine PME), phosphatidylglycerol (PG), phosphatidylinositol (PI), ninhydrin–positive lipid (NPL), five unidentified phospholipids (PLs) and four unidentified lipids (Ls) were observed as the polar lipids in cells (Appendix G, Figure 1 and 2). The chemotaxonomic data indicated that RCPT1–4 represented a member of the genus *Streptomyces*.

Table 4.33 Cellular fatty acid composition of strain RCPT1–4, and the closest relatives. Cultures were grown in ISP 2 broth on a rotary shaker at 30 °C for 5 days.

Fatty acid (%)	RCPT1–4	*S. fumigatiscleroticus* NBRC 12999^T	*S. spiralis* NBRC 14215^T
Anteiso–$C_{15:0}$	25.4	19.2	21.2
Iso–$C_{16:0}$	18.7	16.2	25.0
Anteiso–$C_{17:0}$	19.5	22.4	21.8
Iso–$C_{14:0}$	4.5	1.4	1.1
Iso–$C_{15:0}$	9.7	9.0	9.8
$C_{16:0}$	5.6	9.5	10.0
H–iso–$C_{16:1}$	0.8	0.2	-
$C_{17:0}$	1.4	1.3	0.8
Iso–$C_{17:0}$	8.5	9.7	7.9
Anteiso–$C_{17:1}$ (ω9c)	0.6	1.1	-
$C_{18:0}$	0.5	0.5	0.1
Iso–$C_{18:0}$	0.7	0.9	-
3OH–$C_{18:0}$	0.8	-	-
$C_{18:1}$ (ω9c)	0.6	3.8	0.2
10–Methyl C17:0 cyclo	0.4	0.2	-
$C_{16:1}$ (ω6c) and/or $C_{16:1}$ (ω7c)	0.3	0.5	0.6
$C_{18:2}$ (ω6, 9c) and/or $C_{18:0}$	-	1.6	0.9
$C_{19:1}$ (ω6c) and/or $C_{16:1}$ (ω7c) and/or $C_{19:0}$ cyclo (ω10c)	-	0.6	-
$C_{18:1}$ (ω6c) and/or $C_{18:1}$ (ω7c)	0.2	0.4	0.2
$C_{16:0}$ 10–Methyl and/or *iso*–$C_{17:1}$ (ω9c)	0.9	1.2	-

4.14.1.3 Genotypic characteristics

An almost complete 16S rRNA gene sequence (1413 nt) of RCPT1–4 was analyzed using the EzBioCloud (http://www.ezbiocloud.net/) server. RCPT1–4 shared the highest 16S rRNA gene sequence similarities with *S. fumigatiscleroticus* NBRC 12999^T (99.2%), followed by *S. spiralis* NBRC 14215^T (99.0%), which were slightly higher than the 16S rRNA gene threshold for species differentiation (98.7%) (Richter and Rosselló–Mora, 2009 ; Chun and Rainey, 2014).

Phylogenetic analysis revealed that RCPT1–4 was placed within the cluster of the genus *Streptomyces*. When the sequence of RCPT1–4 was compared with corresponding 16S rRNA gene sequences of 23 close relatives in the genus *Streptomyces* found in the EzTaxon-e database and the 16S rRNA gene sequence of *Allostreptomyces psammosilenae* YIM DR4008^T (as an outgroup), the branches for RCPT1–4 were separated from *S. fumigatiscleroticus* NBRC 12999^T, and *S. spiralis* NBRC 14215^T in both ML and NJ trees, but low bootstrap values (<50%) were detected (Figure 4.79).

The genome–based taxonomic characterization of RCPT1–4 was evaluated to clarify the taxonomic position of the strain. The genome size of RCPT1–4 was 7.35 Mb with 73.1 mol% DNA G+C content determined by *in silico* genome sequencing, which was within the range for members of the genus *Streptomyces* (Kämpfer, 2012). Other genomic details of RCPT1–4 and other related type strains of species of the genus *Streptomyces* are summarized in Table 4.34. Base on the results of dDDH analysis, *S. spiralis* NBRC 14215^{T} (32.1%), *Streptomyces kebangsaanensis* SUK12^{T} (31.2%), *Streptomyces naganishii* JCM 4654^{T} (31.1%), *Streptomyces triticisoli* NEAU DSCPA1-4-4^{T} (31.1%) and *Streptomyces anandii* JCM 4720^{T} (30.7%), shared higher dDDH values with RCPT1–4 than *S. fumigatiscleroticus* NBRC 12999^{T} (27.8%). Furthermore, the phylogenomic tree reconstruction by automatic selection of the most closely related type strains by TYGS indicated that RCPT1–4 was closely related to these neighbours, but the position of RCPT1–4 was separated from the branches carrying *S. fumigatiscleroticus* NBRC 12999^{T} (Figure 4.80). In addition, the ANI, AAI, and Tetra values of RCPT1–4 with closely related species were in the range of 82.8–86.0% for ANIb, 87.1–88.6% for ANIm, 78.7–82.8 % for AAI, and 0.94566–0.99002 for Tetra values which are significantly below the suggested cutoff values for the species delineation (Wayne *et al*., 1987 ; Teeling *et al*., 2004 ; Richter and Rosselló–Mora, 2009 ; Thompson *et al*., 2013 ; Konstantinidis *et al*., 2017), indicating that RCPT1–4 could be considered to represent a novel species of the genus *Streptomyces* (Table 4.35).

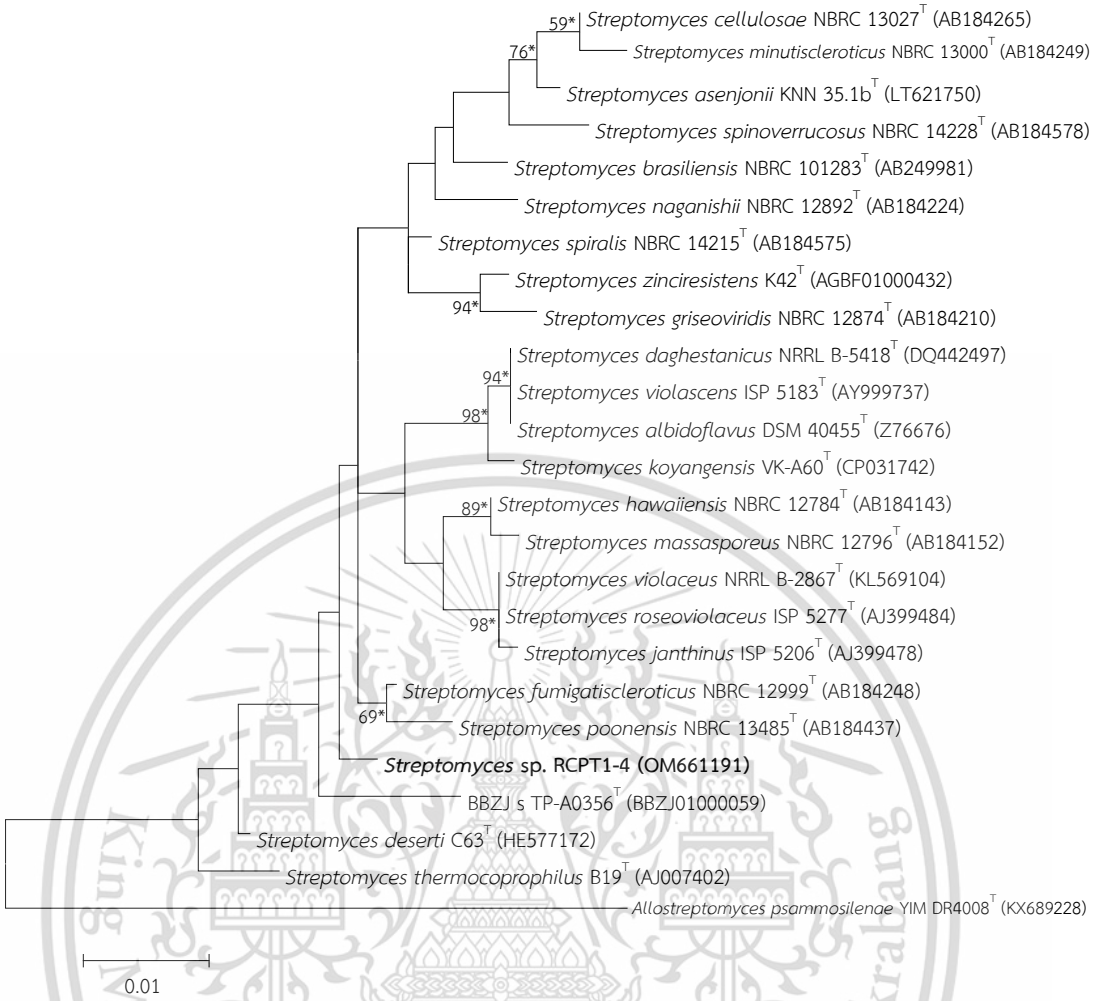


Figure 4.79 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain RCPT1–4 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

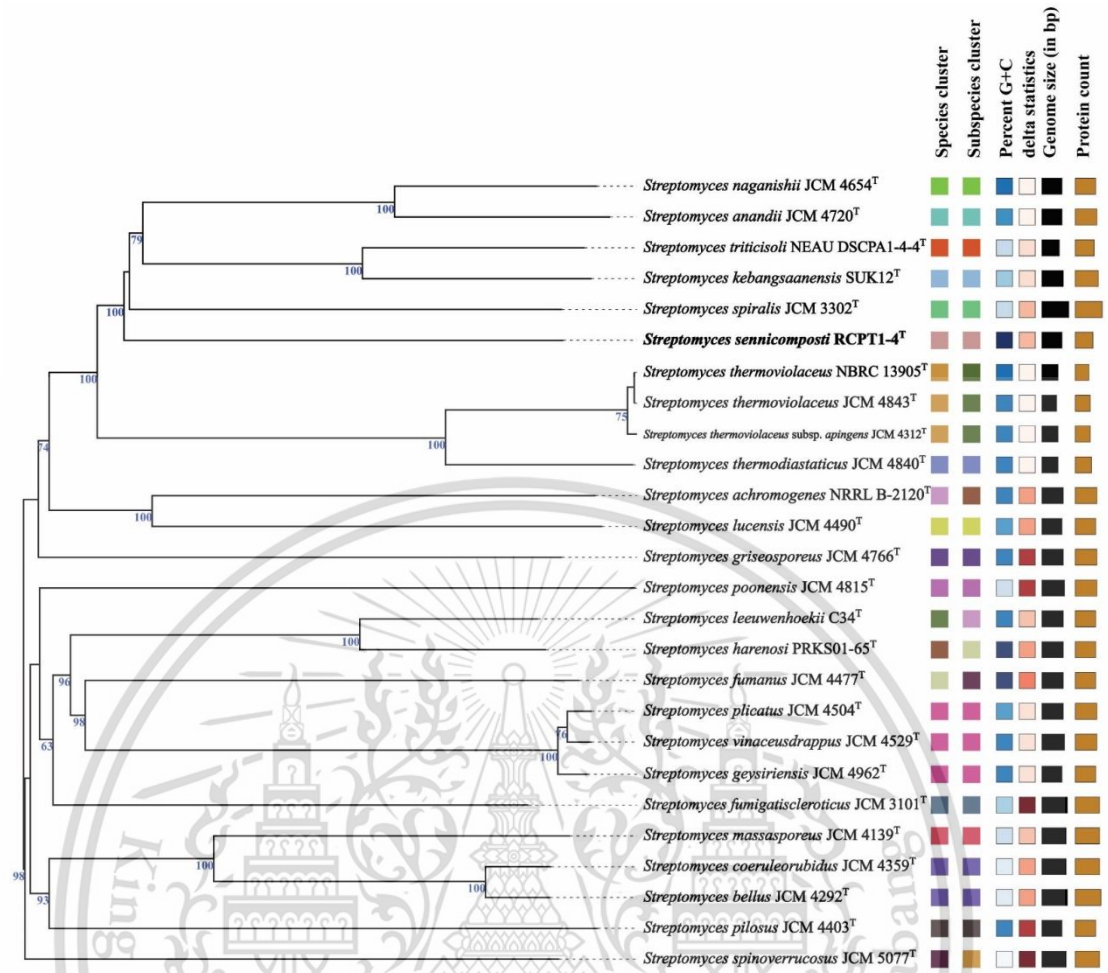


Figure 4.80 The phylogenomic tree of strain RCPT1–4 and their related type strains of the genus *Streptomyces* obtained from TYGS.

Table 4.34 General features of the genome sequences of strain RCPT1-4 and related type strains.

Features	RCPT1–4	*S. fumigatiscleroticus* NBRC 12999^{T}	*S. spiralis* NBRC 14215^{T}	*S. kebangsaanensis* SUK12^{T}	*S. naganishii* JCM 4654^{T}	*S. triticisoli* NEAU DSCPA1-4-4^{T}	*S. anandii* JCM 4720^{T}
Bioproject	PRJNA758547	PRJDB10510	PRJDB10510	PRJNA269542	PRJDB10510	PRJNA454853	PRJDB10510
Accession no.	JAIOAB000000000	BMQF00000000	BNBC00000000	JUJA00000000	BMVF00000000	QFDQ00000000	BMVJ00000000
Genome coverage	50x	118x	116x	170x	59x	299x	145x
N50	95,602	291,204	85,236	17,211	346,038	33,934	15,391
Number of Contigs	150	438	278	782	76	298	908
Genome size (Mb)	7.35	9.61	9.81	8.24	7.81	6.84	7.93
DNA G+C content (%)	73.1	71.0	71.2	71.5	72.6	71.4	72.3
Number of genes	6,297	8,410	8,903	8,126	6,928	6,554	7,627
Protein coding genes	6,103	7,909	8,367	7,161	6,640	5,997	7,023
Number of RNAs	78	115	81	82	81	84	74
rRNA	7	26	7	13	6	6	5
tRNA	68	86	71	66	72	75	66
Other RNA	3	3	3	3	3	3	3
Pseudogene	116	386	455	883	207	473	530

Table 4.35 ANIb, ANIm, tetra values, AAI, and dDDH of strain RCPT1–4 and related type strains.

Query genome	Reference genome	ANIb (%)	ANIm (%)	Tetra values	AAI (%)	Digital DNA–DNA hybridization relatedness				G+C difference
						Formula 2				
						% dDDH	Model C.I. (%)	Distance	Prob. DDH ≥ 70 (same species)	
RCPT1–4	*S.fumigatiscleroticus* NBRC 12999^{T}	82.8	87.1	0.94566	78.7	27.8	25.5 – 30.3	0.1548	0.04	2.1
	S. spiralis NBRC 14215^{T}	86.0	88.6	0.98803	82.7	32.1	29.7 – 34.6	0.1312	0.24	1.9
	S. kebangsaanensis SUK12^{T}	85.1	88.2	0.98566	81.0	31.2	28.8 – 33.7	0.1360	0.17	1.6
	S. naganishii JCM 4654^{T}	85.4	88.4	0.98947	82.6	31.1	28.7 – 33.6	0.1363	0.16	0.5
	S. triticisoli NEAU DSCPA1-4-4^{T}	85.1	88.2	0.98557	81.1	31.1	28.7 – 33.6	0.1362	0.17	1.7
	S. anandii JCM 4720^{T}	85.3	88.3	0.99002	82.8	30.7	28.3 – 33.2	0.1382	0.14	0.8

The genome annotation results obtained by rapid annotations using subsystems technology (RAST) (http://rast.nmpdr.org/) (Aziz *et al*., 2008 ; Overbeek *et al*., 2014) revealed that RCPT1–4 possessed 310 subsystems belonging to 23 categories (Figure 4.81). Among these subsystems, 'amino acids and derivatives' was the largest subsystem (378 feature counts), followed by 'carbohydrates' (315 feature counts), 'protein metabolism' (229 feature counts), 'cofactors, vitamins, prosthetic groups, pigments' (161 feature counts), 'fatty acids, lipids, and isoprenoids' (143 feature counts) and 'nucleosides and nucleotides' (112 feature counts).

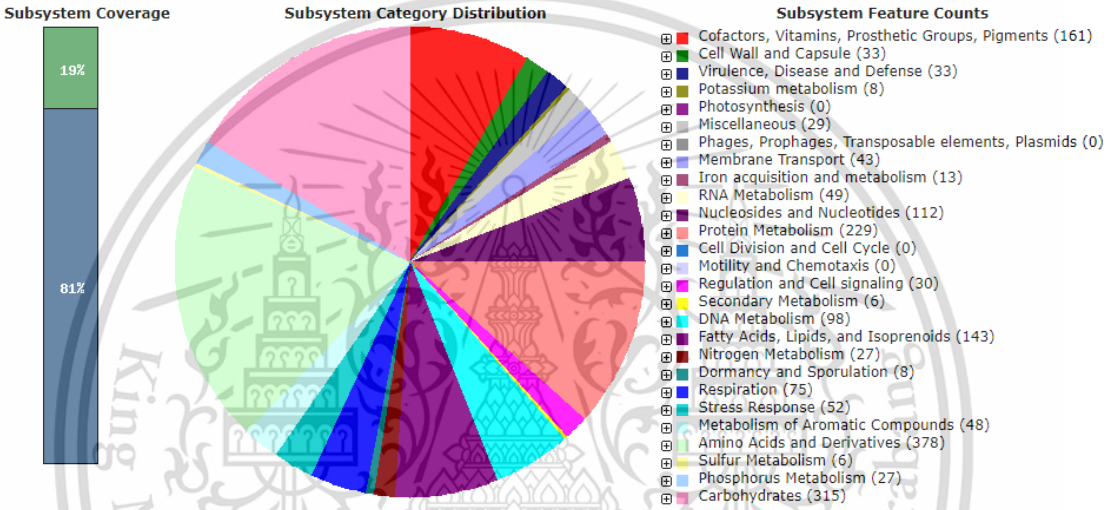


Figure 4.81 Subsystem category distribution of *Streptomyces* sp. RCPT1–4 based on RAST annotation server (https://rast.nmpdr.org/).

4.14.1.4 Differential characteristics

Differential characteristics between strains RCPT1–4 and related type strains are shown in Table 4.36. Moreover, chemotaxonomic and genotypic characteristics were described the section 4.14.1.2–4.14.1.3.

These results from taxonomic data supported that RCPT1–4 could be judged to represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces sennicomposti* sp. nov. is proposed. The type strain, RCPT1–4^{T} (=TBRC 11260^{T}=NBRC 114303^{T}), is an actinomycete isolated from a *Senna siamea* (Lam.) compost sample in Rayong province, Thailand.

Table 4.36 Differential characteristics of strain RCPT1–4 and the closest phylogenetic relatives.

Characteristics	RCPT1–4	*S. fumigatiscleroticus* NBRC 12999^T	*S. spiralis* NBRC 14215^T
Colour of aerial spore mass on ISP 2	Light grey	Pale greenish yellow	Greyish white
Spore surface ornamentation	Hairy	Smooth	Smooth
Maximum NaCl tolerance (%w/v)	6	5	7
Temperature range for growth (°C)	15–47	15–45	14–45
The pH range for growth	5–10	6–10	6–10
Urea hydrolysis	+	+	-
Milk coagulation	+	+	-
Nitrate reduction	+	-	-
Carbon utilization (1.0% w/v):			
Dextran	+	-	-
Raffinose	-	+	+
L–Rhamnose	-	+	+
D–Ribose	+	-	w
Sucrose	w	-	w
Trehalose	+	-	+
Acid production from (1.0% w/v):			
Dextran	+	-	-
myo–Inositol	+	-	-
Inulin	+	-	-
D–Mannitol	-	+	+
Melibiose	-	+	+
Raffinose	-	+	-
L-Rhamnose	-	+	+
Sucrose	-	+	+
D–Xylose	+	+	-
Nitrogen utilization (1.0% w/v):			
DL–2–Aminobutyric acid	+	+	-
L–Arginine	+	-	+
4–Hydroxyproline	+	+	-
L–Methionine	+	+	-
L–Phenylalanine	+	+	-
Decomposition (1.0% w/v) of:			
Hypoxanthine	+	-	+
L–Tyrosine	+	-	+
Enzyme activity with API ZYM:			
N-Acetyl–β–glucosaminidase	+	-	w
Lipase (C14)	w	-	-
α–Mannosidase	+	-	w
Trypsin	w	-	-

Note: + = positive, – = negative, w = weakly positive.

4.14.2 Strain CH5–8

4.14.2.1 Phenotypic characteristics

Strain CH5–8 was aerobic, gram–stain–positive filamentous actinomycete that formed well–developed and nonfragmented branched substrate mycelia. Grew well on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7, and nutrient agar. Grew moderately on glucose–asparagine and Czapek's sucrose agar. Vivid greenish yellow substrate mycelium and medium grey aerial spore mass were observed on ISP 2 after 14 days of cultivation at 30 °C. Brilliant yellow, brownish–black and dark brown diffusible pigments were detected on ISP 2, ISP 6, and ISP 7 media, respectively (Table 4.37). Spiral chains of spores with rough surfaces were observed easily on any media tested, and spores were non-motile (Figure 4.82). This spore morphology was consistent with the description of spore–chain morphology for the members of the genus *Streptomyces* (Waksman and Henrici, 1943 ; Pridham *et al*., 1958). In contrast, spiral spore chains with spiny spore surfaces have been reported in *Streptomyces echinatus* CGMCC 4.1642^{T} (Corbaz *et al*., 1957) and *Streptomyces actinomycinicus* RCU–197^{T} (Tanasupawat *et al*., 2015), the closest relative according to the results of 16S rRNA gene analysis.

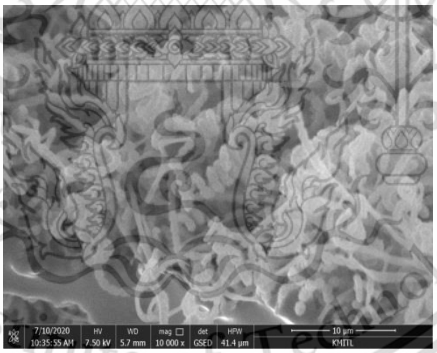


Figure 4.82 Scanning electron microscopy image of strain CH5–8 grown on ISP 2 medium at 30 °C for 14 days.

Table 4.37 Cultural characteristic of strain CH5–8 and the closest relatives.

Strains	Media	Growth	Substrate mycelium	Aerial spore mass	Soluble pigment
CH5–8	ISP 2	Good	Vivid greenish yellow	Medium grey	Brilliant yellow
	ISP 3	Good	Moderate yellowish brown	Light brownish grey	-
	ISP 4	Good	Light greyish olive	Brownish grey	-
	ISP 5	Good	Brilliant greenish yellow	Light greenish grey	-
	ISP 6	Good	Greyish brown	Light grey	Brownish black
	ISP 7	Good	Dark greyish yellowish	Yellow grey	Dark brown
	Glucose–asparagine	Moderate	Brilliant greenish yellow	Brilliant greenish yellow	-
	Czapek's Sucrose	Moderate	Light greyish brown	Light greyish brown	-
	NA	Good	Yellowish grey	Light grey	-
S. echinatus JCM 4144^{T}	ISP 2	Good	Brilliant orange yellow	Yellow grey	Strong yellow
	ISP 3	Good	Moderate yellowish brown	Light grey	-
	ISP 4	Good	Deep greenish yellow	Brownish grey	Brilliant greenish yellow
	ISP 5	Good	Brilliant yellow	Light brownish grey	-
	ISP 6	Moderate	Dark olive	Light grey	Brownish black
	ISP 7	Moderate	Dark olive	Light olive grey	Deep greenish yellow
	Glucose–asparagine	Moderate	Light greenish yellow	Light greenish yellow	Brilliant greenish yellow
	Czapek's Sucrose	Moderate	Light grayish brown	Light greyish brown	-
	NA	Good	Brilliant yellow	Yellowish white	Brilliant yellow
S. actinomycinicus RCU–197^{T}	ISP 2	Good	Brilliant orange yellow	Yellow grey	Strong yellow
	ISP 3	Good	Moderate olive brown	Dark greyish yellow	Light olive brown
	ISP 4	Good	Deep greenish yellow	Brownish grey	Brilliant greenish yellow
	ISP 5	Good	Brilliant yellow	Light brownish grey	Brilliant yellow
	ISP 6	Moderate	Greyish olive	Light greyish olive	Brownish black
	ISP 7	Moderate	Dark olive	Light olive grey	Deep greenish yellow
	Glucose–asparagine	Moderate	Light greyish yellow	Light greyish yellow	Brilliant greenish yellow
	Czapek's Sucrose	Moderate	Light greyish brown	Light greyish brown	-
	NA	Good	Brilliant yellow	Yellowish white	Brilliant yellow

The coagulation of milk and catalase activities were positive, and the reduction of nitrate was weakly positive. Negative for hydrolysis of starch, gelatin liquefaction, peptonization of milk, urease, and oxidase activities. Utilized adonitol, L–arabinose, cellobiose, dextran, D–fructose, D–galactose, D–glucose, glycerol, *myo*–inositol, inulin, lactose, D–mannitol, D–mannose, melezitose, melibiose, raffinose, L–rhamnose, D–ribose, salicin, sucrose, trehalose, xylitol, and D–xylose as sole carbon sources. Utilized DL–2–aminobutyric acid, L–arginine,

L–asparagine, L–cysteine, L–histidine, 4–hydroxyproline, L–methionine, L–phenylalanine, L–proline, L–serline, L–threonine, and L–valine as a sole nitrogen sources. Decomposed adenine, hypoxanthine, and L–tyrosine but not cellulose and xanthine. The growth temperature was between 20 and 45 °C while the optimum range was 25–30 °C. Maximum NaCl concentration for growth was 7% (w/v). The pH range for growth was 6–10. According to the API ZYM system, displayed alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, α–chymotrypsin, acid phosphatase, β–galactosidase, and N–acetyl–β–glucosaminidase activities. Esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, naphthol–AS–BI–phosphohydrolase, α–galactosidase, and α–mannosidase activities were weak. Showed no activities of β–glucuronidase, α–glucosidase, β–glucosidase or α–fucosidase.

4.14.2.2 Chemotaxonomic characteristics

Cell wall peptidoglycan contained *LL*–diaminopimelic acid. Strain CH5–8 was found to have MK–9(H_8) (53.8%), MK–9(H_6) (28.7%), and MK–9(H_4) (13.4%) as the major menaquinones, while MK–10(H_4) (3.3%), MK–10(H_2) (0.8%) were also observed. The major menaquinone of CH5–8 was similar to the major menaquinones found in *S. actinomycinicus* RCU–197^T (Tanasupawat *et al*., 2015). Galactose, glucose, mannose, and ribose were detected as whole–cell sugars. The major fatty acids (>10%) were anteiso–$C_{15:0}$ (25.6%), iso–$C_{16:0}$ (22.0%), and anteiso–$C_{17:0}$ (17.1%), while iso–$C_{15:0}$ (9.1%), $C_{16:0}$ (6.6%), iso–$C_{17:0}$ (5.1%), iso–$C_{14:0}$ (4.3%), $C_{17:0}$ (1.4%) and anteiso–$C_{17:1}$ (ω9c) (1.1%) were also detected. The pattern of major fatty acids in the cells of CH5–8 was similar to those predominant fatty acids in *S. echinatus* JCM 4144^T and *S. actinomycinicus* RCU–197^T, the closest relatives (Table 4.38), with different proportions. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), hydroxyphosphatidylethanolamine (OH–PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylinositol mannoside (PIM), four unidentified phospholipids (PLs) and two unidentified lipids (Ls) were observed as the polar lipids in cells (Appendix G, Figure 3). The chemotaxonomic data indicated that CH5–8 represented a member of the genus *Streptomyces*.

Table 4.38 Cellular fatty acid composition of strain CH5–8, and the closest relatives. Cultures were grown in ISP 2 broth on a rotary shaker at 30 °C for 5 days.

Fatty acid (%)	CH5–8	*S. echinatus* JCM 4144^T	*S. actinomycinicus* RCU–197^T
Anteiso–$C_{15:0}$	25.6	33.5	33.5
Iso–$C_{16:0}$	22.0	7.9	23.5
Anteiso–$C_{17:0}$	17.1	20.4	9.9
Iso–$C_{13:0}$	0.5	0.6	0.5
$C_{14:0}$	0.6	0.8	0.6
Iso–$C_{14:0}$	4.3	1.5	5.5
Iso–$C_{15:0}$	9.1	10.3	13.7
$C_{16:0}$	6.6	10.0	8.5
Iso–$C_{16:1}$ H	0.7	0.2	0.8
$C_{17:0}$	1.4	1.2	0.8
Iso–$C_{17:0}$	5.1	5.5	6.5
Anteiso–$C_{17:1}$ (ω9c)	1.1	1.3	0.9
$C_{18:0}$	0.4	0.5	0.4

4.14.2.3 Genotypic characteristics

An almost complete 16S rRNA gene sequence (1425 nt) of CH5–8 was analyzed using the EzBioCloud (http://www.ezbiocloud.net/) server. CH5–8 shared the highest 16S rRNA gene sequence similarities with *S. echinatus* NBRC 12763^T (98.9%), followed by *S. actinomycinicus* RCU–197^T (98.9%), which were slightly higher than the 16S rRNA gene threshold for species differentiation (98.7%) (Richter and Rosselló–Mora, 2009 ; Chun and Rainey, 2014).

Phylogenetic analysis revealed that CH5–8 was placed within the cluster of the genus *Streptomyces*. When the sequence of CH5–8 was compared with corresponding 16S rRNA gene sequences of 24 close relatives in the genus *Streptomyces* found in the EzTaxon-e database and the 16S rRNA gene sequence of *Micromonospora echinospora* DSM 43816^T (as an outgroup), CH5–8 formed a monophyletic line with *S. actinomycinicus* RCU–197^T and *S. echinatus* NBRC 12763^T in both ML and NJ trees (Figure 4.83).

The genome–based taxonomic characterization of CH5–8 was evaluated to clarify the taxonomic position of the strain. The genome size of CH5–8 was 9.36 Mb with 72.1 mol% DNA G+C content determined by *in silico* genome sequencing, which was within the range for members of the genus *Streptomyces* (Kämpfer, 2012). Other genomic details of CH5–8 and other related type strains of species of the genus *Streptomyces* are summarized in Table 4.39. The dDDH values between the genomes of CH5–8 and the two reference type strains, *S. echinatus* CECT 3313^T and *S. actinomycinicus* RCU–197^T, were 47.4 and 36.6%, respectively.

These values are lower than the threshold of 70% used to define species (Wayne *et al*., 1987 ; Thompson *et al*., 2013). Furthermore, the phylogenomic tree reconstruction by selection of the most closely related type strains by TYGS indicated that CH5–8 was closely related to *S. echinatus* CECT 3313^{T} (Figure 4.84). In addition, the ANI, AAI, and Tetra values of CH5–8 with closely related species were in the range of 87.4–91.6% for ANIb, 89.9–93.0% for ANIm, 85.9–90.8% for AAI, and 0.99830–0.99893 for Tetra values which are significantly below the suggested cutoff values for the species delineation (Teeling *et al*., 2004 ; Richter and Rosselló–Mora, 2009 ; Konstantinidis *et al*., 2017), indicating that CH5–8 could be considered to represent a novel species of the genus *Streptomyces* (Table 4.40).

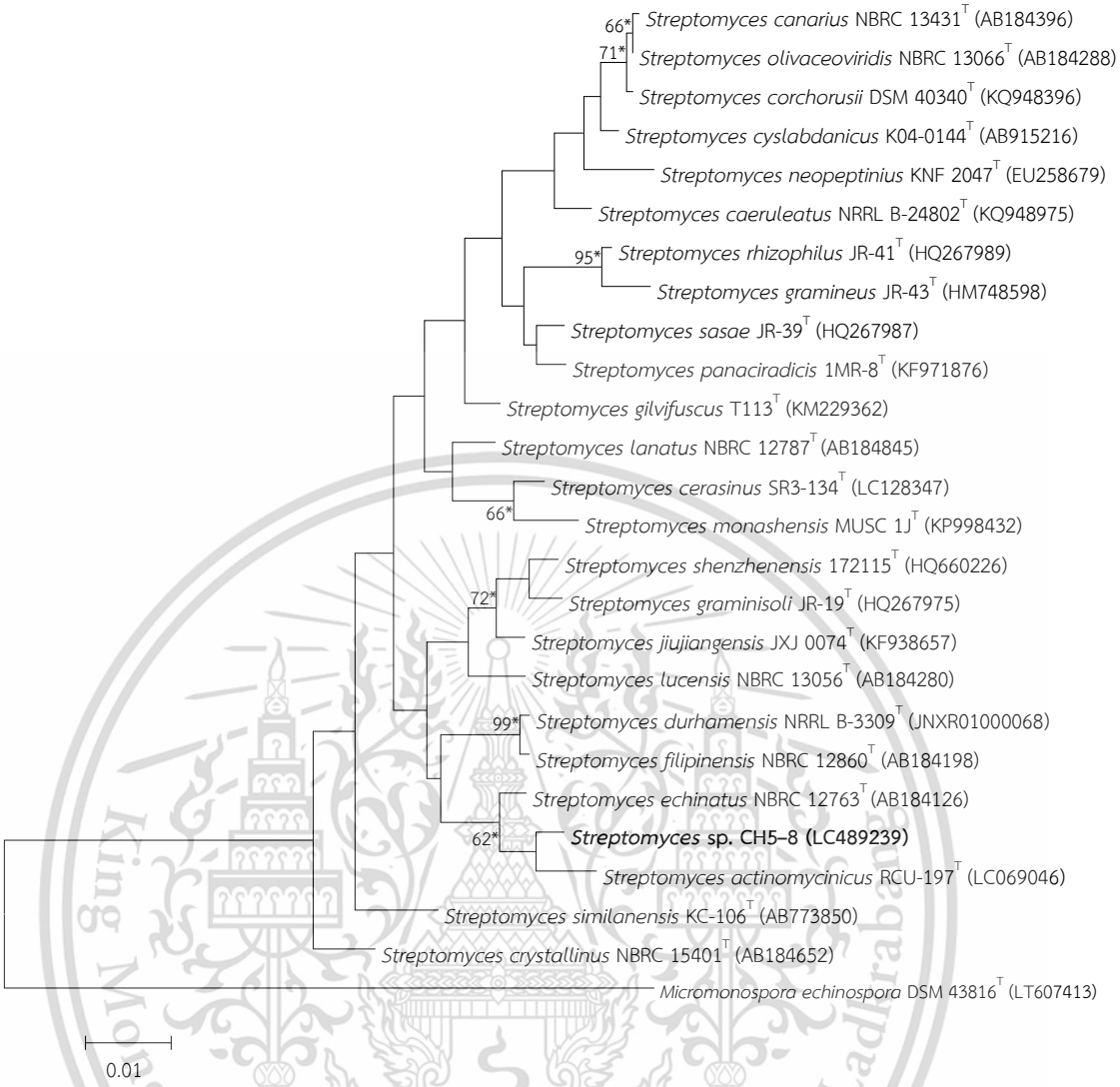


Figure 4.83 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain CH5–8 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

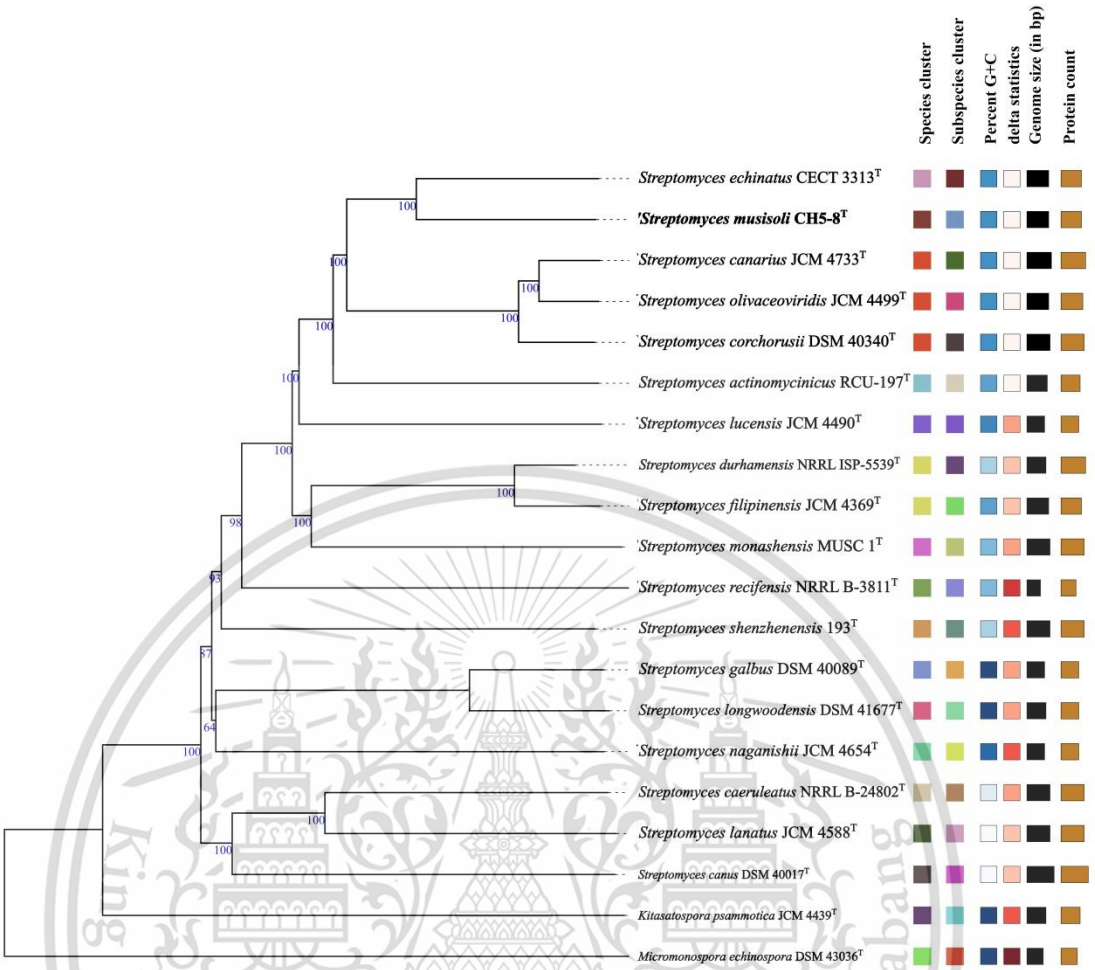


Figure 4.84 The phylogenomic tree of strain CH5–8 and their related type strains of the genus *Streptomyces* obtained from TYGS.

Table 4.39 General features of the genome sequences of strain CH5–8 and related type strains.

Features	CH5–8	*S. echinatus* CECT 3313^{T}	*S. actinomycinicus* RCU–197^{T}
Bioproject	PRJNA690886	PRJNA546735	PRJNA690886
Accession no.	JAERRH000000000	JACHJK000000000	JAERRK000000000
Genome coverage	400x	160x	120x
N50	398,829	404,223	270,358
Number of Contigs	148	65	106
Genome size (Mb)	9.36	9.39	8.79
DNA G+C content (%)	72.1	72.0	71.9
Number of genes	8,213	8,368	7,795
Protein coding genes	7,573	7,675	7,157
Number of RNAs	77	85	82
rRNA	4	9	4
tRNA	70	73	75
Other RNA	3	3	3
Pseudogene	563	608	556

Table 4.40 ANIb, ANIm, tetra values, AAI, and dDDH of strain CH5–8 and related type strains.

Query genome	Reference genome	ANIb (%)	ANIm (%)	Tetra values	AAI (%)	Digital DNA–DNA hybridization relatedness				G+C difference
						Formula 2				
						% dDDH	Model C.I. (%)	Distance	Prob. DDH ≥ 70 (same species)	
CH5–8	*S.echinatus* CECT 3313^{T}	91.6	93.0	0.99893	90.8	47.4	44.8 – 50.0	0.0780	12.66	0.1
	S.actinomycinicus RCU–197^{T}	87.4	89.9	0.99830	85.9	36.6	34.2 – 39.1	0.1116	1.08	0.2

The genome annotation results obtained by rapid annotations using subsystems technology (RAST) (http://rast.nmpdr.org/) (Aziz *et al*., 2008 ; Overbeek *et al*., 2014) revealed that CH5–8 possessed 343 subsystems belonging to 24 categories (Figure 4.85). Among these subsystems, 'amino acids and derivatives' was the largest subsystem (449 feature counts), followed by 'carbohydrates' (403 feature counts), 'protein metabolism' (242 feature counts), 'cofactors, vitamins, prosthetic groups, pigments' (210 feature counts), 'fatty acids, lipids, and isoprenoids' (173 feature counts), 'respiration' (137 feature counts), 'nucleosides and nucleotides' (131 feature counts) and 'DNA metabolism' (112 feature counts).

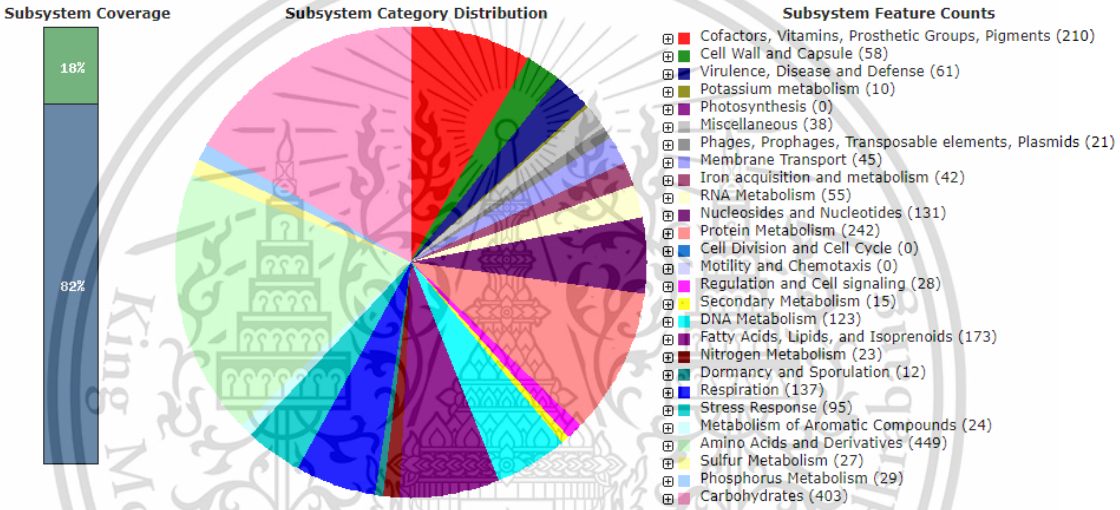


Figure 4.85 Subsystem category distribution of strain CH5–8 based on RAST annotation server (https://rast.nmpdr.org/).

4.14.2.4 Differential characteristics

Differential characteristics between strains CH5–8 and related type strains are shown in Table 4.41. Moreover, chemotaxonomic and genotypic characteristics were described in section 4.14.2.2–4.14.2.3.

These results from taxonomic data supported that CH5–8 could be judged to represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces musisoli* sp. nov. is proposed. The type strain, CH5–8^{T} (=TBRC 9950^{T}=NBRC 113997^{T}), is an actinomycete isolated from soil around a banana plant in Ratchaburi province, Thailand.

Table 4.41 Differential characteristics of strain CH5–8 and the closest phylogenetic relatives.

Characteristics	CH5–8	*S. echinatus* JCM 4144^T	*S. actinomycinicus* RCU–197^T
Colour of aerial spore mass on ISP 2	Vivid greenish yellow	Brilliant orange yellow	Brilliant orange yellow
Spore surface ornamentation	Rough	Spiny	Spiny
Maximum NaCl tolerance (%w/v)	7	3	4
Temperature range for growth (°C)	20–45	20–42	20–42
The pH range for growth	6–10	6–9	6–9
Hydrolysis of starch	-	w	+
Gelatin liquefaction	-	-	+
Milk coagulation	+	-	+
Milk coagulation	-	+	w
Nitrate reduction	w	-	-
Carbon utilization (1.0% w/v):			
Dextran	w	-	-
Inulin	+	-	-
L–Rhamnose	+	-	-
Sucrose	+	-	w
D–Salicin	+	-	-
D–Xylose	+	-	-
Acid production from (1.0% w/v):			
Lactose	-	w	w
Melezitose	-	+	+
L–Rhamnose	+	-	-
D–Ribose	-	w	w
Salicin	+	-	-
Enzyme activity with API ZYM:			
N–Acetyl–β–glucosaminidase	+	-	+
α–Chymotrypsin	+	-	+
Cystine arylamidase	w	-	+
α–Galactosidase	w	-	+
β–Galactosidase	+	-	+
α–Glucosidase	-	-	+
Trypsin	w	-	+
Valine arylamidase	+	-	+

Note: + = positive, – = negative, w = weakly positive.

4.14.3 Strain CH9–7

4.14.3.1 Phenotypic characteristics

Strain CH9–7 was aerobic, gram–stain–positive filamentous actinomycete that formed well–developed and nonfragmented branched substrate mycelia. Grew well on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7, glucose–asparagine, Czapek's sucrose, and nutrient agar. Light yellowish brown substrate mycelium, light grey aerial spore mass, and pale yellow diffusible pigment were observed on ISP 2 after 14 days of cultivation at 30 °C. Moderate olive brown was detected on ISP 7 media (Table 4.42). On ISP 2 medium, spore chains were spiral with smooth surfaces after 14 days of cultivation at 30 °C, and spores were non–motile (Figure 4.86). This spore morphology was consistent with the description of spore–chain morphology for the members of the genus *Streptomyces* (Waksman and Henrici, 1943 ; Pridham *et al*., 1958). In contrast, spiral spore chains with spiny spore surfaces have been reported in *Streptomyces chattanoogensis* ISP 5002^{T} (Kim *et al*., 2012), one of the closest relatives according to the results of 16S rRNA gene analysis.

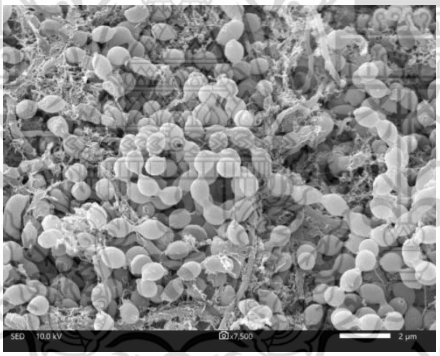


Figure 4.86 Scanning electron microscopy image of strain CH9–7 grown on ISP 2 medium at 30 °C for 14 days.

Table 4.42 Cultural characteristic of strain CH9–7 and the closest relatives.

Strains	Media	Growth	Substrate mycelium	Aerial spore mass	Soluble pigment
CH9–7	ISP 2	Good	Light yellowish brown	Light grey	Pale yellow
	ISP 3	Good	Strong brown	Light grey	-
	ISP 4	Good	Dark green	Medium grey	-
	ISP 5	Good	Pale yellow	Yellowish white	-
	ISP 6	Good	Light yellow	Yellowish white	-
	ISP 7	Good	Greyish yellowish brown	Medium grey	Moderate olive brown
	Glucose–asparagine	Good	Yellowish white	Yellowish white	-
	Czapek's Sucrose	Good	Pale yellow	White	-
	NA	Good	Pale yellow	Greyish pink	-
S. lydicus NBRC 13058^{T}	ISP 2	Good	Light yellow	Light grey	-
	ISP 3	Moderate	Pale yellow	White	-
	ISP 4	Good	Pale yellowish brown	Light grey	-
	ISP 5	Moderate	Light orange	Vivid greenish yellow	Vivid orange yellow
	ISP 6	Poor	Light olive	Light olive	-
	ISP 7	Moderate	Pale orange yellow	Light yellowish brown	-
	Glucose–asparagine	Poor	Light greenish yellow	Yellowish white	Pale greenish yellow
	Czapek's Sucrose	Poor	White	White	-
	NA	Moderate	Pale yellow	Light grey	-
S. chattanoogensis NBRC 12754^{T}	ISP 2	Good	Deep yellow	Strong yellow	Brilliant yellow
	ISP 3	Moderate	Moderate yellow	Light yellow green	-
	ISP 4	Good	Light yellow green	Pale yellowish green	-
	ISP 5	Good	Light yellow green	Pale yellowish green	Very pale green
	ISP 6	Poor	Light olive	Light olive	-
	ISP 7	Good	Moderate greenish yellow	Light greenish yellow	Pale greenish yellow
	Glucose–asparagine	Moderate	Yellowish white	Yellowish white	-
	Czapek's Sucrose	Poor	Yellowish white	Yellowish white	-
	NA	Moderate	Pale yellow	Yellowish white	-

Hydrolysis of starch and urea, liquefaction of gelatin, coagulation, and peptonization of milk, and production of catalase, and oxidase were positive. Negative results were observed for nitrate reduction, hydrogen sulfide, and melanin production. Decomposed adenine, hypoxanthine, and L–tyrosine but not cellulose and xanthine. Utilized L–arabinose, cellobiose, dextran, D–fructose, D–galactose, D–glucose, glycerol, *myo*–inositol, inulin, lactose, D–mannitol, D–mannose, melezitose, melibiose, raffinose, L–rhamnose, sucrose, trehalose, xylitol, and D–xylose; weakly utilized D–ribose, as sole carbon sources. Utilized DL–2–aminobutyric acid, L–arginine, L–asparagine, L–cysteine, L–histidine, 4–hydroxyproline,

L–methionine, L–phenylalanine, L–proline, L–serine, L–threonine, and L–valine as sole nitrogen sources. Produced acid from L–arabinose, cellobiose, D–fructose, D–galactose, D–glucose, glycerol, *myo*–inositol, inulin, lactose, D–mannose, melezitose, melibiose, raffinose, D–ribose, sucrose, trehalose, xylitol, and D–xylose. The growth temperature was between 20 and 45 °C, while the optimum range was 25–30 °C. Maximum NaCl concentration for growth was 7% (w/v). The pH range for growth was 6–10. According to the API ZYM system, cells can produce acid phosphatase, cystine arylamidase, esterase (C4), α–galactosidase, β–galactosidase, α–glucosidase, β–glucosidase, leucine arylamidase, N–acetyl–β–glucosaminidase, naphthol–AS–BI–phosphohydrolase and valine arylamidase. Cells displayed weak activities of alkaline phosphatase, esterase lipase (C8), and lipase (C14) and no activities of cells on α–chymotrypsin, α–fucosidase, β–glucuronidase, α–mannosidase and trypsin were observed.

4.14.3.2 Chemotaxonomic characteristics

Cell wall peptidoglycan contained *LL*–diaminopimelic acid. Strain CH9–7 was found to have MK–9(H_6) (71.7%) and MK–9(H_4) (12.8%) as the major menaquinones, while MK–9(H_2) (8.4%) and MK–9(H_8) (7.1%) were also observed. Glucose, galactose, ribose, and xylose were detected as whole–cell sugars. The major fatty acids (>10%) were iso–$C_{16:0}$ (20.1%), anteiso–$C_{15:0}$ (14.6%), iso–$C_{15:0}$ (13.0%) and $C_{16:0}$ (10.1%). The pattern of major fatty acids in the cells of CH9–7 was similar to those predominant fatty acids in *S. lydicus* NBRC **1**3058^T and *S. chattanoogensis* NBRC 12754^T, the closest relatives (Table 4.43), with different proportions. In contrast, a major fatty acid component, iso–$C_{14:0}$, was detected in *S. lydicus* NBRC **1**3058^T. Phosphatidylethanolamine (PE), hydroxyphosphatidylethanolamine (OH–PE), phosphatidylinositol (PI), glycolic acid (GL), ninhydrin–positive lipid (NPL), two unidentified phospholipids (PLs) and unidentified lipids (Ls) were observed as the polar lipids in cells (Appendix G, Figure 4). The chemotaxonomic data indicated that CH9–7 represented a member of the genus *Streptomyces*.

Table 4.43 Cellular fatty acid composition of strain CH9–7, and the closest relatives. Cultures were grown in ISP 2 broth on a rotary shaker at 30 °C for 5 days.

Fatty acid (%)	CH9–7	*S. lydicus* NBRC 13058^T	*S. chattanoogensis* NBRC 12754^T
Iso–$C_{16:0}$	20.1	24.9	25.8
Anteiso–$C_{15:0}$	14.6	17.7	13.7
Iso–$C_{15:0}$	13.0	13.1	18.0
$C_{16:0}$	10.1	10.1	10.1
Iso–$C_{13:0}$	0.5	0.9	0.6
$C_{14:0}$	0.5	1.1	0.6
Iso–$C_{14:0}$	4.1	10.1	7.4
Iso–$C_{16:1}$ H	1.2	0.3	0.3
$C_{17:0}$	0.6	0.4	0.4
Anteiso–$C_{17:0}$	8.6	6.1	6.2
Iso–$C_{17:0}$	9.5	7.1	7.6
Anteiso–$C_{17:1}$ (ω9c)	1.3	0.2	0.5
$C_{18:0}$	0.5	0.2	0.2
3OH–$C_{18:0}$	0.9	0.4	0.1
Iso–$C_{18:0}$	0.6	0.4	0.4
$C_{18:1}$ (ω9c)	1.9	1.0	0.5
$C_{17:0}$ cyclo	0.8	0.6	-
$C_{16:1}$ (ω6c) and/or $C_{16:1}$ (ω7c)	2.3	0.9	3.6
$C_{18:2}$ (ω6, 9c) and/or $C_{18:0}$	1.1	0.6	0.5
$C_{19:1}$ (ω6c) and/or $C_{16:1}$ (ω7c) and/or $C_{19:0}$ cyclo (ω10c)	0.9	0.3	0.6
$C_{16:0}$ 10–Methyl and/or *iso*–$C_{17:1}$ (ω9c)	3.2	0.3	0.5

4.14.3.3 Genotypic characteristics

An almost complete 16S rRNA gene sequence (1414 nt) of CH9–7 was analyzed using the EzBioCloud (http://www.ezbiocloud.net/) server. CH9–7 shared the highest 16S rRNA gene sequence similarities with *S. lydicus* NBRC 13058^T (99.9%), and *S. chattanoogensis* NBRC 12754^T (99.9%), which were very higher than the 16S rRNA gene threshold for species differentiation (98.7%) (Richter and Rosselló–Mora, 2009 ; Chun and Rainey, 2014).

Phylogenetic analysis revealed that CH9–7 was placed within the cluster of the genus *Streptomyces*. When the sequence of CH9–7 was compared with corresponding 16S rRNA gene sequences of 25 close relatives in the genus *Streptomyces* found in the EzTaxon-e database and the 16S rRNA gene sequence of *Micromonospora echinospora* DSM 43816^T (as an outgroup), the branches for CH9–7

were separated from *S. lydicus* NBRC 13058^{T} and *S. chattanoogensis* NBRC 12754^{T} in both ML and NJ trees, but low bootstrap values (<50%) were detected (Figure 4.87).

The genome–based taxonomic characterization of CH9–7 was evaluated to clarify the taxonomic position of the strain. The genome size of CH9–7 was 8.81 Mb with 71.7 mol% DNA G+C content determined by *in silico* genome sequencing, which was within the range for members of the genus *Streptomyces* (Kämpfer, 2012). Other genomic details of CH9–7 and other related type strains of species of the genus *Streptomyces* are summarized in Table 4.44. The dDDH values between the genomes of CH9–7 and the two reference type strains, *S. lydicus* NBRC 13058^{T}, and *S. chattanoogensis* NBRC 12754^{T}, were 31.7 and 28.4%, respectively. These values are lower than the threshold of 70% used to define species (Wayne *et al*., 1987 ; Thompson *et al*., 2013). Furthermore, the phylogenomic tree reconstruction by selection of the most closely related type strains by TYGS indicated that CH9–7 was closely related to *S. lydicus* ATCC 25470^{T} (Figure 4.88). In addition, the ANI, AAI, and Tetra values of CH9–7 with closely related species were in the range of 83.4–85.7% for ANIb, 87.5–88.6% for ANIm, 80.6–84.6% for AAI, and 0.98919–0.99692 for Tetra values which are significantly below the suggested cutoff values for the species delineation (Teeling *et al*., 2004 ; Richter and Rosselló–Mora, 2009 ; Konstantinidis *et al*., 2017), indicating that CH9–7 could be considered to represent a novel species of the genus *Streptomyces* (Table 4.45).

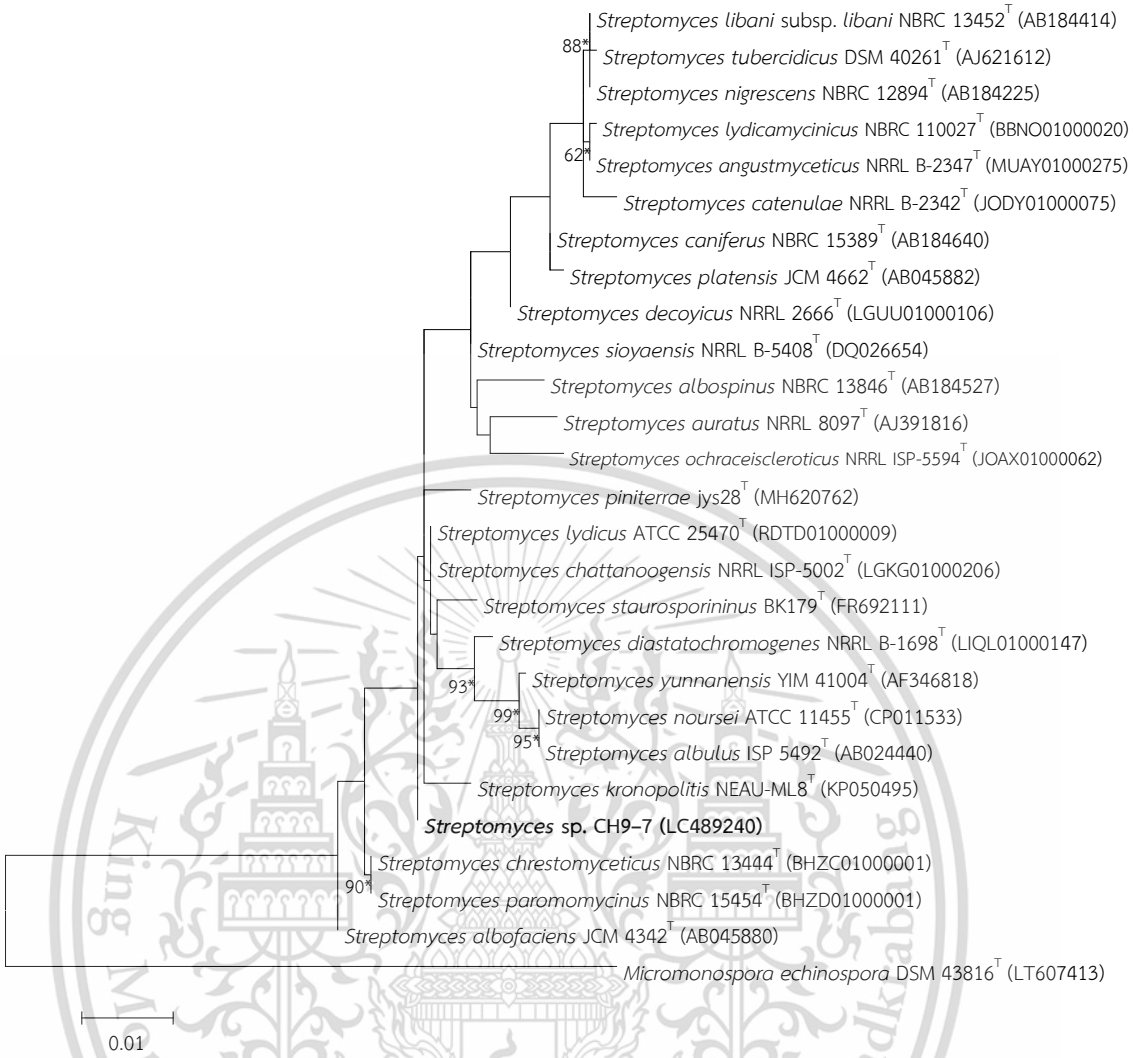


Figure 4.87 Maximum–likelihood tree based on almost complete 16S rRNA gene sequences showing the relationships between strain CH9–7 and other members of the genus *Streptomyces*. Asterisk (*) indicates branches that were recovered in the neighbor–joining method.

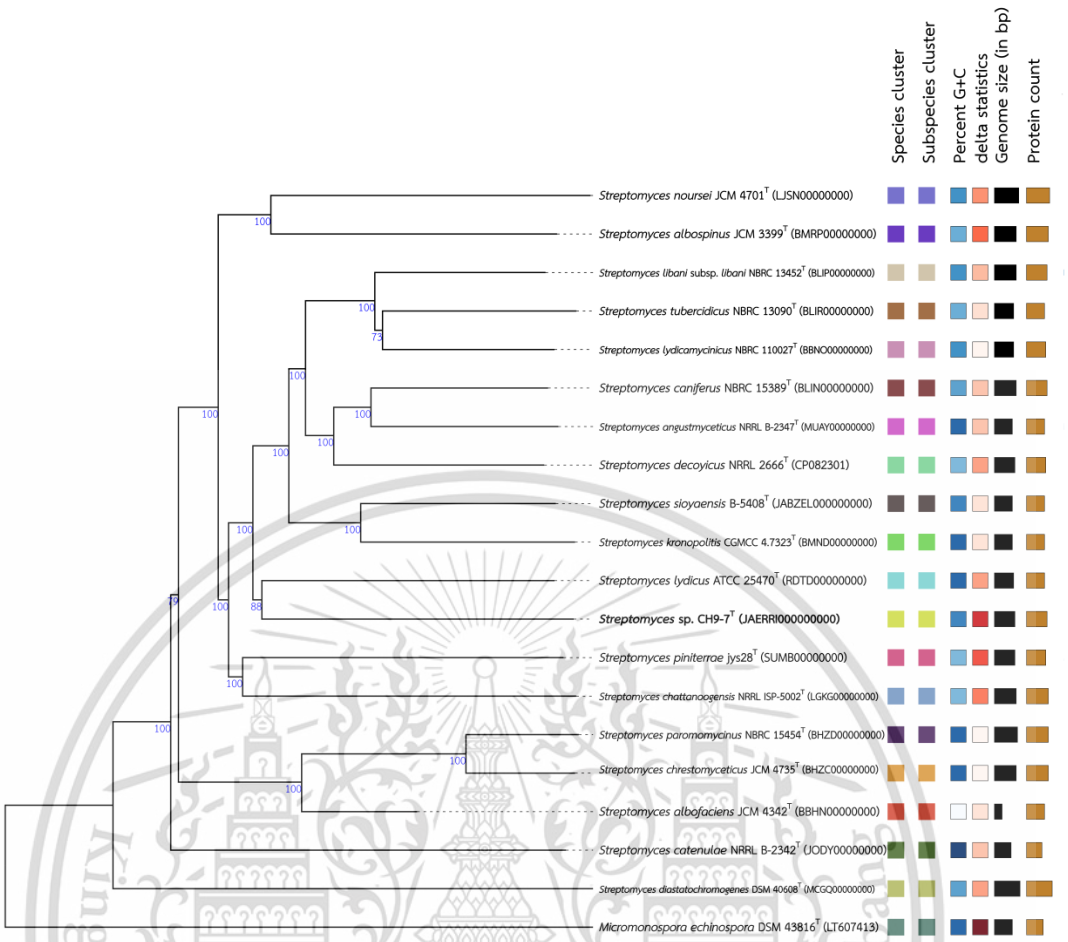


Figure 4.88 The phylogenomic tree of strain CH9–7 and their related type strains of the genus *Streptomyces* obtained from TYGS.

Table 4.44 General features of the genome sequences of strain CH9–7 and related type strains.

Features	CH9–7	*S. lydicus* ATCC 25470^T	*S. chattanoogensis* NRRL ISP-5002^T
Bioproject	PRJNA690886	PRJNA495934	PRJNA238534
Accession no.	JAERRI000000000	RDTD00000000	LGKG00000000
Genome coverage	375x	123x	142x
N50	381,329	4,205,013	166,063
Number of Contigs	82	20	217
Genome size (Mb)	8.81	7.94	9.13
DNA G+C content (%)	71.7	72.0	70.9
Number of genes	7,664	7,336	8,284
Protein coding genes	7,294	7,040	7,838
Number of RNAs	75	91	81
rRNA	3	21	10
tRNA	69	67	68
Other RNA	3	3	3
Pseudogene	295	205	365

Table 4.45 ANIb, ANIm, tetra values, AAI, and dDDH of strain CH9–7 and related type strains.

Query genome	Reference genome	ANIb (%)	ANIm (%)	Tetra values	AAI (%)	Digital DNA–DNA hybridization relatedness				G+C difference
						Formula 2				
						% dDDH	Model C.I. (%)	Distance	Prob. DDH ≥ 70 (same species)	
CH9–7	*S. lydicus* ATCC 25470^{T}	85.7	88.6	0.99692	84.6	31.7	29.3 – 34.3	0.1330	0.21	0.3
	S. chattanoogensis NRRL ISP–5002^{T}	83.4	87.5	0.98919	80.6	28.4	26.0 – 30.9	0.1512	0.05	0.8

The genome annotation results obtained by rapid annotations using subsystems technology (RAST) (http://rast.nmpdr.org/) (Aziz *et al*., 2008 ; Overbeek *et al*., 2014) revealed that CH9–7 possessed 346 subsystems belonging to 25 categories (Figure 4.89). Among these subsystems, 'amino acids and derivatives' was the largest subsystem (480 feature counts), followed by 'carbohydrates' (366 feature counts), 'protein metabolism' (254 feature counts), 'cofactors, vitamins, prosthetic groups, pigments' (240 feature counts), 'fatty acids, lipids, and isoprenoids' (181 feature counts), 'respiration' (136 feature counts), 'nucleosides and nucleotides' (131 feature counts) and 'DNA metabolism' (103 feature counts).

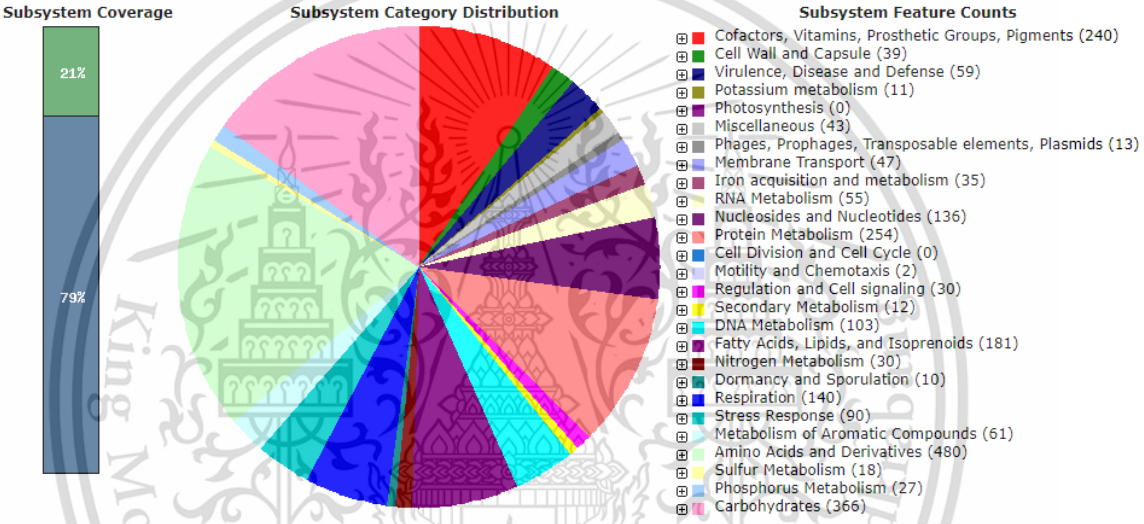


Figure 4.89 Subsystem category distribution of strain CH9–7 based on RAST annotation server (https://rast.nmpdr.org/).

4.14.3.4 Differential characteristics

Differential characteristics between strains CH9–7 and related type strains are shown in Table 4.46. Moreover, chemotaxonomic and genotypic characteristics were described in section 4.14.3.2–4.14.3.3.

These results from taxonomic data supported that CH9–7 could be judged to represent a novel species of the genus *Streptomyces*.

Table 4.46 Differential characteristics of strain CH9–7 and the closest phylogenetic relatives.

Characteristics	CH9–7	*S. lydicus* NBRC 13058^T	*S. chattanoogensis* NBRC 12754^T
Colour of substrate mycelium on ISP 3	Strong brown	Pale yellow	Moderate yellow
Spore surface ornamentation	Smooth	Smooth	Spiny
Maximum NaCl tolerance (%w/v)	7	2	2
Temperature range for growth (°C)	20–45	20–37	20–37
The pH range for growth	6–10	6–9	6–9
Starch hydrolysis	+	-	-
Urea hydrolysis	+	+	-
Milk coagulation	+	-	-
Milk peptonization	+	-	-
Oxidase activity	+	+	-
Carbon utilization (1.0% w/v):			
L–Arabinose	+	w	w
D–Cellobiose	+	w	w
Dextran	+	w	w
D–Fructose	+	w	+
D–Galactose	+	-	w
Glycerol	+	w	w
Inulin	+	w	w
Lactose	+	w	w
D–Mannitol	+	w	w
D–Mannose	+	w	w
D–Melezitose	+	w	w
D–Melibiose	+	w	w
Raffinose	+	w	w
L–Rhamnose	+	w	w
Sucrose	+	w	+
Trehalose	+	w	w
Xylitol	+	w	w
D–Xylose	+	w	w
Acid production from (1.0% w/v):			
D–Cellobiose	+	-	+
D–Galactose	+	-	+
Inulin	+	-	+
D–Mannitol	-	+	-
Nitrogen utilization (1.0% w/v):			
DL–2–Aminobutyric acid	+	w	w
L–Arginine	+	+	w
L–Cysteine	+	w	w
4–Hydroxyproline	+	w	+
Decomposition (1.0% w/v) of:			
L–Tyrosine	+	-	+
Enzyme activity with API ZYM:			
Alkaline phosphatase	w	+	+
α–Chymotrypsin	-	-	+
Cystine arylamidase	+	+	w

Table 4.46 Differential characteristics of strain CH9–7 and the closest phylogenetic relatives (continued).

Characteristics	*Streptomyces* sp. CH9–7	*S. lydicus* NBRC 13058^T	*S. chattanoogensis* NBRC 12754^T
Esterase (C4)	+	w	+
Esterase lipase (C8)	w	w	+
α–Galactosidase	+	-	-
β–Galactosidase	+	+	-
α–Glucosidase	+	w	+
β–Glucosidase	+	w	-
Lipase (C14)	w	-	+
N–Acetyl–β–glucosaminidase	+	w	+
Trypsin	-	-	+

Note: + = positive, – = negative, w = weakly positive.

Chapter 5
Conclusions and suggestions

5.1 Conclusions

In the research of product development for Fusarium wilt disease control in banana and plant growth promoting activity from actinomycetes, one hundred and seventy–three actinomycetes were isolated from the compost types: four samples from aerobic bio–sludge compost, two samples from anaerobic bio–sludge compost, five samples from agricultural waste compost, and two samples from the soil collected from Rayong, Suphanburi, Ratchaburi, Pichit and Nakhon Sawan provinces. Among these isolates, twenty–four isolates showed antagonistic activity against *Fusarium oxysporum* f.sp. *cubense* (*Foc*) with a percentage of inhibition of radial growth values of more than 21% and at least 50% of *Foc* strains. Based on of 16S rRNA gene sequencing, they were identified to the genus *Streptomyces*.

All twenty–four strains were able to fix gaseous nitrogen, and produce indole acetic acid, gibberellin, cytokinin, and siderophore. Only four strains, RCPT1–4, CH5–8, CH9–7, and NKS15, were able to solubilize phosphate. Eleven isolates were found to be capable of potassium solubilization with the highest solubilization efficiency in the strain RCPT1–4. Among all isolates, no presence of actinomycetes produced HCN. Only one strain (SBST2–5) was found to produce H_2S.

Most of twenty–four strains showed a broad spectrum of ACC deaminase, urease, amylase, gelatinase, caseinase, and lipase production. They tolerated stress conditions (pH, NaCl, temperature, and osmotic pressure). Moreover, 24 actinomycetes were able to grow with chemical fertilizer and fungicide 2.0% benzimidazole.

All actinomycetes were able to grow in the solid medium using solid–state fermentation that tended to be better than in a liquid medium.

Three actinomycete products with eight strains were formulated based on antifungal activity against *Foc*, plant–growth promoting traits, and their properties (extracellular enzyme production, tolerance, fast growth, and non–toxic to host plant or non–pathogenic to humans). ATU–Bio 1 consisted of the strains RBST1–4, RCPT3–28, and CH9–7, ATU–Bio 2 consisted of the strains CH5–8, RCPT1–4, and RBST2–54 and ATU–Bio 3 consisted of the strains RCPT1–4, RCPT3–40, and RBST2–21, respectively.

Actinomycete products group enhanced growth of banana in pot experiment that higher than the control group (no supplement) and commercial PGPR–2. ATU–Bio 1 and ATU–Bio 2 showed plant growth promoters better than ATU–Bio 3.

For the banana protective experiment, the final severity index, for leaf symptom (LSI) and rhizome discoloration (RDI) of the actinomycete products group were reduced with maximum values of about 63 and 57% of ATU–Bio 1, 50 and 50% of ATU–Bio 2, and 50 and 46% of ATU–Bio 3, respectively, compared to untreated plantlets (no supplement). For plant growth promoters under *Foc* infection, actinomycete products still enhanced banana growth but growth parameters showed lower than no–*Foc* treatments.

For the banana curative experiment, the final severity index, for LSI and RDI of the actinomycete products group were reduced with maximum values of about 21 and 25% of ATU–Bio 1, 15 and 9% of ATU–Bio 2, and 11 and 7% of ATU–Bio 3, respectively, compared to untreated plantlets (no supplement). The actinomycete products had a poor–efficiency capacity to cure *Foc* infection and plant growth promoters in banana.

Chitinase production from strains RCPT1–4, CH5–8, and CH9–7 and CMCase from strains RCPT1–4 and CH5–8 were one of an antifungal mechanism of fungal cell wall degradation. Moreover, alboclycline from strain CH5–8 and nocardamine from strain CH9–7 as secondary metabolites exhibited antifungal activity.

Three strains such as RCPT1–4, CH5–8, and CH9–7 were selected based on anti–*Foc* activity, plant growth promoting traits, and 16S rRNA gene sequences analysis. They were identified using a polyphasic taxonomy approach. All three strains were represented also new species of the genus *Streptomyces*. Strains RCPT1–4^{T} and CH5–8^{T} were proposed to represent the novel species as *Streptomyces sennicomposti* and *Streptomyces musisoli*, respectively. In addition, a strain CH9–7^{T} was proposed to represent a novel species as *Streptomyces* sp. CH9-7^{T}.

The actinomycete products, especially ATU–Bio 1 and ATU–Bio 2, could be useful for the biological control of Fusarium wilt disease and plant growth promoter of banana.

5.2 Suggestions

1) Due to these experiments were performed in a short period, the biocontrol effect of the actinomycete products needs to be evaluated for a longer growth period.

2) For utilization of actinomycete products and avoiding the cross–contamination, single–use systems such as directly add to the soil, root dipping, and/or compost making were suggested.

3) Rhizome or stem injection with secondary metabolite was suggested for protection or curation the incidence of Fusarium wilt of banana.

4) Production of the secondary metabolite production by solid–state fermentation with various substrates may be producing the different kind of antifungal bioactive compounds.

5) Biocontrol and production in the banana industry can be promoted by the application of these actinomycete products.

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Appendix/Appendices

Appendix A
Culture Media

Aleksandrov agar

$MgSO_4.7H_2O$	0.5	g
$CaCO_3$	0.1	g
$AlKO_6Si_2$	2.0	g
Glucose	5.0	g
$FeCl_3.6H_2O$	0.005	g
$Ca_3(PO_4)_2$	2.0	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Basal inorganic nitrogen medium (acid production medium)

$(NH_4)_2HPO_4$	1.0	g
KCl	0.2	g
$MgSO_4.7H_2O$	0.2	g
Agar	18.0	g
Carbohydrate	10.0	g
0.04% Bromocresol purple	15.0	ml
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at a pressure of 110 °C for 10 min.

Basal medium for nitrogen source

Glucose	10.0	g
$MgSO_4.7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4.7H_2O$	0.01	g
KH_2PO_4	1.0	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at a pressure of 110 °C for 10 min.

Bouillon gelatin broth

Peptone	1.0	g
Meat extract	0.5	g
NaCl	0.5	g
Gelatin	15.0	g
Distilled water	100	ml
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Caboxymethycellulose (CMC) agar

$MgSO_4.7H_2O$	0.1	g
$CaCl_2.2H_2O$	0.2	g
$FeSO_4.7H_2O$	0.04	g
NaCl	0.2	g
KH_2PO_4	0.3	g
K_2HPO_4	0.5	g
Yeast extract	0.1	g
Caboxymethycellulose	10.0	g
Agar	18.0	g
Distilled water	100	ml
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Chitin agar

KH_2PO_4	0.3	g
K_2HPO_4	0.7	g
$MgSO_4.7H_2O$	0.5	g
$FeSO_4.7H_2O$	0.01	g
$ZnSO_4.7H_2O$	0.01	g
$MnCl_2.4H_2O$	0.01	g
$(NH_4)_2SO_4$	0.25	g
Yeast extract	0.2	g
Colloidal chitin	10.0	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Colloidal chitin preparation

About 5 g of chitin was mixed with 30 ml of HCl acid (35.5%) and incubated overnight at 4 °C. The colloidal chitin was precipitated by slowly adding 250 ml of chilled ethanol (50%), with constant mixing at room temperature, and left overnight. The colloidal chitin was centrifuged at 10,000 g for 20 min, and sterile distilled water was used to wash the pellets until the pH value is neutral.

Christensens's urea agar

Peptone	1.0	g
Glucose	1.0	g
NaCl	5.0	g
KH_2PO_4	0.3	g
Phenol red	0.012	g
Agar	18.0	g
Distilled water	900	ml
20% Urea solution*	100	ml

*Dissolve 20 g urea in 100 ml distilled water, filter–sterilize and add aseptically to cooled basal medium (121 °C for 15 min). Mix. Final pH, 6.8 ± 0.1.

Chrome azurol S (CAS) agar

Basal medium (ISP 2 agar)	900	ml
Casamino acid s	0.45	g
Blue dye solution*	100	ml

* Blue dye solution

I. Solution I: 1 mM $FeCl_3.6H_2O$ 5 ml + 10 mM HCl 5 ml

II. Solution II: Chrome Azurol S 60.5 g in distilled water 50 ml

III: Solution III: Hexadecyltrimethylammonium bromide 72.9 mg in distilled water 40 ml

Mix, sterilize by autoclaving at pressure of 110 °C for 10 min, and add aseptically to cooled basal medium (121 °C for 15 min).

Czapek's sucrose agar

Sucrose	30.0	g
$NaNO_3$	2.0	g
K_2HPO_4	1.0	g
$MgSO_4.7H_2O$	0.5	g
KCl	0.5	g
$FeSO_4.7H_2O$	0.01	g

Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Dworkin and Foster's salt minimal–1–aminocyclopropane–1–carboxylate (DF–ACC) agar

$(NH_4)_2SO_4$	2.0	g
KH_2PO_4	4.0	g
Na_2HPO_4	6.0	g
$MgSO_4.7H_2O$	0.2	g
$FeSO_4.7H_2O$	0.001	g
H_3BO_3	10.0	µg
$MnSO_4.H_2O$	10.0	µg
$ZnSO_4.7H_2O$	70.0	µg
$CuSO_4.5H_2O$	50.0	µg
$MoO_3.H_2O$	10.0	µg
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

*Prepare 0.5 mol/L of ACC, filter–sterilize and add aseptically to cooled basal medium with final concentration at 3 mmol/L. Mix. Final pH, 6.8 ± 0.1.

Gelatin agar

Glucose	4.0	g
Yeast extract	4.0	g
Malt extract	10.0	g
Gelatin	6–8	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Glucose–asparagine agar

Glucose	10.0	g
L–Asparagine	0.5	g
K_2HPO_4	0.5	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Glycerol–asparagine agar (ISP 5)

Glycerol	10.0	g
L–Asparagine	1.0	g
K_2HPO_4	1.0	g
Trace salts solution	1.0	ml
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Trace salts solution

$FeSO_4.7H_2O$	0.1	g
$MnCl_2.4H_2O$	0.1	g
$ZnSO_4.7H_2O$	0.1	g
Distilled water	100	ml

Inorganic salts starch agar (ISP 4)

Soluble starch	10.0	g
K_2HPO_4	1.0	g
$MgSO_4.7H_2O$	1.0	g
NaCl	1.0	g
$(NH_4)_2SO_4$	2.0	g
$CaCO_3$	2.0	g
Trace salts solution	1.0	ml
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Carbon utilization agar (ISP 9)

Carbohydrate	10.0	g
$(NH_4)_2SO_4$	2.64	g
K_2HPO_4	5.65	g
KH_2PO_4	2.38	g
$MgSO_4.7H_2O$	1.0	g
Pridham and Gottlieb trace salts	1.0	ml
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

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

M9 medium

NH_4Cl	1.0	g
K_2HPO_4	6.0	g
KH_2PO_4	3.0	g
NaCl	0.5	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Modified soil extract agar (A9) agar

$CaSO_4.H_2O$	0.5	g
$Ca(NO_3)_2.4H_2O$	0.25	g
$MgSO_4.7H_2O$	0.05	g
K_2SO_4	0.03	g
KH_2PO_4	0.02	g
$NaHCO_3$	0.1	g
Trace element mix I	0.3	ml
$CaCl_2.2H_2O$	0.02	g
Yeast extract	0.1	g

Casamino acids	0.1	g
Glucose	0.2	g
Soil extract	100	ml
Agar	18.0	g
Distilled water	900	ml
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Allow the media to cool down to 50 °C before the addition of filter–sterilize trace element mix I.

Soil extract

Humic soil	1	kg
Distilled water	1	L

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Allow it to sediment for a few hours at room temperature. Centrifuge the supernatant.

Trace element mix I

$CaCl_2.2H_2O$	4.0	g
$ZnSO_4.7H_2O$	2.0	g
$Na_2B_4O_7.10H_2O$	0.1	g
$FeSO_4.7H_2O$	5.0	g
KI	0.05	g
$CoCl_2.6H_2O$	0.5	g
$CuSO_4.5H_2O$	0.2	g
$MnCl_2.4H_2O$	2.0	g
$Na_2MoO_4.2H_2O$	0.05	g
H_2SO_4 (95–97% p.a.)	1.0	ml
Distilled water	1	L

National Botanical Research Institute's phosphate growth medium (NBRIP) agar

Glucose	10.0	g
$Ca_3(PO_4)_2$	5.0	g
$MgCl_2.6H_2O$	0.25	g
KCl	0.2	g
$(NH_4)_2SO_4$	0.1	g
Bromophenol blue	0.025	g
Agar	18.0	g

Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Nitrogen–free bromothymol blue (NFb) agar

Malic acid	5.0	g
K_2HPO_4	0.5	g
$MgSO_4.7H_2O$	0.2	g
NaCl	0.1	g
$CaCl_2.2H_2O$	0.02	g
Micronutrient solution	2.0	ml
Bromothymol blue (5 g/L in 0.2 N KOH)	2.0	ml
FeEDTA solution (16.4 g/L)	4.0	ml
Vitamin solution	1.0	ml
KOH	4.5	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Micronutrient solution

$CuSO_4.5H_2O$	0.04	g
$ZnSO_4.7H_2O$	0.12	g
H_3BO_3	1.4	g
$Na_2MoO_4.2H_2O$	1.0	g
MnSO4.H2O	1.175	g
Distilled water	1	L

Vitamin solution

Biotin	10.0	mg
Pyridoxal–HCl	20.0	mg
Distilled water	1	L

Allow the media to cool down to 50 °C before the addition of filter–sterilize vitamin solution.

Nitrogen–free bromothymol blue (NFb) semi–solid medium

NFb medium with agar 6–8 g/L

Nutrient agar

Beef extract	3.0	g
Peptone	5.0	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Oatmeal agar (ISP 3)

Oatmeal	20.0	g
Trace salts solution	1.0	ml
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Peptone potassium nitrate broth

Peptone	1.0	g
KNO_3	0.1	g
NaCl	0.5	g
Distilled water	100	ml
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Peptone water

Peptone	10.0	g
NaCl	5.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Peptone–yeast extract iron agar (ISP 6)

Peptone iron agar	36.0	g
Yeast extract	1.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Peptonization test medium

Solution A

Skim milk	5.0	g
Distilled water	50.0	ml

Sterilize by autoclaving at a pressure of 110 ℃ for 10 min.

Solution B

Agar	1.0	g
Distilled water	50.0	ml

Sterilize by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

Allow the media to cool down to 45 ℃ before the addition the solution A.

Pikovskaya's agar

Yeast extract	0.5	g
Glucose	10.0	g
$Ca_3(PO_4)_2$	5.0	g
$(NH_4)_2SO_4$	0.5	g
K_2SO_4	0.2	g
$MnSO_4.H_2O$	0.1	g
$FeSO_4.7H_2O$	0.0001	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

Potato dextrose agar (PDA)

Potatoes, infusion form	200	g
Dextrose	20.0	g
Agar	15.0	g
Distilled water	1	L
Final pH	5.6 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

Potato dextrose broth (PDB)

Potatoes, infusion form	200	g
Dextrose	20.0	g
Distilled water	1	L
Final pH	5.6 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

10% Skim milk broth

Skim milk	10.0	g
Distilled water	100	ml

Sterilize by autoclaving at a pressure of 110 ℃ for 10 min.

Skim milk agar

Skim milk	2.0	g
Glucose	1.0	g
K_2HPO_4	0.2	g
$FeSO_4.7H_2O$	Trace	
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at a pressure of 110 ℃ for 10 min.

Tributyrin agar

Peptone	5.0	g
Yeast extract	3.0	g
Tributyrin	10.0	ml
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

Triple sugar iron (TSI) agar

Beef extract	3.0	g
Peptone	20.0	g
Yeast extract	3.0	g
Lactose	10.0	g
Sucrose	10.0	g
Dextrose	1.0	g
$FeSO_4.7H_2O$	0.2	g
NaCl	5.0	g
$Na_2S_2O_3.5H_2O$	0.3	g
Phenol red	0.024	g
Agar	12.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (115 ℃) for 15 min.

Tween 80 agar

Peptone	10.0	g
NaCl	5.0	g
$CaCl_2.2H_2O$	0.1	g
Tween 80	10.0	ml
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Tyrosine agar (ISP 7)

Glycerol	15.0	g
L–Tyrosine	0.5	g
L–Asparagine	1.0	g
K_2HPO_4	0.5	g
$MgSO_4 \cdot 7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4 \cdot 7H_2O$	0.01	g
Trace salts solution	1.0	ml
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Yeast extract malt extract agar (ISP 2)

Yeast extract	4.0	g
Glucose	4.0	g
Malt Extract	10.0	g
Agar	18.0	g
Distilled water	1	L
Final pH	7.2 ± 0.2	

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Zhang's starch soil extract (ZSSE) agar

Soluble starch	5.0	g
KNO_3	1.0	g
Agar	18.0	g
Soil extract solution	1	L

Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Appendix B
Reagents and Buffer

1 N H_2SO_4

Conc. H_2SO_4	1.0	ml
Distilled water	34.0	ml

Add conc. H_2SO_4 into distilled water.

6 N HCl

Conc. HCl	60.0	ml
Distiller water	60.0	ml

Add conc. HCl into the distilled water.

5 N Acetic acid

Glacial acetic acid	28.74	ml
Distilled water	71.26	ml

Add gracial acetic acid into the distilled water.

0.4% Ninhydrin solution

Ninhydrin	0.4	g
n–Butanol saturated with water	100	ml

Aniline phthalate

Phthalic acid	3.25	g
Water–saturated n–butanol	100	ml
Aniline	2.0	ml

Reagents for nitrate reduction analysis

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	ml

N, N–dimethyl–1–naphtylamine reagent

N, N–dimethyl–1–naphtylamine	0.5	g
5 N Acetic acid	100	ml

Reagents for fatty acid analysis

Reagent 1 (Sponification reagent)

NaOH	15.0	g
Methanol (HPLC grade)	50.0	ml
Milli Q water	50.0	ml

Dissolve NaOH pellets in milli–Q water and add methanol.

Reagent 2 (Methylation reagent)

6 N HCl	65.0	ml
Methanol (HPLC grade)	55.0	ml

pH below 1.5.

Reagent 3 (Extraction solvent)

N–Hexane (HPLC grade)	50.0	ml
Methyl–3–Buthyl ether (HPLC grade)	50.0	ml

Reagent 4 (Base wash reagent)

NaOH	1.2	g
Milli Q water	100	ml

Reagent 5 (Saturated solution chloride)

NaOH saturated in mili–Q water

Reagents for polar lipid analysis

Anisaldehyde reagent

Ethanol	90.0	ml
Conc. H_2SO_4	5.0	ml
p–Anisaldehyde	5.0	ml
Acetic acid	1.0	ml

Dragendorff's reagent

Solution A:

Basic bismuth nitrate	1.7	g
Acetic acid	20.0	ml
Distilled water	80.0	ml

Solution B:

KI	40.0	g
Distilled water	100	ml

Before spraying, solution A (10 ml) plus solution B (10 ml) and acetic acid (10 ml), were mixed.

Phosphomolybdic acid reagent

Phosphomolybdic acid	5.0	g
Absolute ethanol	100.0	ml

Salkowski's reagent

0.5 M $FeCl_3.6H_2O$	2.0	ml
35% Perchloric acid	98.0	ml

Iodine solution

Iodine	5.0	g
KI	10.0	g
Distilled water	100	ml

Acid mercuric chloride solution

$HgCl_2$	15.0	g
Conc. HCl	20.0	ml
Distilled water	100	ml

Nessler's reagent

KI	50.0	g
Saturated $HgCl_2$ solution	20.0	ml
50% KOH	400	ml
Distilled water	580	ml

50X TAE buffer

Tris base	24.2	g
Glacial acetic acid	57.1	ml
0.5 M EDTA	10.0	ml
Add milli Q water up to	100	ml

TE buffer

10 mM Tris–HCl (pH 8)	10.0	ml
1 mM Na_2–EDTA (pH 8)	10.0	ml
Milli Q water	980	ml

The solution was sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

TES solution

10 mM Tris–HCl (pH 8)	10.0	ml
10 mM Na_2–EDTA (pH 8)	1.0	ml
2% SDS	20.0	ml
Milli Q water	69.0	ml

The solution was sterilized by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

5 M ammonium acetate

NH_4Ac	385.4	g
Milli Q water	1	L

The solution was sterilized by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

1 M Tris–HCl (pH 8)

Tris	121.1	g
Milli Q water	800	ml

The 1 M Tris was prepared by dissolving 121.1 g of Tris base in 800 ml of distilled water. The pH was adjusted to the desired value by adding conc. HCl (pH 8). The solution was cooled to room temperature before making a final adjustment to the desired pH. The volume of the solution was adjusted to 1 liter with Milli Q water and sterilized by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

1 mM Na2–EDTA (pH 8) Saline-EDTA

EDTA	292.24	mg
Distilled water	700	ml

292.24 mg of EDTA was dissolved in distilled water and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8 with NaOH pellets. The volume was adjusted with Milli Q water to 1 liter and sterilized by autoclaving at 15 lbs pressure (121 ℃) for 15 min.

Phosphate buffer (pH 8)

1 M K_2HPO_4	94.0	ml
1 M KH_2PO_4	6.0	ml

10% Sodium dodecyl sulfate (SDS)

The stock solution of 10% SDS was prepared by dissolving 10 g of sodium dodecyl sulfate in 100 ml Milli Q water. Sterilization is not required for the preparation of this stock solution.

5 M NaCl

NaCl	292.2	g
Milli Q water	1	L

CTAB/NaCl

CTAB	10	g
NaCl	4.1	g
Milli Q water	100	ml

The solution was sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Phenol:Chloroform (1:1 v/v)

Crystalline phenol was liquified in distilled water at 65 °C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a light–tight bottle at 4 °C.

Lysozyme (50 mg/ml)

Lysozyme	0.5	g
1 M Tris–HCl (pH 8)	10.0	ml

Proteinase K

Proteinase K	4.0	mg
50 mM Tris–HCl (pH 8)	1.0	ml

Use freshly prepared solution.

RNase A solution

RNase A	20	mg
0.15 M NaCl	10	ml

Dissolve 20 mg of RNase A in 10 ml 0.15 M NaCl and heat at 95 °C for 5–10 minutes. Keep RNase A solution in –20 °C.

Agarosea gel 0.8% (stained gel)

Agarose	0.8	g
1X TBE or TAE buffer	100	ml
SYBR® safe DNA gel stain	1.0	µl

Appendix C
Standard Curve

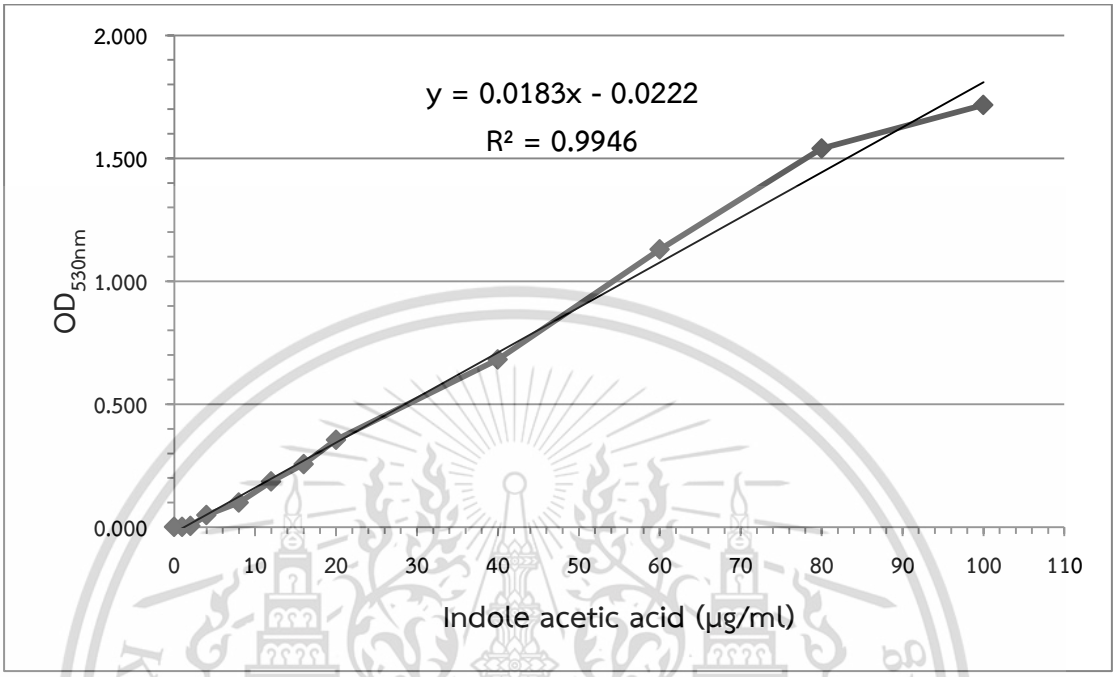

Figure 1 Standard curve of indole acetic acid.

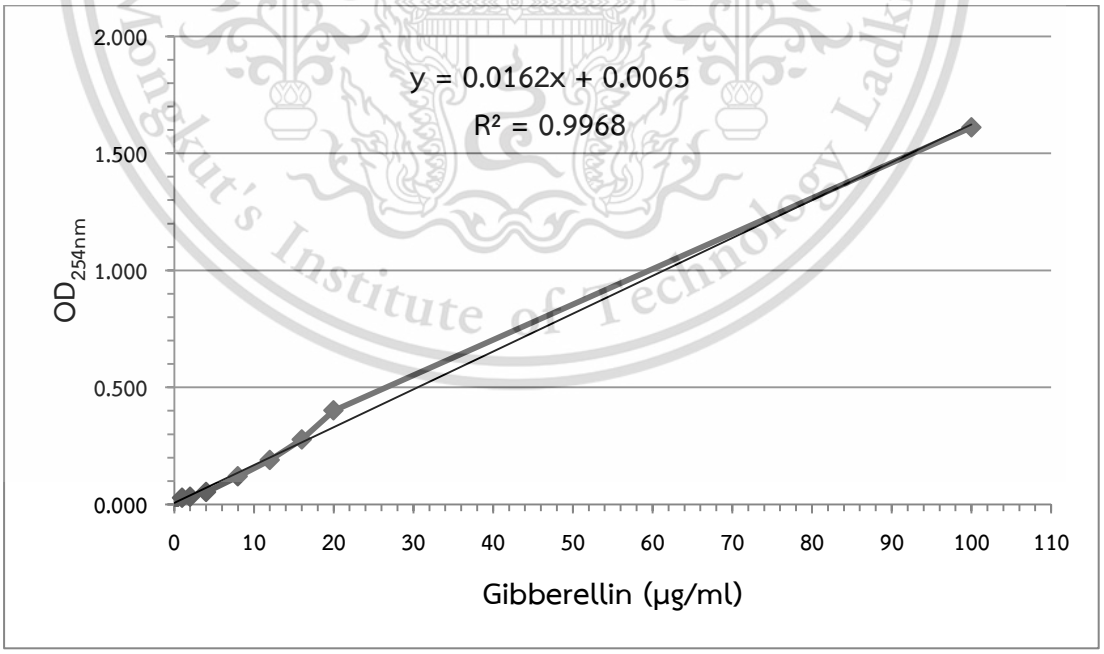

Figure 2 Standard curve of Gibberellin.

Appendix D
Soil Composition

กลุ่มวิเคราะห์ดิน สำนักงานพัฒนาที่ดินเขต 1

ผู้อำนวยการกลุ่ม น.ส. กัญจน์รัชต์ ลชิตาวงศ์

ชนิด/จำนวนตัวอย่าง ดิน 1 ตัวอย่าง — เลขที่รับ L221 /62

ผู้ส่งตัวอย่าง นายฐิติกร ดวงอุปมา — เลขที่ตัวอย่าง L62 6263

จังหวัด/ที่มาของตัวอย่าง โครงการวิจัยเรื่อง การใช้เชื้อแบคทีเรียในการส่งเสริมการเจริญของพืช — วันที่รับตัวอย่าง 5 กรกฎาคม 2562

ลำดับที่	เลขที่ปฏิบัติการ	รายละเอียด	ระดับ	พิกัด		[1] pH ดิน:น้ำ 1:1	[2] LR *$CaCO_3$ (กก./ไร่)	[3] EC (dS/m) ดิน:น้ำ 1:5	[4] OM (%) Walkley and Black	ไนโตรเจน (%) Kjeldahl	ฟอสฟอรัส ($mg\ kg^{-1}$) Bray II	โพแทสเซียม ($mg\ kg^{-1}$) NH_4OAc pH 7.0
				X	Y							
1	L62-6563	ตัวอย่าง 1				6.4	-	0.995	0.62	0.031	7	48

1.ความเป็นกรด/ด่าง (pH) ดิน : น้ำ อัตราส่วน 1: 1 2.ความต้องการปูน (Lime Requirement) Woodruff Buffer 3.ค่าการนำไฟฟ้า (Electrical Conductivity) ดิน : น้ำ อัตราส่วน 1: 5 4.อินทรียวัตถุ (Organic matter) วิธี Walkley and Black Total Nitrogen (N) วิธี Kjeldahl Available Phosphorus (P) สกัดด้วย Bray II Available- Potassium (K), สกัดด้วย NH_4OAc pH 7.0

* ค่าความต้องการปูนได้แก่ $CaCO_3$ 100 กก. เทียบเท่ากับปริมาณปูนขาว 78 กก. หรือปูนมาร์ล 120 กก. หรือหินปูนบด 150 กก. หรือปูนโดโลไมท์ 109 กก.

ค่ามาตรฐานที่ใช้เปรียบเทียบสมบัติทางเคมีของดิน

ระดับ	อินทรียวัตถุ (OM,%) Walkley and Black	ระดับธาตุอาหารหลัก ธาตุอาหารรอง รูปที่เป็นประโยชน์ต่อพืชในดิน						
		ไนโตรเจน (N,%) วิธี Kjeldahl	ฟอสฟอรัส (P,$mg\ Kg^{-1}$) สกัดด้วย Bray II	สกัดด้วย NH_4OAc pH 7.0				ซัลเฟอร์ (S,$mg\ Kg^{-1}$) สกัดด้วย NH_4OAc pH 5.0
				โพแทสเซียม (K,$mg\ Kg^{-1}$)	แคลเซียม (Ca,$mg\ Kg^{-1}$)	แมกนีเซียม (Mg,$mg\ Kg^{-1}$)	โซเดียม (Na,$mg\ Kg^{-1}$)	
ต่ำมาก	<0.5	< 0.10	<3	<30	<400	<36	<25	<5
ต่ำ	0.5-1.5	0.10 - 0.30	3-10	30-60	401-1000	36-120	25-70	5-10
ปานกลาง	1.5-2.5	0.31 - 0.60	11-15	61-90	1001-2000	121-365	70-160	11-20
สูง	2.5-4.5	0.61 - 1.0	16-45	91-120	2001-4000	366-975	160-450	21-30
สูงมาก	>4.5	> 1.0	>45	>120	>4000	>975	>450	>30

ที่มา: Standard rating of USDA อ้างโดย [illegible] (2547)

Figure 1 Soil composition for *in planta* experiment.

Appendix E
Plant Growth Promotion and Disease Control Experimental Data

Protective experiment

Table 1 Leaf symptom index (LSI) from the protective effect of actinomycete products with *Foc* strains on banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	3.25 ± 1.26^{cd}	3.25 ± 1.26^{cd}	4.00 ± 1.16^{d}	1.00 ± 0.50^{a}
Carrier (well compost)	3.25 ± 1.26^{cd}	2.75 ± 1.50^{bcd}	3.25 ± 1.26^{cd}	1.00 ± 0.00^{a}
2% Carbendazim	2.75 ± 1.50^{bcd}	2.50 ± 1.73$^{a–d}$	2.75 ± 1.50^{bcd}	1.00 ± 0.00^{a}
Commercial *Trichoderma harzianum*	2.25 ± 1.89^{abc}	2.50 ± 0.58$^{a–d}$	2.50 ± 1.00$^{a–d}$	1.00 ± 0.00^{a}
ATU–Bio 1	1.75 ± 0.50^{abc}	2.00 ± 0.00^{abc}	1.50 ± 0.58^{ab}	1.00 ± 0.00^{a}
ATU–Bio 2	2.00 ± 0.82^{abc}	2.00 ± 0.00^{abc}	2.25 ± 0.96^{abc}	1.00 ± 0.00^{a}
ATU–Bio 3	1.75 ± 0.96^{abc}	2.25 ± 0.96^{abc}	2.00 ± 0.00^{abc}	1.00 ± 0.00^{a}

Note: Mean ± Standard deviation. Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

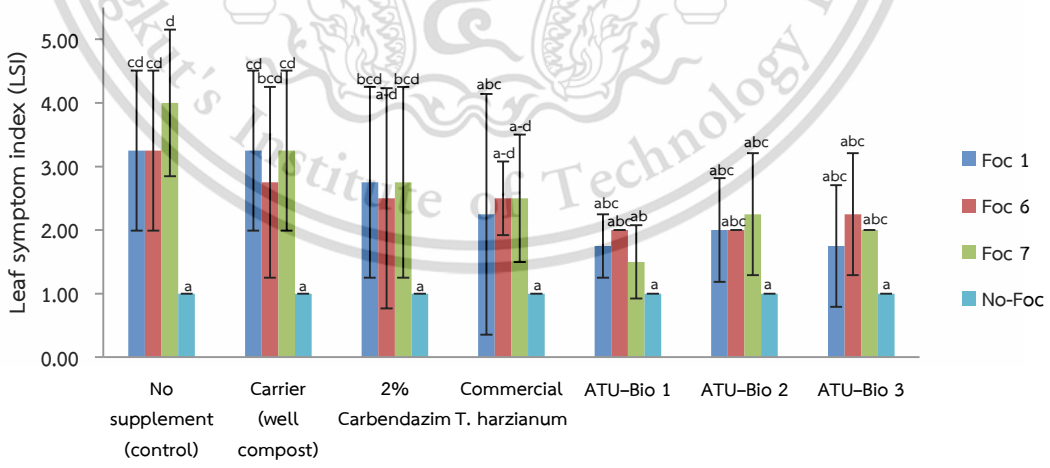

Figure 1 Leaf symptom index (LSI) from the protective effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation of four replications.

Table 2 Rhizome discoloration index (RDI) from the protective effect of actinomycete products with *Foc* strains on banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	6.50 ± 1.00^{def}	6.75 ± 1.26^{ef}	7.00 ± 1.16^{f}	1.00 ± 0.00^{a}
Carrier (well compost)	6.75 ± 1.71^{ef}	5.25 ± 1.89^{b-f}	5.50 ± 1.73^{b-f}	1.00 ± 0.00^{a}
2% Carbendazim	5.00 ± 2.00^{b-f}	4.75 ± 2.22^{b-f}	4.50 ± 2.38^{b-f}	1.00 ± 0.00^{a}
Commercial *Trichoderma harzianum*	4.50 ± 2.65^{b-f}	4.75 ± 2.06^{b-f}	5.00 ± 1.41^{b-f}	1.00 ± 0.00^{a}
ATU–Bio 1	3.25 ± 0.50^{abc}	4.00 ± 0.82^{bcd}	3.00 ± 2.00^{ab}	1.00 ± 0.00^{a}
ATU–Bio 2	4.00 ± 1.83^{bcd}	4.25 ± 1.26^{b-e}	3.50 ± 2.38^{abc}	1.00 ± 0.00^{a}
ATU–Bio 3	3.50 ± 1.92^{abc}	4.00 ± 1.83^{bcd}	4.00 ± 0.82^{bcd}	1.00 ± 0.00^{a}

Note: Mean ± Standard deviation. Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

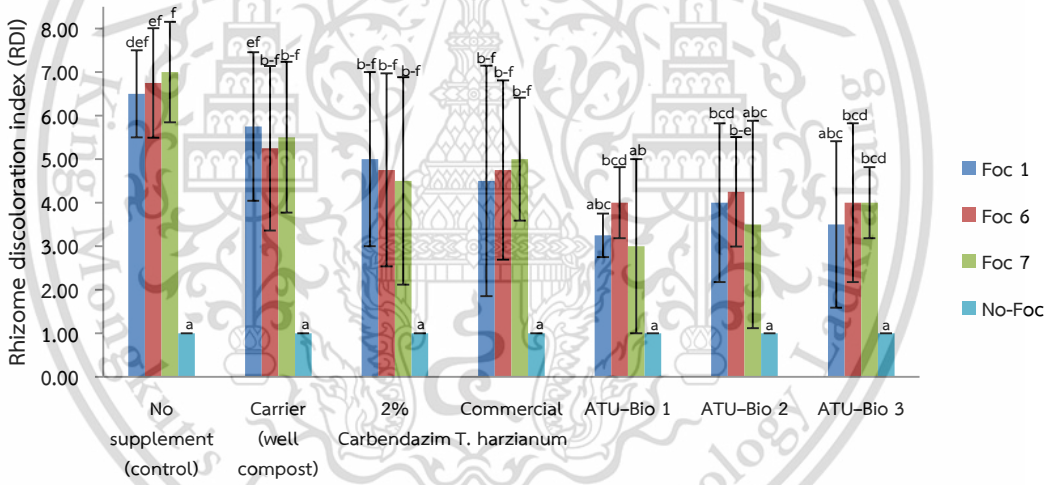

Figure 2 Rhizome discoloration index (RDI) from the protective effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation of four replications.

Table 3 Protective effect of actinomycete products with *Foc* strains on plant height (cm) of banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	4.60 ± 0.20^{j}	4.40 ± 0.79^{j}	3.65 ± 0.35^{j}	6.98 ± 0.21^{i}
Carrier (well compost)	9.40 ± 0.69^{fg}	9.03 ± 0.68^{fg}	7.70 ± 0.46^{hi}	12.70 ± 1.31^{b}
2% Carbendazim	7.23 ± 1.24^{i}	7.23 ± 0.98^{i}	7.50 ± 0.56^{i}	7.18 ± 0.26^{i}
Commercial *Trichoderma harzianum*	8.85 ± 0.50^{gh}	7.30 ± 0.14^{i}	7.23 ± 0.25^{i}	9.80 ± 1.10^{efg}
ATU–Bio 1	12.10 ± 0.56^{bc}	12.07 ± 0.65^{bc}	11.33 ± 0.72^{cd}	14.88 ± 0.46^{a}
ATU–Bio 2	11.10 ± 0.42^{cde}	9.40 ± 0.53^{fg}	10.35 ± 0.21^{def}	13.18 ± 1.08^{b}
ATU–Bio 3	9.70 ± 0.76^{fg}	9.80 ± 1.13^{efg}	9.20 ± 0.44^{fg}	12.75 ± 0.44^{b}

Note: Mean ± Standard deviation. Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

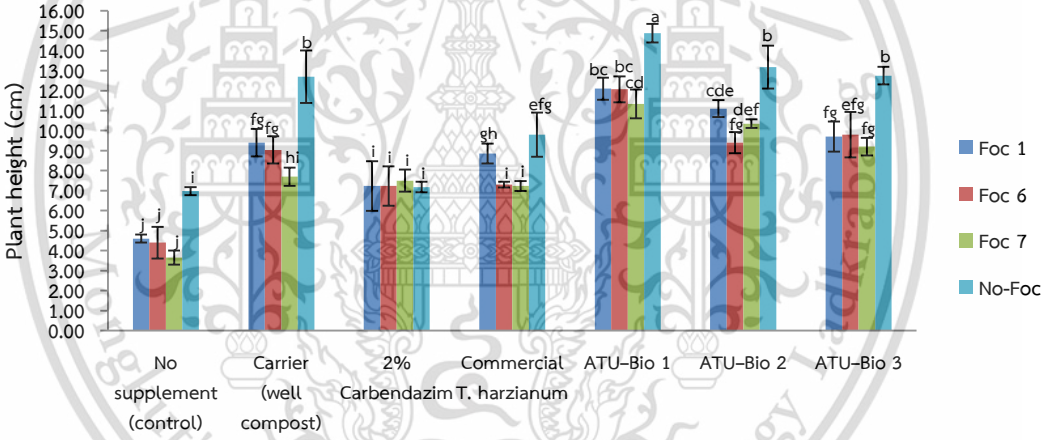

Figure 3 Plant height (cm) of banana plantlets from the protective effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

Table 4 Protective effect of actinomycete products with *Foc* strains on root length (cm) of banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	6.03 ± 1.53^{j}	5.23 ± 0.59^{j}	4.50 ± 0.42^{j}	8.88 ± 0.44^{i}
Carrier (well compost)	10.83 ± 0.29^{ghi}	11.00 ± 0.89^{f-i}	9.97 ± 0.55^{hi}	19.15 ± 2.22^{bc}
2% Carbendazim	11.73 ± 1.62^{f-i}	10.10 ± 1.02^{hi}	11.17 ± 0.86^{fgh}	12.08 ± 0.94^{fgh}
Commercial *Trichoderma harzianum*	11.60 ± 1.98^{fgh}	15.50 ± 0.71^{de}	13.13 ± 1.21^{efg}	13.55 ± 1.06^{ef}
ATU–Bio 1	20.73 ± 1.42^{ab}	20.83 ± 1.76^{ab}	17.33 ± 0.76^{cd}	22.58 ± 1.10^{a}
ATU–Bio 2	18.90 ± 0.85^{bc}	16.93 ± 2.64^{cd}	17.85 ± 1.91^{cd}	22.58 ± 1.31^{a}
ATU–Bio 3	18.03 ± 2.05^{cd}	16.75 ± 1.35^{cd}	22.50 ± 0.87^{a}	22.60 ± 2.05^{a}

Note: Mean ± Standard deviation. Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

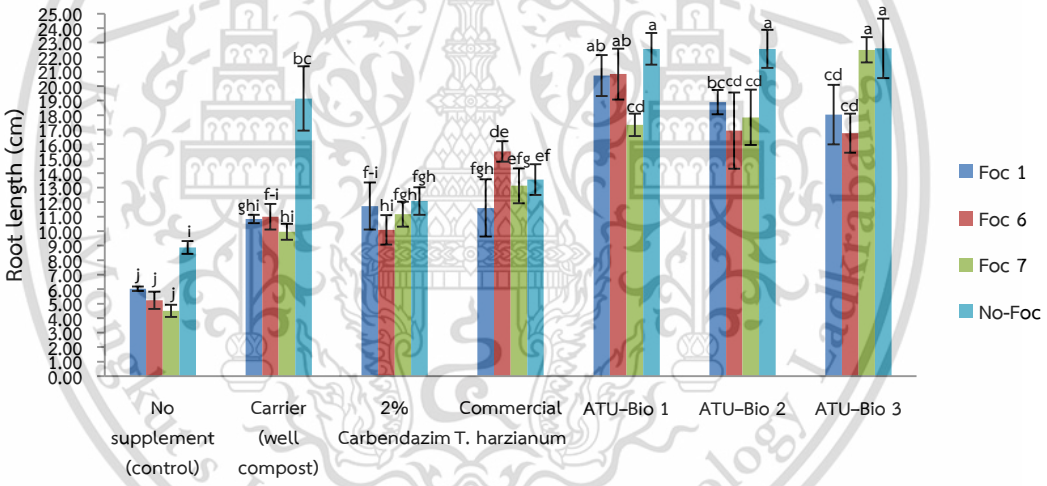

Figure 4 Root length (cm) of banana plantlets from the protective effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

Table 5 Protective effect of actinomycete products with *Foc* strains on stem diameter (cm) of banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	0.45 ± 0.05^{g}	0.50 ± 0.10^{g}	0.40 ± 0.10^{g}	0.80 ± 0.12^{def}
Carrier (well compost)	0.77 ± 0.12^{ef}	0.67 ± 0.12^{fg}	0.67 ± 0.58^{fg}	1.03 ± 0.21^{a-e}
2% Carbendazim	0.80 ± 0.10^{def}	0.80 ± 0.10^{def}	0.80 ± 0.10^{def}	0.80 ± 0.10^{def}
Commercial *Trichoderma harzianum*	0.85 ± 0.07^{c-f}	0.95 ± 0.07^{b-f}	0.90 ± 0.10^{b-f}	0.85 ± 0.13^{c-f}
ATU–Bio 1	1.00 ± 0.27^{a-e}	0.93 ± 0.32^{b-f}	0.97 ± 0.15^{a-e}	1.25 ± 0.19^{a}
ATU–Bio 2	1.00 ± 0.14^{a-e}	1.00 ± 0.14^{a-e}	1.15 ± 0.07^{ab}	1.10 ± 0.18^{abc}
ATU–Bio 3	1.00 ± 0.10^{a-e}	0.90 ± 0.14^{b-f}	0.90 ± 0.17^{b-f}	1.08 ± 0.10^{a-d}

Note: Mean ± Standard deviation. Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

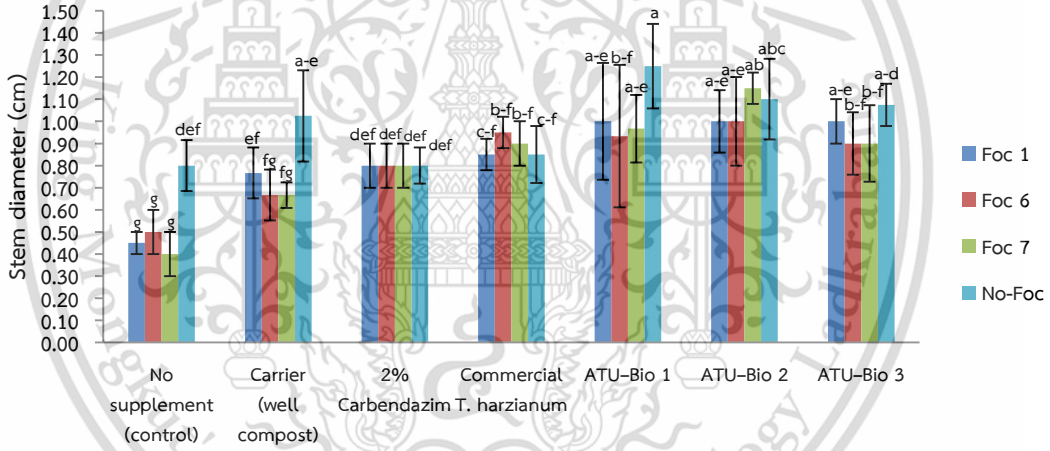

Figure 5 Stem diameter (cm) of banana plantlets from the protective effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

Table 6 Protective effect of actinomycete products with *Foc* strains on fresh weight (g) of banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	1.33 ± 0.21^{j}	1.17 ± 0.15^{j}	1.25 ± 0.07^{j}	2.85 ± 0.34^{i}
Carrier (well compost)	3.10 ± 0.14^{hi}	3.10 ± 0.27^{hi}	3.27 ± 0.31^{ghi}	4.95 ± 0.74^{cd}
2% Carbendazim	3.97 ± 0.15^{ef}	4.00 ± 0.10^{ef}	3.87 ± 0.25^{efg}	4.38 ± 0.21^{de}
Commercial *Trichoderma harzianum*	3.60 ± 0.57^{fgh}	3.65 ± 0.21^{fgh}	3.60 ± 0.53^{fgh}	4.80 ± 0.41^{cd}
ATU–Bio 1	5.10 ± 0.30^{c}	5.10 ± 0.30^{c}	4.97 ± 0.25^{cd}	7.05 ± 0.42^{a}
ATU–Bio 2	4.50 ± 0.42^{cde}	4.87 ± 0.15^{cd}	4.90 ± 0.28^{cd}	6.53 ± 0.46^{ab}
ATU–Bio 3	4.67 ± 0.42^{cd}	4.75 ± 0.35^{cd}	4.97 ± 0.15^{cd}	6.13 ± 0.22^{b}

Note: Mean ± Standard deviation. Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

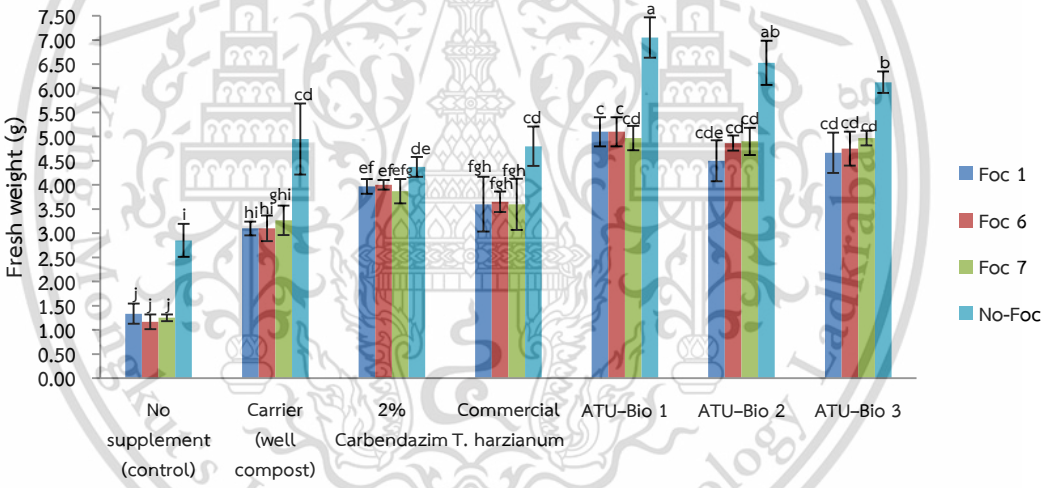

Figure 6 Fresh weight (g) of banana plantlets from the protective effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

Table 7 Protective effect of actinomycete products with *Foc* strains on dry weight (g) of banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	0.13 ± 0.02^{h}	0.12 ± 0.01^{h}	0.13 ± 0.01^{h}	0.31 ± 0.04^{fg}
Carrier (well compost)	0.31 ± 0.04^{fg}	0.31 ± 0.02^{fg}	0.33 ± 0.03^{efg}	0.51 ± 0.10^{b}
2% Carbendazim	0.40 ± 0.03^{de}	0.37 ± 0.01^{efg}	0.38 ± 0.03^{efg}	0.41 ± 0.01^{cde}
Commercial *Trichoderma harzianum*	0.37 ± 0.04^{efg}	0.36 ± 0.01^{efg}	0.38 ± 0.07^{def}	0.50 ± 0.06^{b}
ATU–Bio 1	0.52 ± 0.04^{b}	0.49 ± 0.04^{b}	0.50 ± 0.02^{b}	0.66 ± 0.02^{a}
ATU–Bio 2	0.45 ± 0.05^{bcd}	0.49 ± 0.01^{b}	0.51 ± 0.01^{b}	0.62 ± 0.02^{a}
ATU–Bio 3	0.47 ± 0.05^{bc}	0.50 ± 0.01^{b}	0.50 ± 0.02^{b}	0.60 ± 0.02^{a}

Note: Mean ± Standard deviation. Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

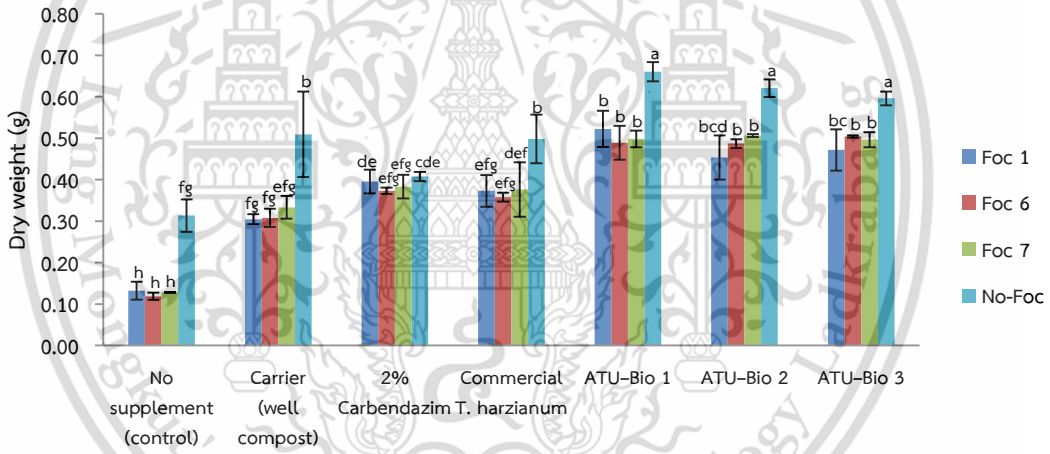

Figure 7 Dry weight (g) of banana plantlets from the protective effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation.

Curative experiment

Table 8 Leaf symptom index (LSI) from the curative effect of actinomycetes products with *Foc* strains on banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	4.75 ± 0.50^{bc}	5.00 ± 0.00^{d}	4.75 ± 0.50^{bc}	1.00 ± 0.00^{a}
Carrier (well compost)	4.75 ± 0.50^{bc}	4.75 ± 0.00^{bc}	4.50 ± 0.58^{bc}	1.00 ± 0.00^{a}
2% Carbendazim	3.75 ± 0.96^{b}	4.50 ± 1.00^{bc}	4.25 ± 0.96^{bc}	1.00 ± 0.00^{a}
Commercial *Trichoderma harzianum*	4.25 ± 0.96^{bc}	4.50 ± 1.00^{bc}	5.00 ± 1.00^{d}	1.00 ± 0.00^{a}
ATU–Bio 1	4.50 ± 1.00^{bc}	3.75 ± 0.96^{b}	3.75 ± 0.96^{b}	1.00 ± 0.00^{a}
ATU–Bio 2	5.00 ± 0.00^{d}	4.25 ± 0.96^{bc}	4.50 ± 1.00^{bc}	1.00 ± 0.00^{a}
ATU–Bio 3	5.00 ± 0.00^{d}	4.50 ± 1.00^{bc}	4.25 ± 0.96^{bc}	1.00 ± 0.00^{a}

Note: Mean ± Standard deviation. Different lower-case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

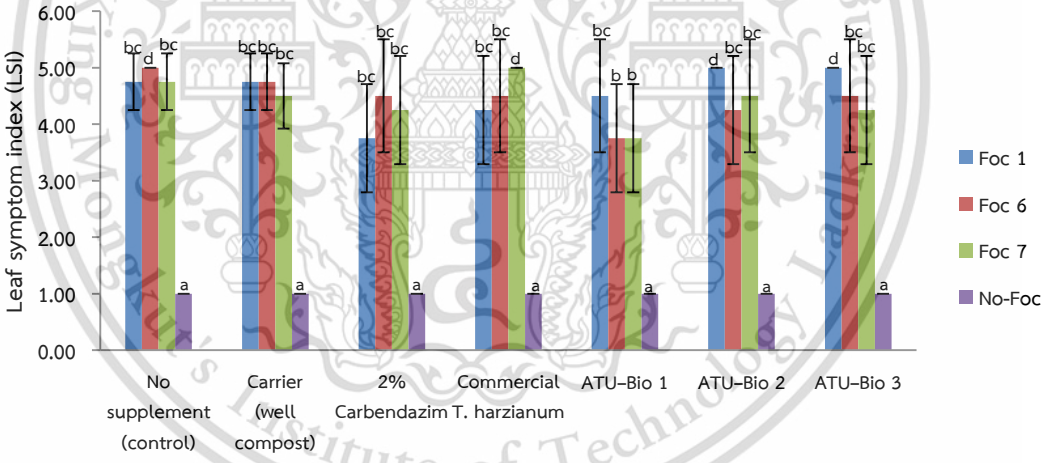

Figure 8 Leaf symptom index (LSI) from the curative effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation of four replications.

Table 9 Rhizome discoloration index (RDI) from the curative effect of actinomycetes products with *Foc* strains on banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	7.75 ± 0.50^{cd}	8.00 ± 0.00^{d}	7.75 ± 0.50^{cd}	1.00 ± 0.00^{a}
Carrier (well compost)	7.75 ± 0.50^{cd}	7.75 ± 0.50^{cd}	7.25 ± 0.96^{cd}	1.00 ± 0.00^{a}
2% Carbendazim	6.50 ± 1.29^{bc}	7.50 ± 1.00^{cd}	7.25 ± 0.96^{cd}	1.00 ± 0.00^{a}
Commercial *Trichoderma harzianum*	7.25 ± 0.46^{cd}	7.50 ± 1.00^{cd}	8.00 ± 0.00^{d}	1.00 ± 0.00^{a}
ATU–Bio 1	7.50 ± 1.00^{cd}	6.00 ± 1.41^{b}	6.50 ± 1.29^{bc}	1.00 ± 0.00^{a}
ATU–Bio 2	8.00 ± 0.00^{d}	7.25 ± 0.96^{cd}	7.50 ± 1.00^{cd}	1.00 ± 0.00^{a}
ATU–Bio 3	8.00 ± 0.00^{d}	7.50 ± 1.00^{cd}	7.25 ± 0.96^{cd}	1.00 ± 0.00^{a}

Note: Mean ± Standard deviation. Different lower–case letters indicated a significant difference at the *P<0.05* levels by Duncan's new multiple range test (DMRT).

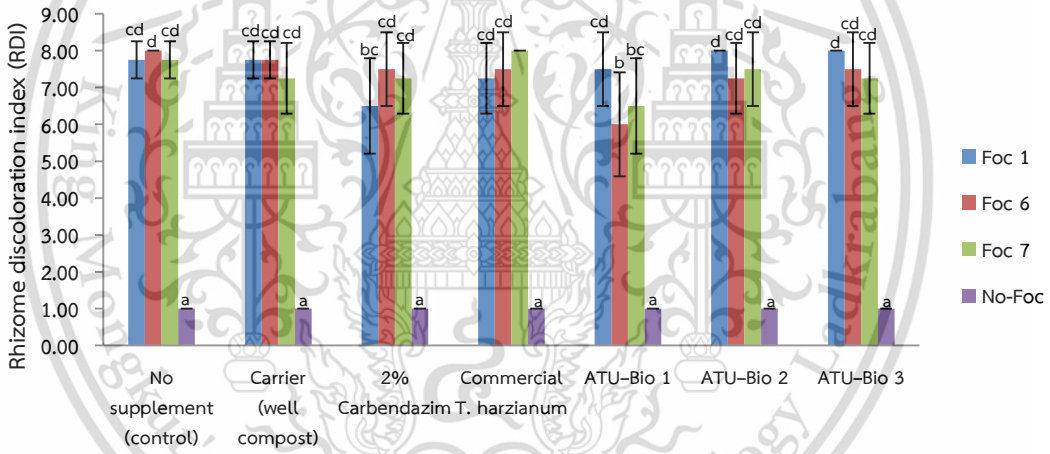

Figure 9 Rhizome discoloration index (RDI) from the curative effect of actinomycete products with *Foc* strains on banana plantlets. In each figure, bars with significantly different values by Duncan's new multiple range test (DMRT) (P<*0.05)* are marked with different letters. Error bars indicate the standard deviation of four replications.

Table 10 Curative effect of actinomycete products with *Foc* strains on plant height (cm) of remaining banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	2.80 ± 0.00	0.00 ± 0.00	2.50 ± 0.00	6.65 ± 0.39
Carrier (well compost)	4.50 ± 0.00	3.20 ± 0.00	3.30 ± 0.28	12.43 ± 0.94
2% Carbendazim	3.87 ± 0.31	3.50 ± 0.00	3.65 ± 0.08	7.08 ± 0.18
Commercial *Trichoderma harzianum*	3.90 ± 0.28	3.80 ± 0.00	0.00 ± 0.00	9.65 ± 0.94
ATU–Bio 1	4.60 ± 0.00	4.67 ± 0.38	4.60 ± 0.95	14.43 ± 0.56
ATU–Bio 2	0.00 ± 0.00	4.45 ± 0.07	4.30 ± 0.00	13.05 ± 0.81
ATU–Bio 3	0.00 ± 0.00	4.00 ± 0.06	4.25 ± 0.07	12.78 ± 0.53

Note: Mean ± Standard deviation.

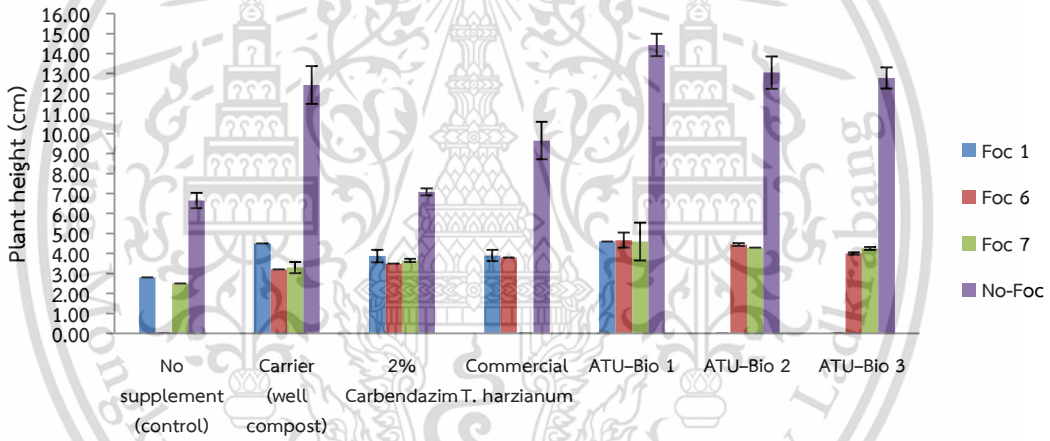

Figure 4.10 Plant height (cm) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* strains on banana plantlets. Error bars indicate the standard deviation.

Table 11 Curative effect of actinomycete products with *Foc* strains on root length (cm) of remaining banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	3.20 ± 0.00	0.00 ± 0.00	3.00 ± 0.00	8.73 ± 0.49
Carrier (well compost)	5.20 ± 0.00	3.60 ± 0.00	4.15 ± 0.21	18.38 ± 1.60
2% Carbendazim	5.03 ± 0.25	4.40 ± 0.00	4.90 ± 0.14	11.98 ± 0.87
Commercial *Trichoderma harzianum*	4.90 ± 0.14	5.00 ± 0.00	0.00 ± 0.00	13.60 ± 0.91
ATU–Bio 1	6.00 ± 0.90	6.07 ± 0.90	6.47 ± 1.37	22.20 ± 0.48
ATU–Bio 2	0.00 ± 0.00	5.85 ± 0.21	5.60 ± 0.00	22.48 ± 1.21
ATU–Bio 3	0.00 ± 0.00	5.20 ± 0.00	5.60 ± 0.28	22.55 ± 2.14

Note: Mean ± Standard deviation.

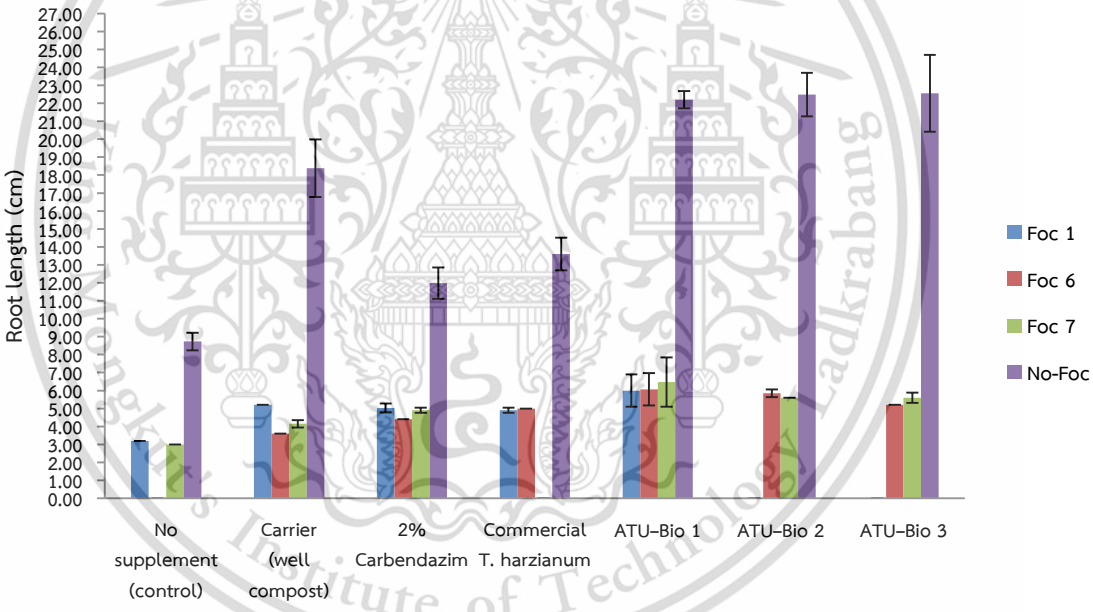

Figure 4.11 Root length (cm) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* strains on banana plantlets. Error bars indicate the standard deviation.

Table 12 Curative effect of actinomycete products with *Foc* strains on stem diameter (cm) of remaining banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	0.30 ± 0.00	0.00 ± 0.00	0.30 ± 0.00	0.80 ± 0.08
Carrier (well compost)	0.40 ± 0.00	0.40 ± 0.00	0.35 ± 0.07	0.90 ± 0.18
2% Carbendazim	0.43 ± 0.15	0.40 ± 0.00	0.35 ± 0.07	0.88 ± 0.10
Commercial *Trichoderma harzianum*	0.50 ± 0.00	0.50 ± 0.00	0.00 ± 0.00	0.88 ± 0.05
ATU–Bio 1	0.60 ± 0.00	0.57 ± 0.06	0.53 ± 0.12	1.15 ± 0.13
ATU–Bio 2	0.00 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	1.05 ± 0.17
ATU–Bio 3	0.00 ± 0.00	0.50 ± 0.00	0.55 ± 0.07	1.10 ± 0.08

Note: Mean ± Standard deviation.

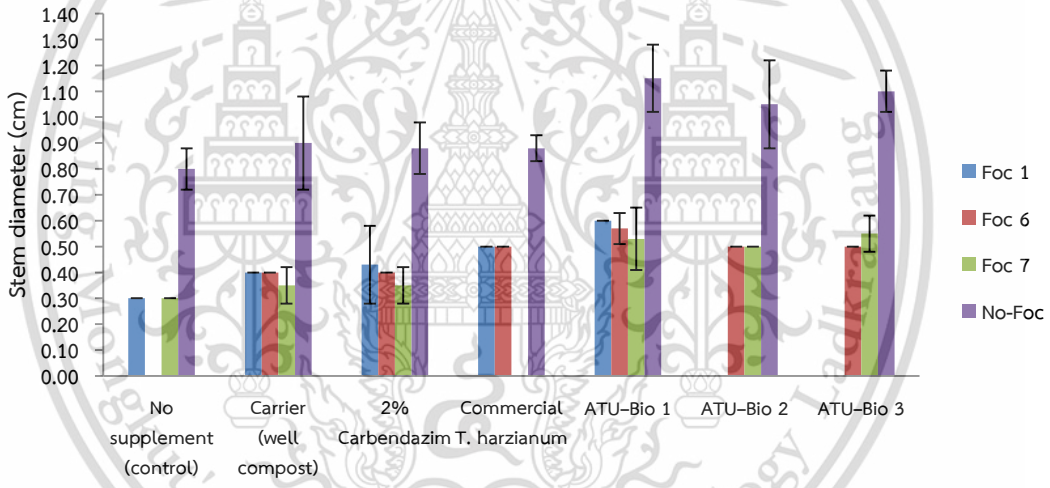

Figure 4.12 Stem diameter (cm) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* strains on banana plantlets. Error bars indicate the standard deviation.

Table 13 Curative effect of actinomycete products with *Foc* strains on fresh weight (g) of remaining banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	0.80 ± 0.00	0.00 ± 0.00	1.00 ± 0.00	2.78 ± 0.34
Carrier (well compost)	1.30 ± 0.00	1.30 ± 0.00	1.30 ± 0.14	4.90 ± 0.59
2% Carbendazim	1.23 ± 0.21	1.30 ± 0.00	1.25 ± 0.07	4.28 ± 0.28
Commercial *Trichoderma harzianum*	1.35 ± 0.07	1.40 ± 0.00	0.00 ± 0.00	4.65 ± 0.33
ATU–Bio 1	1.50 ± 0.00	1.53 ± 0.01	1.50 ± 0.10	6.98 ± 0.33
ATU–Bio 2	0.00 ± 0.00	1.45 ± 0.07	1.40 ± 0.00	6.70 ± 0.85
ATU–Bio 3	0.00 ± 0.00	1.40 ± 0.00	1.35 ± 0.07	6.13 ± 0.15

Note: Mean ± Standard deviation.

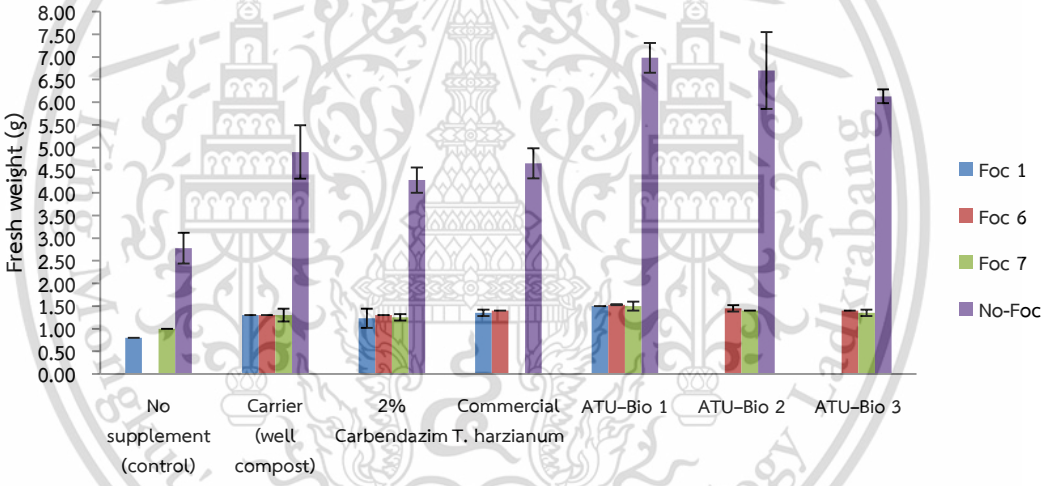

Figure 4.13 Fresh weight (g) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* strains on banana plantlets. Error bars indicate the standard deviation.

Table 14 Curative effect of actinomycete products with *Foc* strains on dry weight (g) of remaining banana plantlets.

Treatments	*Foc* strains			
	Foc 1	*Foc* 6	*Foc* 7	No *Foc* (control)
No supplement (control)	0.08 ± 0.00	0.00 ± 0.00	0.10 ± 0.00	0.31 ± 0.04
Carrier (well compost)	0.13 ± 0.00	0.13 ± 0.00	0.13 ± 0.01	0.50 ± 0.09
2% Carbendazim	0.13 ± 0.02	0.13 ± 0.00	0.14 ± 0.01	0.40 ± 0.02
Commercial *Trichoderma harzianum*	0.14 ± 0.02	0.14 ± 0.00	0.00 ± 0.00	0.48 ± 0.05
ATU–Bio 1	0.16 ± 0.00	0.16 ± 0.01	0.15 ± 0.01	0.65 ± 0.01
ATU–Bio 2	0.00 ± 0.00	0.14 ± 0.01	0.14 ± 0.00	0.64 ± 0.06
ATU–Bio 3	0.00 ± 0.00	0.16 ± 0.00	0.14 ± 0.01	0.60 ± 0.02

Note: Mean ± Standard deviation.

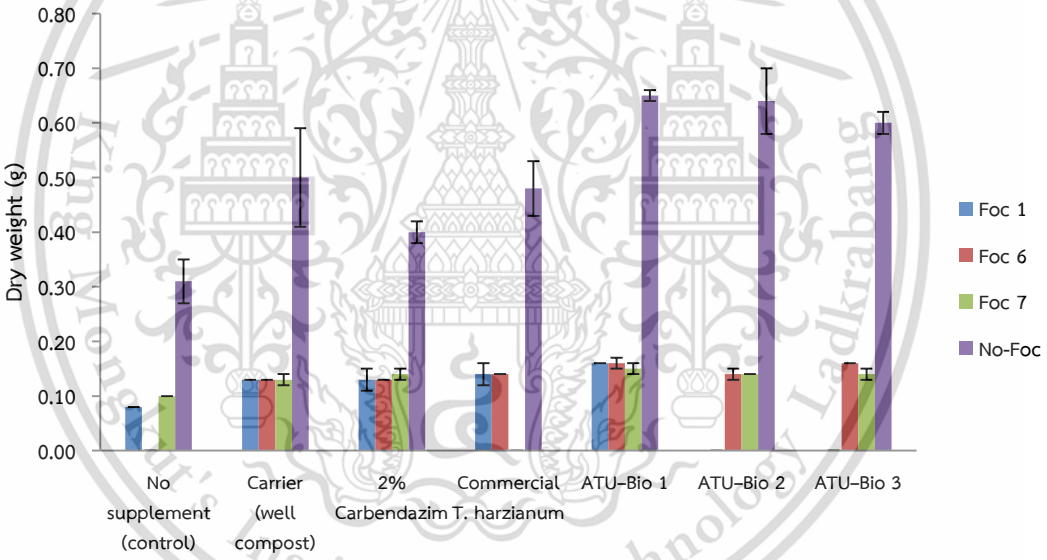

Figure 4.14 Dry weight (g) of remaining banana plantlets from the curative effect of actinomycete products with *Foc* strains on banana plantlets. Error bars indicate the standard deviation.

Appendix F
Spectral Data

Albocycline

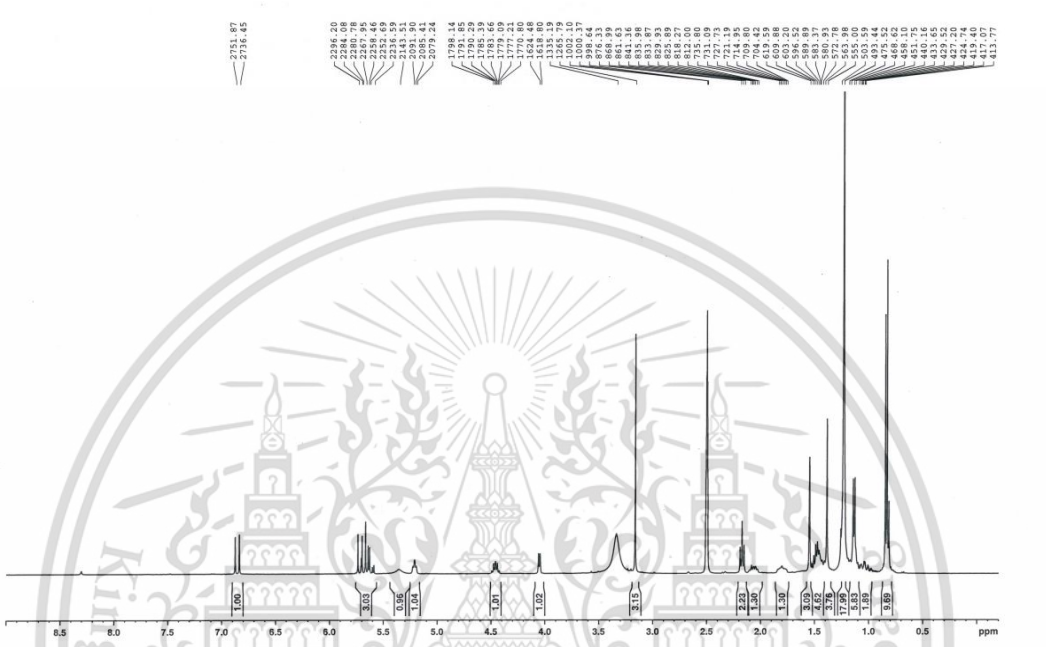

Figure 1 ^{1}H NMR spectrum (400 MHz, DMSO–d_6) of compound A (CH5–8 F2F2H12).

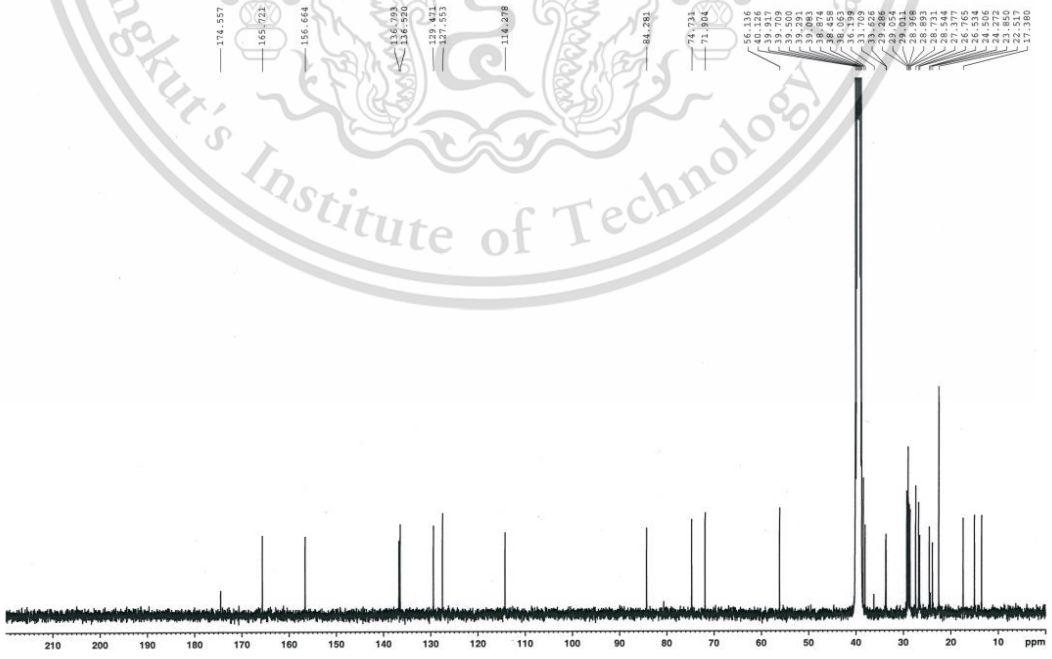

Figure 2 ^{13}C NMR spectrum (100 MHz, DMSO–d_6) of compound A (CH5–8 F2F2H12).

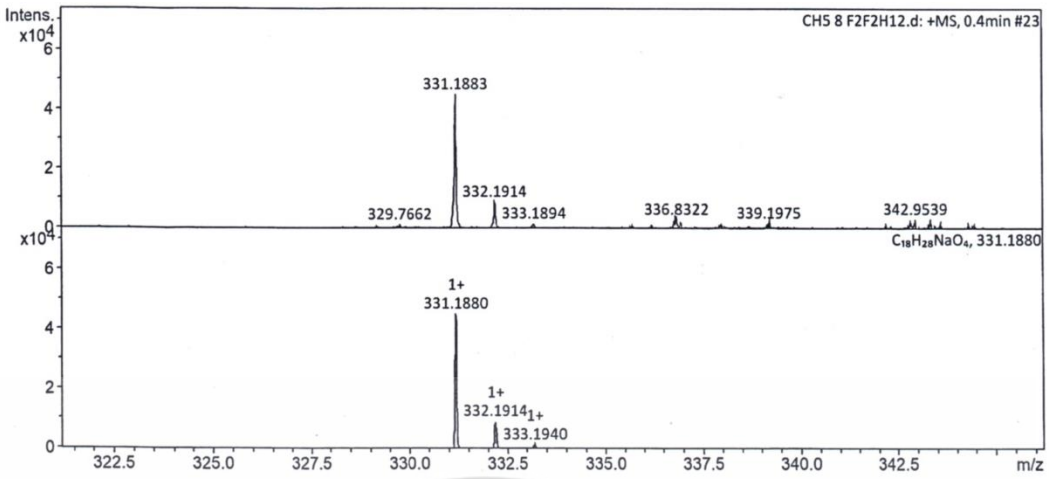

Figure 3 HRESIMS $[M+Na]^+$ spectrum compound A (CH5–8 F2F2H12).

Nocardamine

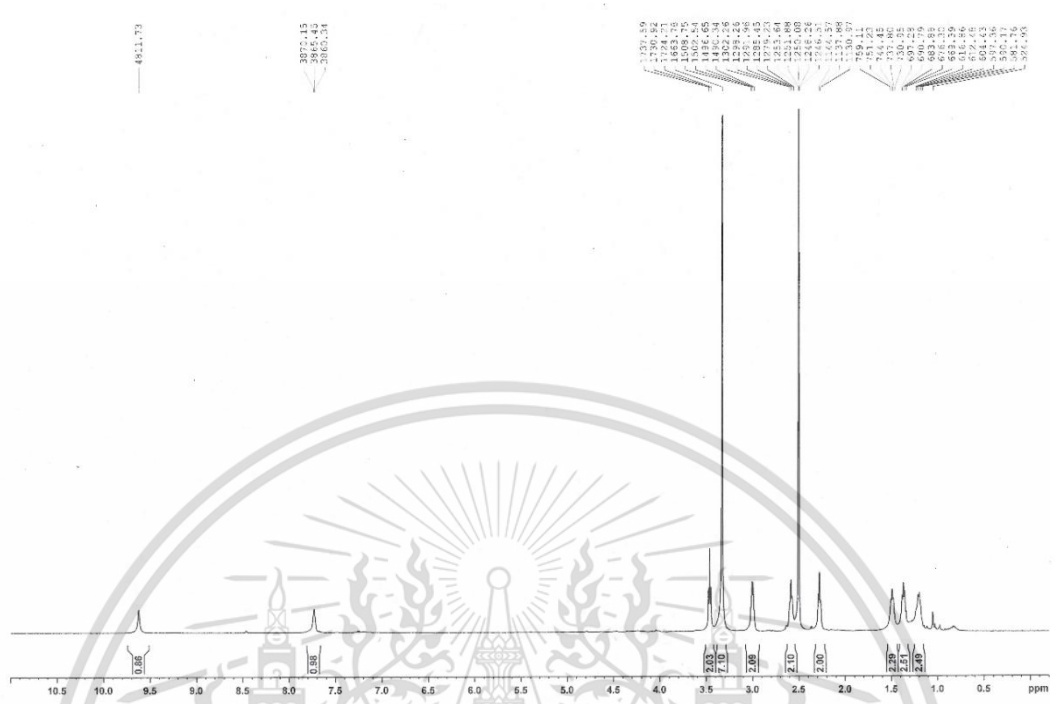

Figure 4 ^{1}H NMR spectrum (500 MHz, DMSO–d_6) of compound B (CH9–7 F1H2).

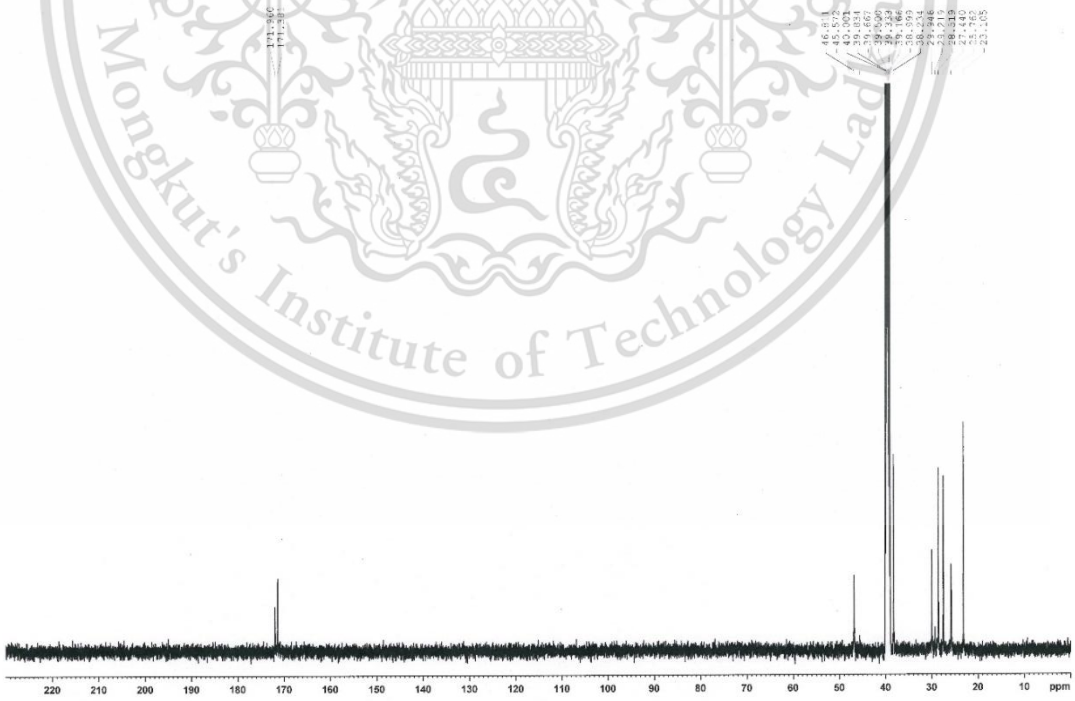

Figure 5 ^{13}C NMR spectrum (125 MHz, DMSO–d_6) of compound B (CH9–7 F1H2).

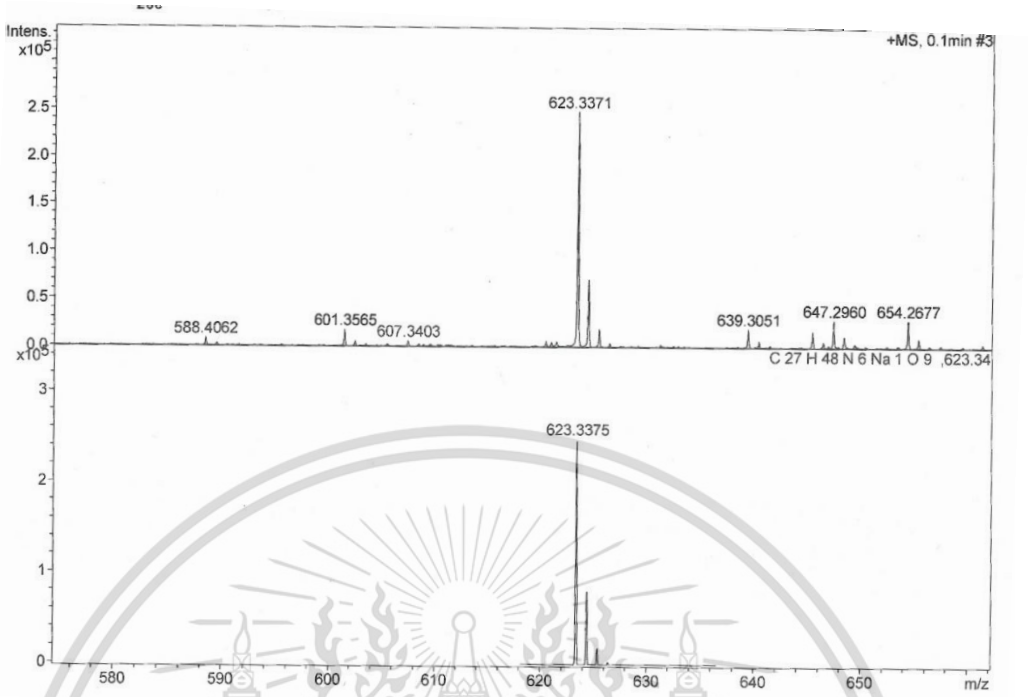

Figure 6 HRESIMS $[M+Na]^+$ spectrum of compound B (CH9–7 F1H2).

Appendix G
Chemotaxonomic Characteristics

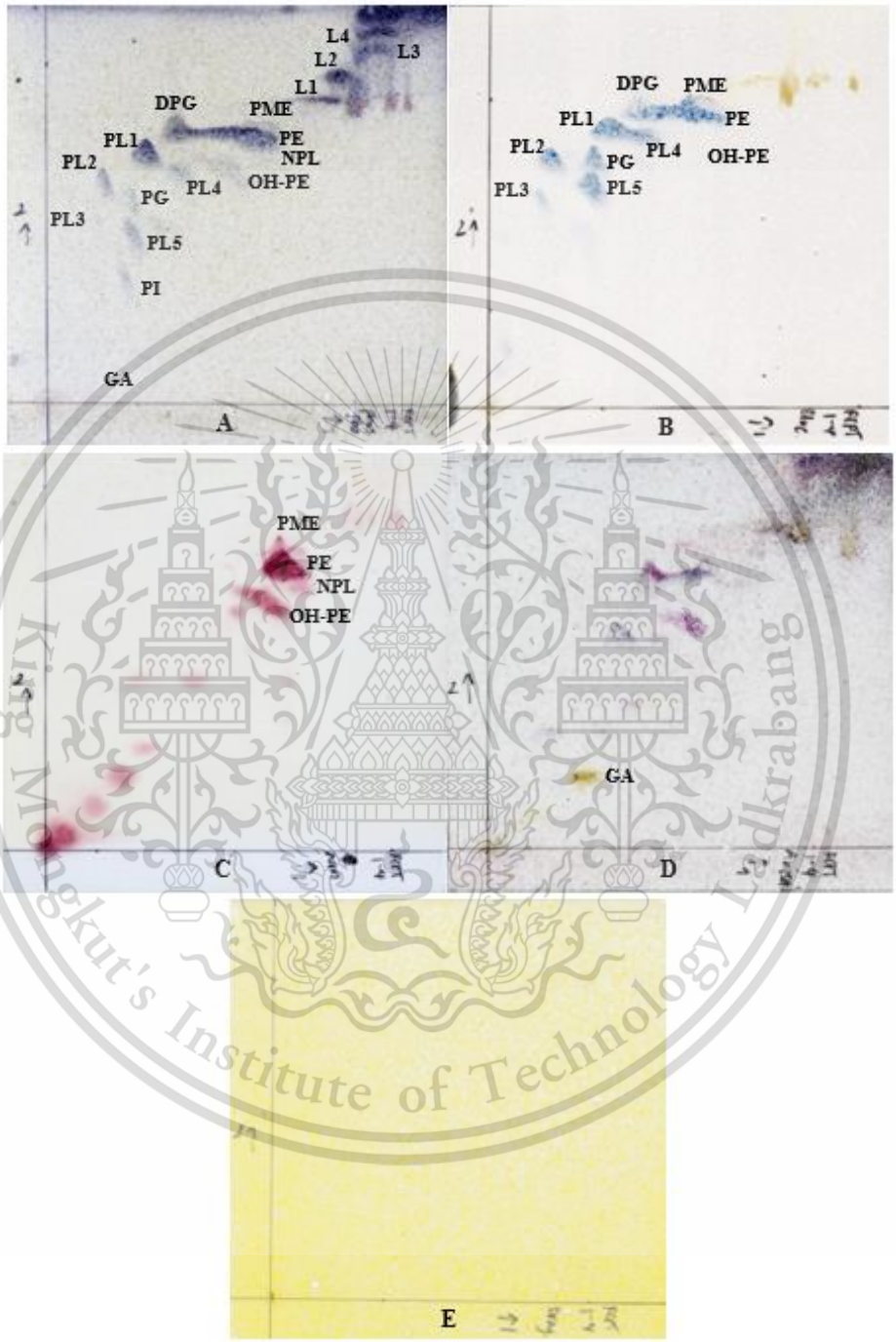

Figure 1 Polar lipids appearing on the two–dimensional thin–layer chromatograms of *Streptomyces* sp. strain RCPT1–4.

Note: (A) Phosphomolybdic acid's TLC chromatogram; (B) Dittmer & Lester's TLC chromatogram; (C) Ninhydrin's TLC chromatogram; (D) Anisaldehyde's TLC chromatogram; € Dragendroff's TLC chromatogram

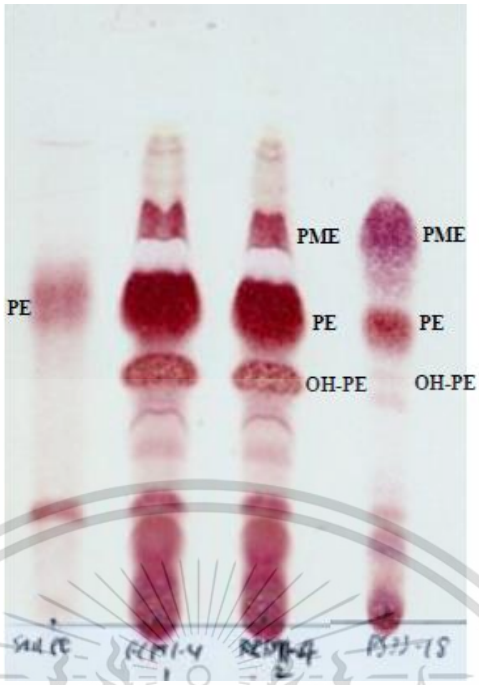

Figure 2 Polar lipids appear on the one–dimensional thin–layer chromatograms of *Streptomyces* sp. RCPT1–4.

Note: Ninhydrin's TLC chromatogram

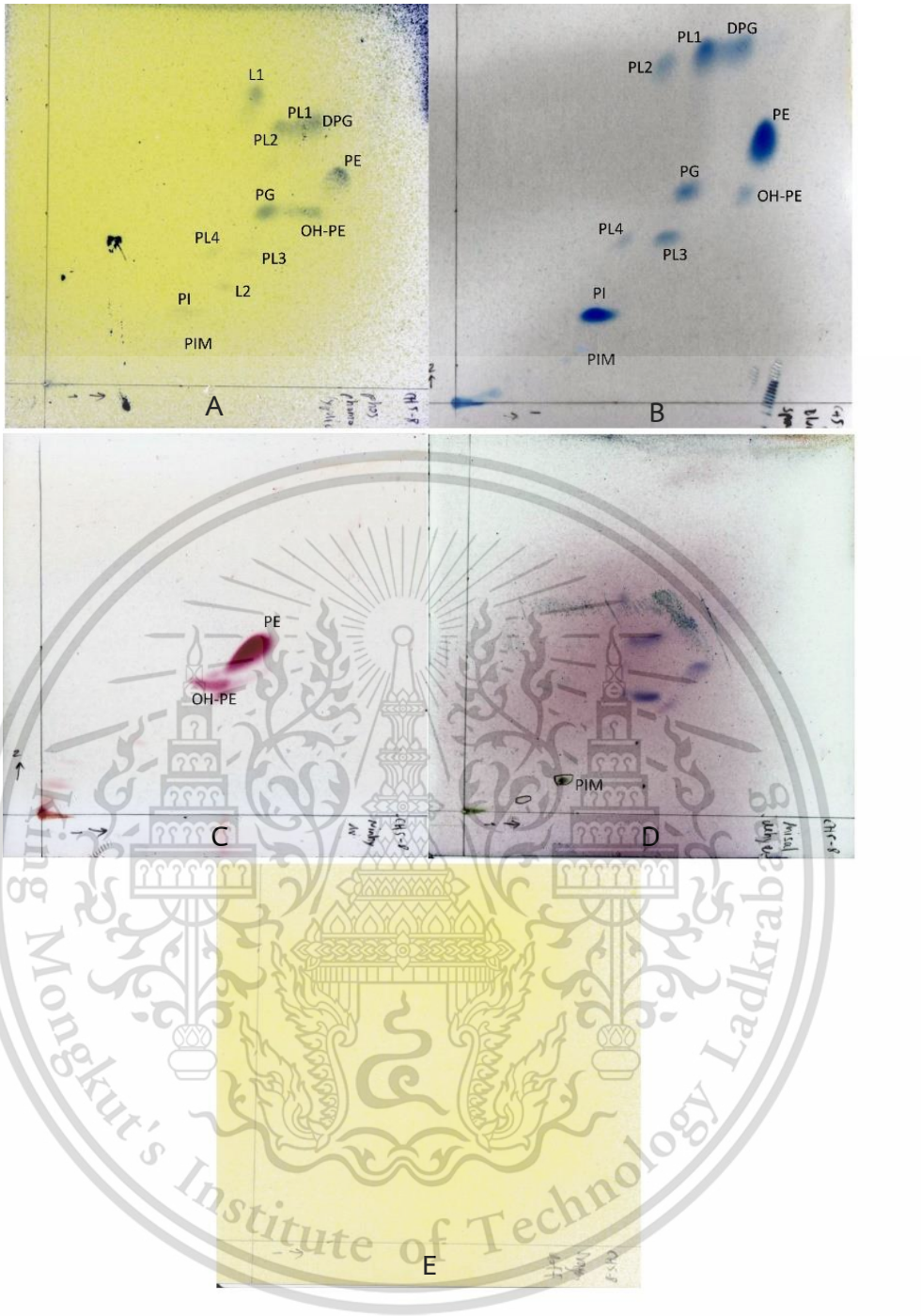

Figure 3 Polar lipids appearing on the two–dimensional thin–layer chromatograms of *Streptomyces* sp. strain CH5–8.

Note: (A) Phosphomolybdic acid's TLC chromatogram; (B) Dittmer & Lester's TLC chromatogram; (C) Ninhydrin's TLC chromatogram; (D) Anisaldehyde's TLC chromatogram; € Dragendroff's TLC chromatogram

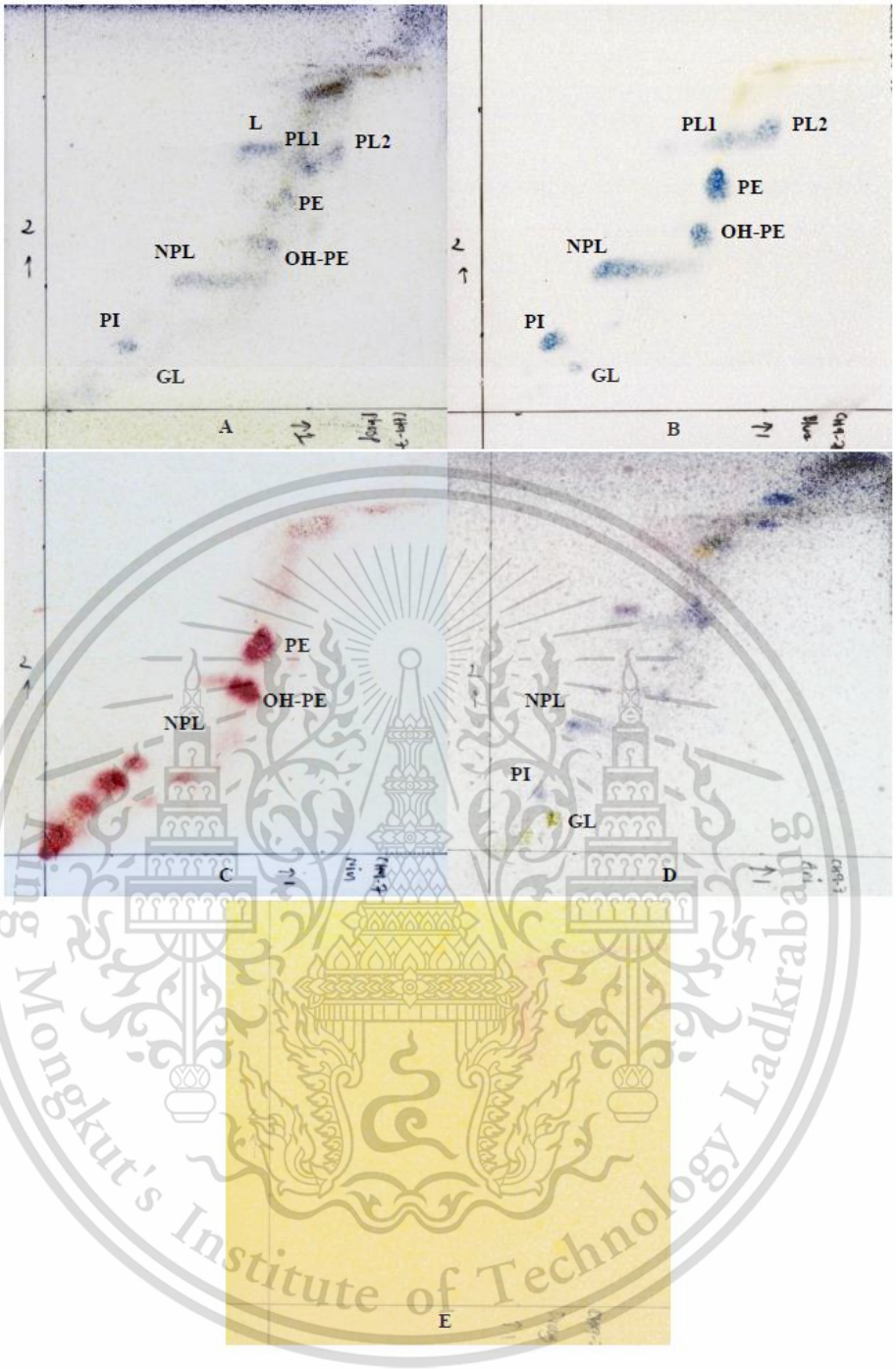

Figure 4 Polar lipids appearing on the two–dimensional thin–layer chromatograms of *Streptomyces* sp. strain CH9–7.

Note: (A) Phosphomolybdic acid's TLC chromatogram; (B) Dittmer & Lester's TLC chromatogram; (C) Ninhydrin's TLC chromatogram; (D) Anisaldehyde's TLC chromatogram; ∈ Dragendroff's TLC chromatogram

Appendix H
Academic Publications

Streptomyces musisoli CH5–8^{T}

INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Duangupama *et al.*, *Int. J. Syst. Evol. Microbiol.* 2021;71:004857
DOI 10.1099/ijsem.0.004857

MICROBIOLOGY SOCIETY

Streptomyces musisoli sp. nov., an actinomycete isolated from soil

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Abstract

An actinobacterium, strain CH5-8^{T}, which formed spiral chains of spore arising from the aerial mycelium, was isolated from rhizosphere soil of *Musa* spp. The organism exhibited vivid greenish yellow substrate mycelium and easily produced the medium grey aerial spore mass on ISP2 medium. The typical chemotaxonomic properties of members of the genus *Streptomyces* were observed for strain CH5-8^{T}, e.g. LL-diaminopimelic acid in cell peptidoglycan, MK-9(H_6), MK-9(H_8), and MK-9(H_4) as major menaquinones and anteiso-$C_{15:0}$, iso-$C_{16:0}$, and anteiso-$C_{17:0}$ as major fatty acids. Diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside were detected in the cells. A combination of morphological and chemotaxonomic data supported the assignment to the genus *Streptomyces*. The analysis result obtained for the 16S rRNA gene sequence confirmed the taxonomic affiliation at the genus level of this strain. The novel strain CH5-8^{T} showed the highest 16S rRNA gene sequence values to *Streptomyces echinatus* NBRC 12763^{T} (98.9%), followed by *Streptomyces actinomycinicus* RCU-197^{T} (98.9%). The average nucleotide identity by blast (ANIb) and digital DNA–DNA hybridization values between CH5-8^{T} and its closest relatives, *S. echinatus* CECT 3313^{T} and *S. actinomycinicus* RCU-197^{T}, were ≤91.6% and ≤47.4%, respectively. The digital DNA G+C content of genomic DNA was 72.1 mol%. On the basis of these phenotypic and genotypic data, strain CH5-8^{T} represents a novel species, for which the name *Streptomyces musisoli* sp. nov. is proposed. The type strain is CH5-8^{T} (=TBRC 9950^{T}=NBRC 113997^{T}).

INTRODUCTION

Many species of the genus *Streptomyces* have been reported as dominant soil microorganisms [1, 2]. Because of their ability to produce valuable secondary metabolites, the members of the genus *Streptomyces* have attracted biotechnologists and microbiologists' attention. Several novel bioactive compounds with diverse biological activities have been reported from species of the genus *Streptomyces* from soil; for example, oligomycin [3], an actinomycin group compound [4], aturanoside A and B and aturanosin [5], an albocycline-type macrolide [6], cyclobutane,2-hexyl-1,1,4-trimethyl; thiophene,2-butyl-5-ethyl; 1-heptyn-3-ol; 8-[*N*-aziridylethylamino]-2-6,dimethyloctene-2; pyrrolo[1,2-a]pyrazine-1,4-dion,hexahydro; octahydro-2H-pyrido(1,2-a) pyrimidin-2-one; 9,9-dimethyl-3,7-diazabicyclo[3.3.1] nonane; 2,4-dihydroxy-6-propylbenzoic acid [7] and julichrome derivatives and gliotoxin [8]. To date, more than 650 species of the genus *Streptomyces* have been reported in the

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Keywords: 16S rRNA gene; actinomycetes; *Musa* spp; soil; *Streptomyces*.
Abbreviations: ANIb, average nucleotide identity-BLAST; ANIm, average nucleotide identity-MUMmer algorithm; DAP, diaminopimelic acid; dDDH, digital DNA–DNA hybridisation; DPG, diphosphatidylglycerol; GGDC, Genome to Genome Distance Calculator; GL, glycolic acid; HPLC, high performance liquid chromatography; MK, menaquinone; OH-PE, hydroxyphosphatidylethanolamine; PE, phosphatidylethanolamine; PIM, phosphatidylinositol mannoside; PL, unidentified phospholipid; tetra, tetranucleotide signature correlation index; TLC, thin layer chromatography.
The DDBJ accession number for the 16S rRNA gene sequence of strain CH5-8^{T} is LC489239. The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession code JAERRH000000000. The version described in this paper is version JAERRH010000000.
Four supplementary figures and three supplementary tables are available with the online version of this article.

List of Prokaryotic names with Standing in Nomenclature (LPSN) (https://lpsn.dsmz.de/genus/streptomyces). Recently, *Streptomyces roseicoloratus* sp. nov. [9], *Streptomyces paludis* sp. nov. [10], *Streptomyces qinzhouensis* sp. nov. [11], *Streptomyces coryli* sp. nov. [12], *Streptomyces cupreus* sp. nov. [13], *Streptomyces montanus* sp. nov. [14] and *Streptomyces scabichelini* sp. nov. [15] have been reported as novel species from soil in different areas. During an investigation of novel actinomycetes from soil, we isolated strain CH5-8^T, which showed morphological characteristics typical of members of the genus *Streptomyces* but exhibited different phenotypic and genotypic properties from those of type strains of species of the genus *Streptomyces* with validly published names. In this study, strain CH5-8^T was taxonomically characterized as representing a novel species of the genus *Streptomyces*.

METHODS

Organisms and cultural conditions

Strain CH5-8^T was isolated from a soil sample collected around *Musa* spp. from Ratchaburi province, Thailand. The soil sample was air-dried at room temperature (approximately 30 °C) for 3 days. Then the air-dried soil was heated at 100 °C for one hour. The diluted 1000-fold soil suspension was prepared by serial dilution with 0,01% sterile sodium dodecyl sulphate (SDS) in distilled water, and an aliquot of 0.1 ml of solution (10^{-3}) was spread onto Zhang's starch soil extract (ZSSE) medium [16] supplemented with 100 mg nystatin l^{-1}. After incubation at 30 °C for 7–14 days, a small grey colony of strain CH5-8^T was selected and purified on yeast extract–malt extract agar (International Streptomyces Project, ISP2 medium) [17]. The purified isolate was preserved on slants of ISP2 and in glycerol [20% (v/v)] suspension at –80 °C and by freeze-drying.

Morphology, cultural, biochemical and physiological tests

Cell morphology of CH5-8^T grown on on ISP2 medium at 30 °C for 14 days was examined by scanning electron microscope (model JSM-6610 LV; JEOL). To test the cultural characteristics, CH5-8^T was cultivated on various International Streptomyces Project (ISP) media (ISP2–7), Czapek's sucrose agar [18], glucose–asparagine agar (ISP5 with 1% glucose replacing glycerol) and nutrient agar (Difco) at 30 °C for 14 days. The Inter-Society Color Council – National Bureau of Standards (ISCC–NBS) colour charts [19] were used for determining the colour of substrate and aerial mycelia, including soluble pigments of the strain. The temperature range for growth (15, 20, 25, 30, 37, 40, 42, 45, 50 °C) and the NaCl tolerances [0–10% (w/v) at increments of 1%] were evaluated on ISP2 agar for 14 days. The effect of pH on growth (4.0–11.0 at an increment of 0.5 pH units) was determined by cultivation at 30 °C in ISP2 broth for 14 days. Hydrolysis of casein (1% w/v skim milk), starch (1%, w/v), xanthine, adenine, hypoxanthine and tyrosine and nitrate reduction, carbon and nitrogen utilization, gelatin liquefaction and acid production from carbon sources, were tested as described previously [20–22]. Catalase and oxidase activities were tested using 3% (v/v) hydrogen peroxide solution and 1% *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride, respectively. The enzyme activities were determined using the API ZYM system (bioMérieux).

Chemotaxonomy

To prepare the cell biomass used for chemotaxonomic analyses, CH5-8^T was cultured in ISP 2 broth on a rotary shaker (200 r.p.m.) at 30 °C for 5 days. The cells were harvested and washed with sterile distilled water (five times) using centrifugation (3700 × g for 10 min at 4 °C, rotor JA18, Beckman Coulter, Galway, Ireland). The cells were freeze-dried in a vacuum freeze-dryer. The general chemotaxonomic characteristics for the genus *Streptomyces*, e.g. the isomer of diaminopimelic acid (DAP), whole-cell reducing sugars and the polar lipids, were determined by the thin layer chromatographic (TLC) technique using standard protocols as described previously [23–26]. To determine the menaquinone type of CH5-8^T, the extraction method of Collins *et al.* [27] was used. The crude menaquinone extracts were analysed by high performance liquid chromatography (HPLC) with a Cosmosil 5C18 column (4.6×150 mm, Nacalai Tesque) [28]. Cellular fatty acid compositions were prepared and analysed using the standard protocol as described previously [29, 30]. The composition of fatty acids was detected by gas chromatography (GC) (model 6890; Hewlett 107 Packard) with Sherlock Aerobic Bacterial Database (TSBA6) in the Microbial Identification software package.

Genotypic characteristics

Genomic DNA of CH5-8^T used for 16SrRNA gene amplification and whole-genome sequencing was extracted and purified according to the method of Tamaoka [31]. The 16S rRNA gene was amplified under the conditions described by Thawai [32]. To calculate the 16S rRNA gene sequence similarity values between CH5-8^T and the type strains of species of the genus *Streptomyces*, the nearly complete 16S rRNA gene sequence of CH5-8^T (1425 bases) was analysed using the available online server EzBioCloud (www.ezbiocloud.net) [33]. Phylogenetic trees were reconstructed using the neighbor-joining [34], maximum-likelihood [35] and maximum-parsimony methods [36] algorithms in mega X software [37]. The confidence values of nodes were evaluated using the bootstrap resampling method with 1000 replicates [38]. The genomic DNA of CH5-8^T was sequenced using a HiSeq 4000 platform (Illumina; Chulalongkorn University, Bangkok, Thailand). The assembled genome was evaluated with SPAdes [39] The genome annotation was implemented using the PROKKA pipeline [40]. The average nucleotide identity (ANI) values, i.e. ANI-BLAST (ANIb), ANIMUMmer (ANIm) and correlation indexes of tetranucleotide signature (Tetra), were calculated using the JSpecies Web Server [41, 42]. The genome-to genome distance calculator (GGDC 2.1 BLAST+ method) with formula 2 (identities/high-scoring segment pairs length) was used for analysing the digital DNA–DNA hybridization (dDDH) values [43]. To identify the

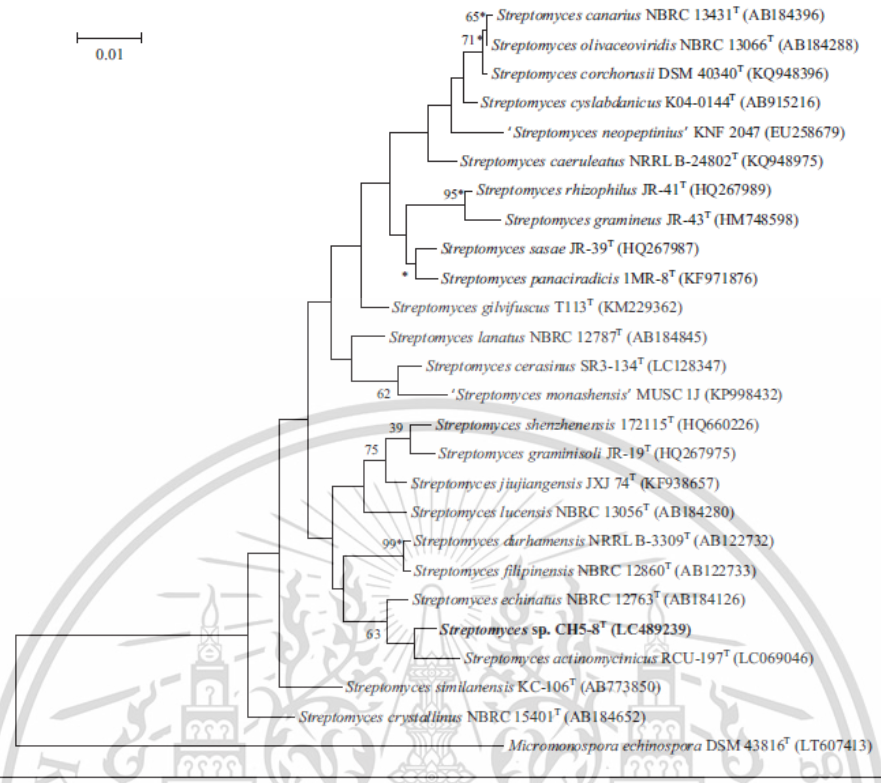

Fig. 1. Maximum-likelihood tree based on almost-complete 16S rRNA gene sequences showing the relationships between strain CH5-8T, and 24 closely related members of the genus *Streptomyces* obtained from the EzBioCloud database. *Micromonospora echinospora* DSM 43816T was used as an outgroup. Asterisks (*) indicate the branches of the tree that were also found using the neighbor-joining and maximum-parsimony methods. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates; only values ≥50% are shown. Bar, 0.01 substitutions per nucleotide position.

secondary metabolite biosynthesis gene clusters (smBGCs), the draft genome sequence of strain CH5-8T was applied to the anti-SMASH platform version 5.0 [44].

RESULTS AND DISCUSSION

Strain CH5-8T was isolated from rhizosphere soil of *Musa* spp. collected from Ratchaburi province, Thailand. A small grey colony of strain CH5-8T was picked up from isolation medium and then purified on ISP2 medium. The purified CH5-8T was used to characterize the taxonomic status using polyphasic taxonomic approaches. Firstly, the 16S rRNA gene sequence of CH5-8T was analysed. The results of the pairwise alignment of the 16S rRNA gene sequence (1425 bp) of CH5-8T indicated that it exhibited the closest similarity to *Streptomyces echinatus* NBRC 12763T (98.9%), followed by *S. actinomycinicus* RCU-197T (98.9%). Other type strains of species of the genus *Streptomyces* available from the EzBioCloud database showed similarity values below 98.5% to CH5-8T. These 16S rRNA gene sequence similarity values were close to the cut-off point (98.7%) for assigning the bacterial strains to the same genomic species [45]. Thus this result led us to predict that CH5-8T has genetic differences from any other type strains of species of the genus *Streptomyces* and may represent a novel species of the genus. Furthermore, the maximum-likelihood and the neighbour-joining trees based on 16S rRNA gene sequence gave the same result indicating that CH5-8T formed a monophyletic line with *S. actinomycinicus* RCU-197T and *S. echinatus* NBRC 12763T (Figs 1 and S1, available in the online version of this article). In contrast, the taxonomic position of strain CH5-8T in the maximum-parsimony tree showed that strain CH5-8T occupied a different cluster from these closely related type strains (Fig. S2). For this reason, *S. echinatus* JCM 4144T, and *S. actinomycinicus* RCU-197T were selected for comparative physiological and biochemical studies.

To confirm the taxonomic affiliation of CH5-8^T at the genus level its chemotaxonomic characteristics were investigated. The isomer of DAP of strain CH5-8^T was the LL form, a common feature of members of the genus *Streptomyces* [46, 47]. The sugars of whole-cell hydrolysate comprised galactose, glucose, mannose and ribose. The major menaquinones were MK-9(H_8) (53.8%), MK-9(H_6) (28.7%), and MK-9(H_4) (13.4%) while MK-10(H_4) (3.3%), MK-10(H_2) (0.8%) were also observed. The major menaquinone of CH5-8^T was similar to the major menaquinones found in *S. actinomycinicus* RCU-197^T [48]. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), hydroxyphosphatidylethanolamine (OH-PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylinositol mannoside (PIM), four unidentified phospholipids (PLs) and two unidentified lipids (Ls) were observed as the polar lipids in cells (Fig. S3). The predominant fatty acids (>10%) detected in cells of CH5-8^T were anteiso-$C_{15:0}$ (25.6%), iso-$C_{16:0}$ (22.0%), and anteiso-$C_{17:0}$ (17.1%); iso-$C_{15:0}$ (9.1%), $C_{16:0}$ (6.6%), iso-$C_{17:0}$ (5.1%), iso-$C_{14:0}$ (4.3%), $C_{17:0}$ (1.4%) and anteiso-$C_{17:1}\omega9c$ (1.1%) were also detected. The pattern of major fatty acids in the cells of CH5-8^T were similar to those predominant fatty acids in *S. echinatus* JCM 4144^T and *S. actinomycinicus* RCU-197^T, the closest relatives (Table S1), with different proportions. The 16S rRNA gene sequence and chemotaxonomic analyses confirmed that CH5-8^T represented a member of the genus *Streptomyces* and may represent a novel species.

To confirm the novelty at the species level of CH5-8^T, the phenotypic characteristics and the genome-based taxonomic characterization of CH5-8^T were performed. CH5-8^T grew well on ISP2, ISP3, ISP4, ISP5, ISP6, ISP7 and nutrient agar. Moderate growth was observed on glucose–asparagine and Czapek's sucrose agar. Vivid greenish yellow substrate mycelium and medium grey aerial spore mass were observed on ISP2 after 14 days of cultivation at 30 °C. Brilliant yellow, brownish-black and dark brown diffusible pigments were detected on ISP2, ISP6 and ISP7 media, respectively (Table S2). Spiral chains of spore with the rough surfaces were observed easily on any media tested (Fig. S4). The phenotypic comparison between CH5-8^T and its two closest relatives, *S. echinatus* JCM 4144^T, and *S. actinomycinicus* RCU-197^T, revealed differential characteristics that enabled CH5-8^T to be readily distinguished from those closest relatives. Unlike *S. echinatus* JCM 4144^T and *S. actinomycinicus* RCU-197^T, CH5-8^T grew in the presence of up to 7% NaCl. The pH for cell growth was in the range of 6–10. The maximum temperature for growth was 45 °C. Moreover, the utilization of L-rhamnose, D-xylose, dextran, salicin, sucrose and inulin as sole carbon sources and the production, or absence of production of acid from lactose, melezitose, L-rhamnose, D-ribose and salicin were the critical characteristics for discriminating between CH5-8^T and both reference type strains. Furthermore, the abilities to produce various enzymes, such as valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase and *N*-acetyl-β-glucosaminidase (Table 1) were also observed as significant phenotypic differences between CH5-8^T and *S. echinatus* JCM 4144^T. The genome sequencing data for CH5-8^T gave results of 9.36 Mbp in size with 148 contigs, an N50 of 398 kb, and genome coverage of 400×. The genomic DNA G+C content of this strain was 72.1 mol%, which was in the range for members of the genus *Streptomyces* [14, 15]. Genome-based taxonomic characterization revealed that CH5-8^T shared the highest ANIb, ANIm and tetra values (91.6, 93.0% and 0.99893, respectively) with *S. echinatus* CECT 3313^T. The genome of *S. actinomycinicus* RCU-197^T showed an ANIb of 87.4%, ANIm of 89.9% and tetra value of 0.9983 to that of CH5-8^T, which were well below an ANI threshold range (95–96%) and the cut-off value of ≥0.999 for membership of the same species [41]. The digital DNA–DNA hybridization (dDDH) values between the genomes of CH5-8^T and the two reference type strains were 47.4 and 36.6%, respectively. These values are lower than the threshold of 70% used to define species [49] (Table S3). A total of 51 secondary metabolite biosynthesis gene clusters in the draft genome of CH5-8^T were predicted using the antiSMASH server. Among these gene clusters, nine clusters showed more than 50% similarity to known biosynthetic gene clusters. There were the geosmin biosynthetic gene cluster (100%), ectoine biosynthetic gene cluster (100%), albaflavenone biosynthetic gene cluster (100%), sceliphrolactam biosynthetic gene cluster (100%), hopene biosynthetic gene cluster (92%), desferrioxamine B/E biosynthetic gene cluster (83%), spore pigment biosynthetic gene cluster (83%), melanin biosynthetic gene cluster (60%) and carotenoid biosynthetic gene cluster (54%). On the basis of these these phenotypic and genotypic characteristics, it is suggested that strain CH5-8^T should be recognized as representing a novel species of the genus *Streptomyces* under the proposed name *Streptomyces musisoli* sp. nov.

DESCRIPTION OF *STREPTOMYCES MUSISOLI* SP. NOV.

Streptomyces musisoli (mu.si.so'li. L. fem. n. *Musa, Musa*; L. neut. n. *solum*, soil; N.L. gen. n. *musisoli*, of *Musa* soil).

Cells are Gram-stain-positive and aerobic. Vivid greenish yellow substrate mycelium is observed on ISP2 medium. A medium grey aerial spore mass is formed on ISP2 medium after 14 days of cultivation. Brilliant yellow, brownish-black, and dark brown diffusible pigments are detected on ISP2, ISP6, and ISP7, respectively. Spiral chains of spores were observed easily on any media tested. The surfaces of the spores are rough. Spores are non-motile. The coagulation of milk and catalase activities are positive; the reduction of nitrate is weakly positive. Negative for hydrolysis of starch, gelatin liquefaction, peptonization of milk, urease and oxidase activities. Utilizes adonitol, L-arabinose, cellobiose, dextran, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, inulin, lactose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, salicin, sucrose, trehalose, xylitol and D-xylose as sole carbon sources. Utilizes DL-2-aminobutane, L-arginine, L-asparagine, L-cysteine, L-histidine, 4-hydroxyproline, L-methionine, L-phenylalanine, L-proline, L-serline, L-threonine, and L-valine as a sole nitrogen sources. Decomposes adenine, hypoxanthine and tyrosine but not cellulose and xanthine. The growth temperature is between 20 and 45 °C while the optimum range is 25–30 °C. Maximum NaCl concentration for growth is 7%

Table 1. Differential characteristics of strain CH5-8^T and its closest phylogenetic relatives

Strains: 1, CH5-8^T; 2, *S. echinatus* JCM 4144^T; 3, *S. actinomycinicus* RCU-197^T. All data were determined during this study. +, Positive; –, negative; w, weakly positive.

Characteristics	1	2	3
Colour of substrate mycelium on ISP2 (14 days culture)	Vivid Greenish Yellow	Brilliant Orange Yellow	Brilliant Orange Yellow
Hydrolysis of starch	–	w	+
Gelatin liquefaction	–	–	+
Coagulation of milk	+	–	+
Peptonization of milk	–	+	w
Nitrate reduction	w	–	–
Maximum NaCl tolerance (%, w/v)	7	3	4
Maximum temperature for growth (°C)	45	42	42
pH range for growth	6–10	6–9	6–9
Carbon source utilization:			
L-rhamnose	+	–	–
D-xylose	+	–	–
Dextran	w	–	–
D-salicin	+	–	–
Sucrose	+	–	w
Inulin	+	–	–
Acid production from:			
Lactose	–	w	w
Melezitose	–	+	+
L-rhamnose	+	–	–
D-ribose	–	w	w
Salicin	+	–	–
D-xylose	+	–	–
Enzyme activity:			
Valine arylamidase	+	–	+
Cystine arylamidase	w	–	+
Trypsin	w	–	+
α-Chymotrypsin	+	–	+
α-Galactosidase	w	–	+
β-Galactosidase	+	–	+
α-Glucosidase	–	–	+
N-acetyl-β-glucosaminidase	+	–	+

(w/v). The pH range for growth is 6–10. According to the API ZYM system, displays alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase, β-galactosidase and *N*-acetyl-β-glucosaminidase activities. Esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase and α-mannosidase activities are weak. Shows no activities of β-glucuronidase, α-glucosidase, β-glucosidase or α-fucosidase.

Cell wall peptidoglycan contains LL-diaminopimelic acid. The major menaquinones are MK-9(H_8), MK-9(H_6), and MK-9(H_4). Glucose, ribose, mannose and galactose are detected as reducing sugars of the cell. The polar lipid profile contains diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, four unidentified phospholipids and two unidentified lipids. The main fatty acids (>10%) are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$.

The type strain, CH5-8^T (=TBRC 9950^T=NBRC 113997^T), is an actinomycete isolated from soil around a banana plant in Ratchaburi province, Thailand. The DNA G+C content of the type strain is 72.1 mol%. The GenBank/EMBL/ DDBJ accession number for the 16S rRNA gene sequence of strain CH5-8^T is LC489239. The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession code JAERRH000000000. The version described in this paper is version JAERRH010000000.

Funding information
The authors gratefully acknowledge the financial support from King Mongkut's Institute of Technology Ladkrabang Research Fund (RE-KRIS/005/64) to C.T.

Acknowledgements
This work was supported by a grant from King Mongkut's Institute of Technology Ladkrabang Research Fund (RE-KRIS/005/64) to C.T. We thank the Actinobacterial Research Unit (ARU), Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, for laboratory support.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Streptomyces sennicomposti RCPT1–4^{T}

INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Duangupama *et al.*, *Int. J. Syst. Evol. Microbiol.* 2022;72:005320
DOI 10.1099/ijsem.0.005320

MICROBIOLOGY SOCIETY

Streptomyces sennicomposti sp. nov., an actinomycete isolated from compost of *Senna siamea* (Lam.)

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Abstract

A member of the genus *Streptomyces*, designated RCPT1-4^{T}, was isolated from compost of *Senna siamea* (Lam.), collected from an agricultural area in Rayong province, Thailand. The spore morphology and the presence of LL-diaminopimelic acid in the peptidoglycan indicate that RCPT1-4^{T} shows the typical properties of members of the genus *Streptomyces*. On the basis of the results of 16S rRNA gene sequence analysis, the strain should be classified as representing a member of the genus *Streptomyces* and was most closely related to *Streptomyces fumigatiscleroticus* NBRC 12999^{T} with the highest 16S rRNA gene sequence similarity of 99.2%, followed by *Streptomyces spiralis* NBRC 14215^{T} (99.0%). In addition, RCPT1-4^{T} shared the highest average nucleotide identity by BLAST (ANIb) (86.0%), and digital DNA–DNA hybridization (dDDH) (32.1%) values with *S. spiralis* NBRC 14215^{T}. Furthermore, several physiological and biochemical differences were observed between RCPT1-4^{T} and the closely related type strains of species with validly published names. These taxonomic data indicated that RCPT1-4^{T} could be considered to represent a novel species of the genus *Streptomyces* and the name *Streptomyces sennicomposti* sp. nov. is proposed for this strain. The type strain is RCPT1-4^{T} (=TBRC 11260^{T}=NBRC 114303^{T}).

Genus *Streptomyces* was first proposed by Waksman and Henrici [1]. It is known to be the largest taxon of the phylum *Actinobacteria*, and contains 682 species with validly published names at the time of writing (https://lpsn.dsmz.de/genus/*Streptomyces*) [2]. Species of the genus *Streptomyces* are Gram-positive bacteria that form an extensively branched substrate mycelium and commonly produce aerial mycelium. They can produce a variety of spore chains with diverse spore ornamentation [3]. In the past, species of the genus *Streptomyces* have been mainly classified using phenotypic and chemotaxonomic traits [4], but these seem insufficient for differentiating the species of the genus *Streptomyces*. Nowadays, the polyphasic taxonomic approach, including 16S rRNA gene analysis, is the generally used to circumscribe species for taxonomic purposes. Furthermore, genome-based taxonomy is a valuable strategy for clarifying the taxonomic status of members of the genus *Streptomyces*. It generates genomic approaches with the cutoff values for delimiting species boundaries, e.g. digital DNA–DNA hybridization (dDDH,<70%) [5], average nucleotide identity (ANI,<95%) [6–8], average amino acid identity (AAI,<95–96%) [9, 10] and tetranucleotide signature correlation index (Tetra,<0.989) [11], that are frequently used as taxonomic criteria for species discrimination. Species of the genus *Streptomyces* are well known as a rich source of antibiotics and bioactive molecules. Thus, they are considered to be a promising biotechnological resource [12–14]. An attempt to investigate novel members of the genus *Streptomyces* from previously unexamined environments is one strategy to discover new bioactive compounds from the microbial resources. Composting is the biological decomposition of organic matter by different phyla of microorganisms, including actinomycetes, under aerobic

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Keywords: *Streptomyces*; actinomycete; 16S rRNA gene; *Senna siamea* (Lam.); compost.
Abbreviations: AAI, average amino-acid Identity; ANIb, average nucleotide identity by BLAST; ANIm, average nucleotide identity-MUMmer algorithm; dDDH, digital DNA–DNA hybridization; ML, maximum-likelihood; NJ, neighbor-joining.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RCPT1-4^{T} is OM661191. The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAIOAB000000000.
Seven supplementary figures and six supplementary tables are available with the online version of this article.

conditions to produce a humus-like product that can be used for planting or as a soil corrective [15, 16]. During the composting process, the environment inside the compost pile has been found to have a high temperature and less oxygen content. Therefore, the viable species of the genus *Streptomyces* in the compost may exhibit some properties that differ from those of the soil-borne members of the genus *Streptomyces*. Species of the genus *Streptomyces* produce extracellular enzymes that can break down organic components, such as protein, lipid, cellulose, hemicellulose and lignin, and these can be used in various industries. For example, *Streptomyces argenteolus* AE58P isolated from natural compost of plant biomass is an interesting candidate biocatalyst-producing bacterium for lignocellulose conversion and production of biochemical and bioenergy [17]. Indigenous actinomycetes from empty fruit bunch compost of oil palm, *Streptomyces violaceorubidus* 6ca11, and *Streptomyces* sp. ac19, potentially produce amylases, cellulases and xylanase that can be applied in the industry [18]. Within the last decade, novel species of the genus *Streptomyces* isolated from compost samples have not been documented. In contrast, other non-Streptomycetes, for example, *Nocardiopsis yanglingensis* [19], *Compostimonas suwonensis* [20], *Thermotunica guangxiensis* [21], *Thermoactinomyces guangxiensis* [22], *Nonomuraea thermotolerans* [23] and *Thermomonospora catenispora* [24] from spent mushroom compost, *Thermasporomyces composti* [25] from mature compost, and *Cellulosimicrobium composti* [26] from sesame husks compost have been reported as novel species from compost. In the course of our investigation of actinomycetes from compost, strain RCPT1-4^T was isolated from the compost of *Senna siamea* (Lam.), collected from an agricultural area in Rayong province, Thailand. The polyphasic taxonomic details of RCPT1-4^T are reported in this study.

ISOLATION AND ECOLOGY

RCPT1-4^T was isolated from a sample of compost from the leaf-and-branch of *Senna siamea* (Lam.), collected from the farmland area (GPS location: 12°52′17″N 101°08′25″E) in Rayong province, Thailand. The compost was produced in a turned (aerated) windrow composting system. The air-dried sample was heated at 100 °C for 1 h. The diluted 1000-fold compost solution was prepared by serial dilution with 0.01% sterile sodium dodecyl sulphate (SDS) in distilled water, and an aliquot of 0.1 ml of solution was taken and spread onto modified Zhang's starch soil extract (ZSSE) medium (5 g soluble starch, 1 g KNO_3, 15 g agar, 100 ml compost extract solution, 900 ml distilled water, pH: 7.2) supplemented with 50 mg nalidixic acid and 50 mg nystatin l^{-1} [27]. To prepare the compost extract solution, 1 kg of *Senna siamea* (Lam.) compost was added to 1000 ml distilled water. The compost extract solution was sterilized by autoclaving (121 °C, 15 min) three times. After that, the compost extract solution was filtered using cellulose nitrate membrane filters (pore size 0.45 µm, Whatman). After incubation at 30 °C for 14 days, a *Streptomyces*-like colony was picked from the sample plates using a micro-needle. It was transferred and purified on the International Streptomyces Project (ISP) medium no.2 (ISP 2 medium) [28] at 30 °C for 14 days. The purified isolate was preserved at 4 °C on ISP 2 agar slants and by freeze-drying.

16S rRNA PHYLOGENY

Genomic DNA was extracted using the protocol of Tamaoka [29]. The 16S rRNA gene amplification and sequencing were conducted using the method suggested by Nakajima *et al.* [30]. The 16S rRNA gene similarity was calculated using the EzTaxon-e server (www.ezbiocloud.net) [31]. The 16S rRNA gene sequence was multiple-aligned with selected sequences available from the GenBank/EMBL/DDBJ databases using CLUSTAL W multiple alignment modes within BioEdit version 7.1.3.0 [32]. Phylogenetic trees were reconstructed by using neighbor-joining (NJ) [33] and maximum-likelihood (ML) [34] algorithms in MEGA version X [35]. The evolutionary distances were calculated by using Kimura's two-parameter model [36]. The Tamura–Nei model [37] was applied to the maximum-likelihood analysis using the Subtree-Pruning-Regrafting-Extensive (SPR level 5) program. The confidence values of nodes were evaluated using the bootstrap resampling method with 1000 replicates [38].

A preliminary strategy to predict the taxonomic position of bacteria, especially species of the genus *Streptomyces* is 16S rRNA gene analysis. The suggested cutoff value for species demarcation is below 98.7% [6, 7]. An almost complete 16S rRNA gene sequence (1413 nt) of RCPT1-4^T was analysed using the EzBioCloud (http://www.ezbiocloud.net/) server. RCPT1-4^T shared the highest 16S rRNA gene sequence similarities with *Streptomyces fumigatiscleroticus* NBRC 12999^T (99.2%), followed by *Streptomyces spiralis* NBRC 14215^T (99.0%), which were slightly higher than the 16S rRNA gene threshold for species differentiation. Phylogenetic analysis revealed that RCPT1-4^T was placed within the cluster of the genus *Streptomyces*. When the sequence of RCPT1-4^T was compared with corresponding 16S rRNA gene sequences of 23 close relatives in the genus *Streptomyces* found in the EzTaxon-e database and the 16S rRNA gene sequence of *Allostreptomyces psammosilenae* YIM DR4008^T (as an outgroup), the branches for RCPT1-4^T were separated from *S. fumigatiscleroticus* NBRC 12999^T, and *S. spiralis* NBRC 14215^T in both ML and NJ trees, but low bootstrap values (<50%) were detected (Figs 1 and S1, available in the online version of this article).

GENOME FEATURES

Genomic DNA for whole-genome sequencing of RCPT1-4^T was extracted from three day old cultures grown in ISP 2 broth at 30 °C and purified following the GeneJET Genomic DNA Kit (Thermo Scientific) purification protocol. Sequencing libraries were prepared using the QIAseq FX DNA Library Kit (Qiagen). The genomic DNA of RCPT1-4^T was sequenced using a Miseq platform

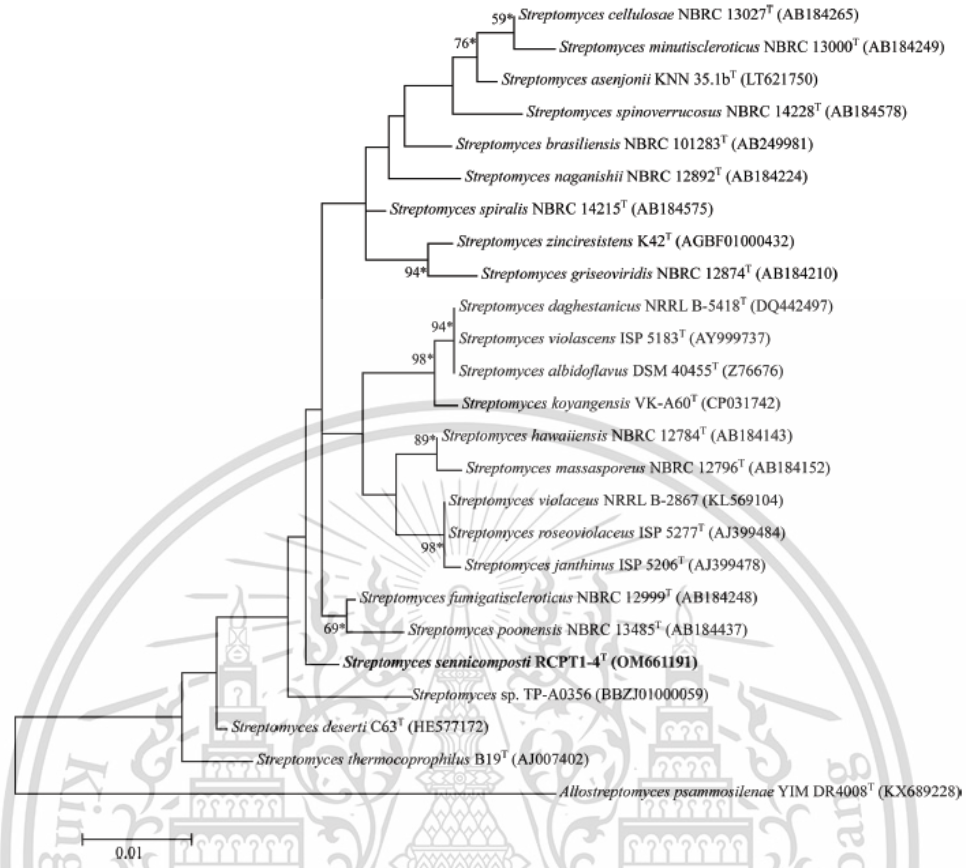

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the relationships between *Streptomyces sennicomposti* RCPT1-4^T and 23 related species with validly published names obtained from the EzBioCloud database. *Allostreptomyces psammosilenae* YIM DR4008^T was used as an outgroup. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates; only values of 50% or more are shown. Bar, 0.01 substitutions per nucleotide position.

(Illumina) with Reagent Kit V3 (600 cycles) using 2×250 bp paired-end reads (Chulalongkorn University, Thailand). Genome assembly was performed using SPAdes [39]. The online server, Rapid Annotations using Subsystems Technology (RAST) (http://rast.nmpdr.org/), was used for genome annotation [40, 41]. The genome was applied to the JSpecies Web Server for calculating the average nucleotide identity (ANI) values [6, 42]. Average amino acid identity (AAI) values were calculated using the Kostas Lab ANI calculator (http://enve-omics.ce.gatech.edu/ani/) [43]. The digital DNA–DNA hybridization (dDDH) values between the genome of RCPT1-4^T, and the most closely related species were calculated using the genome-to-genome distance calculator (GGDC 2.1; BLAST+ method) in which formula 2 (identities/high-scoring segment pair length) was applied to the incomplete draft genome [44]. The phylogenomic tree was reconstructed using the Type (Strain) Genome Server (TYGS) [45]. Determination and comparison of biosynthetic gene clusters (BGCs) were performed using the antiSMASH version 6.0.1 software [46]. To evaluate the genes relating to enzyme production, the genome of RCPT1-4^T was analysed using BLASTP on the Uniprot database with matrix; blosum62 (https://www.uniprot.org/blast) [47].

The genome-based taxonomic characterization of RCPT1-4^T was evaluated to clarify the taxonomic position of the strain. The genome size of RCPT1-4^T was 7.35 Mb with 73.1 mol% DNA G+C content determined by *in silico* genome sequencing, which was withing the range for members of the genus *Streptomyces* [3]. Other genomic details of RCPT1-4^T and other related type strains of species of the genus *Streptomyces* are summarized in Table S1. Recently genome-based taxonomic approaches, i.e., ANI

(<95%) [6], AAI (<95–96%) [8], tetranucleotide signature correlation index (<0.989) [6, 11], and dDDH (<70%) [48, 49] have been used for bacterial species discrimination, the values in parentheses are cut off values. On the basis of the results of dDDH analysis, *S. spiralis* NBRC 14215^T (32.1%), *Streptomyces kebangsaanensis* SUK12^T (31.2%), *Streptomyces naganishii* JCM 4654^T (31.1%), *Streptomyces triticisoli* NEAU DSCPA1-4-4^T (31.1%) and *Streptomyces anandii* JCM 4720^T (30.7%), shared higher dDDH values with RCPT1-4^T than *S. fumigatiscleroticus* NBRC 12999^T (27.8%). Furthermore, the phylogenomic tree reconstruction by automatic selection of the most closely related type strains by TYGS indicated that RCPT1-4^T was closely related to these neighbours, but the position of RCPT1-4^T was separated from the branches carrying *S. fumigatiscleroticus* NBRC 12999^T (Fig. S2). In addition, the ANI, AAI and Tetra values of RCPT1-4^T with closely related species were in the range of 82.8–86.0% for ANIb, 78.7–82.8% for AAI, and 0.94566–0.99002 for Tetra values which are significantly below the suggested cutoff values for the species delineation, indicating that RCPT1-4^T could be considered to represent a novel species of the genus *Streptomyces* (Table S2). The draft genome of RCPT1-4^T comprised 6086 protein-coding genes, 68 tRNA genes, 1 transfer–messenger RNA (tmRNA) gene, and no miscellaneous RNA (misc_RNA) genes. The genome annotation results obtained by rapid annotations using subsystems technology (RAST) (http://rast.nmpdr.org/) [40, 41] revealed that RCPT1-4^T possessed 310 subsystems belonging to 23 categories (Fig. S3). Among these subsystems, 'amino acids and derivatives' was the largest subsystem (378 feature counts), followed by 'carbohydrates' (315 feature counts), 'protein metabolism' (229 feature counts), 'cofactors, vitamins, prosthetic groups, pigments' (161 feature counts), 'fatty acids, lipids, and isoprenoids' (143 feature counts) and 'nucleosides and nucleotides' (112 feature counts). Secondary metabolite biosynthetic gene clusters (smBGCs) of RCPT1-4^T and its close relatives, *S. fumigatiscleroticus* NBRC 12999^T, and *S. spiralis* NBRC 14215^T were analysed and compared using the specialized metabolites prediction pipeline antiSMASH version 6.0.1. The draft genome of RCPT1-4^T contained several smBCGs, most of which relate to the production of antibiotics, siderophores, terpenes and peptides (Fig. S4). They belonged to several cluster categories, e.g. nonribosomal peptide synthases (NRPS), type I polyketide synthase (T1PKS), terpenes, siderophore, heterocyst glycolipid synthase-like PKS (hgIE-KS), post-translationally modified peptide product [ribosomally synthesized and post-translationally modified peptide (RiPP)-like] cluster, lanthipeptide-class III and IV, ectoine, T2PKS, T3PKS, non-alpha poly-amino acids (NAPAA) and butyrolactone. Sixteen smBCGs of strain RCPT1-4^T were found to have a similarity of over 50–100% with known compounds consisting of ectoine, hopene, paenibactin, geosmin, abyssomicin M-X, informatipeptin, albaflavenone, melanin, isofuranonaphoquinone, desferrioxamin B/desferrioxamin E, antimycin, flaviolin, bacillibactin and carotenoid. In contrast, betalactone, lanthipeptide class I and II, and proteusin gene clusters were only found in *S. fumigatiscleroticus* NBRC 12999^T, and redox-cofactor, lassopeptide and furan gene clusters were detected in *S. spiralis* NBRC 14215^T (Table S3). Interestingly, the genome of RCPT1-4^T was found to have several genes encoding protease, lipase, amylase and cellulase that can be applied in industry. Many types of enzymes which could degrade glucan, xylose and xylan were also found in this strain. Moreover, RCPT1-4^T contained a gene encoding chitinase, which could be used to control fungi and insects (Table S4).

PHYSIOLOGY AND CHEMOTAXONOMY

Spore chain morphology and spore-surface ornamentation of RCPT1-4^T were observed after growth on ISP 2 medium at 30 °C for 14 days. To prepare the samples for observation by scanning electron microscopy, a cultured agar block (3 mm × 5 mm) was fixed with the vapour of 2% osmium tetroxide for 2 h. Then, the samples were gently washed with sterile distilled water. The dehydration step was done through a graded ethanol series [30, 50, 70, 95% each for 10–15 min, finally followed by 100% ethanol (10 min) three times]. After that, the dehydrated samples were dried using liquid carbon dioxide in a critical-point dryer (model EM CPD300; Leica). Finally, the specimens were stuck on the stub and were coated with gold using a sputter coater (model SCD040; Balzers). The specimens were observed under a scanning electron microscope (model JSM-6610 LV; JEOL) at the scientific and technological research equipment centre, Chulalongkorn University. The cultural characterization was performed by cultivating the strain on various International Streptomyces Project (ISP) media (ISP 2–7), Czapek's sucrose agar [50], glucose–asparagine agar (ISP 5 with 1% glucose replaced with glycerol) and nutrient agar (Difco) at 30 °C for 14 days. The colours of the substrate and aerial mycelia and diffusible pigments were evaluated by comparison with the Inter-Society Color Council–National Bureau of Standards (ISCC–NBS) colour charts [51]. Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, 40, 45, 47 and 50 °C) and with 0–10% (w/v) NaCl (at single unit intervals) were evaluated on ISP 2 agar after incubation for 14 days. The effect of pH on growth (4.0–12.0 at an increment of 0.5 pH units) was determined by cultivation at 30 °C in ISP 2 broth for 14 days. Catalase activity was observed by bubble production after applying 3% (v/v) hydrogen peroxide solution. Testing for oxidase activity was carried out by oxidation of 1% *N, N, N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride. Carbon and nitrogen utilization (1%, w/v) were examined according to the standard method of Shirling and Gottlieb [28] and Gordon *et al.* [52], respectively. Nitrate reduction, acid production from different carbon sources, decomposition of insoluble compounds, e.g. adenine, cellulose, hypoxanthine, L-tyrosine and xanthine, gelatin liquefaction, hydrolysis of urea, hydrolysis of casein (1%, w/v) and starch (1%, w/v), hydrogen sulfide and melanin production were evaluated by using the methods of Arai [53], Williams and Cross [54] and Gordon *et al.* [52]. The enzyme activities were determined using the API ZYM system (bioMérieux).

Biomass used for chemotaxonomic analyses was collected from cell culture grown in ISP 2 broth on a rotary shaker (200 r.p.m.) at 30 °C for 5 days. The diaminopimelic acid isomers and whole-cell sugars were evaluated based on the methods of Hasegawa

et al. [55], and Komagata and Suzuki [56], respectively. The polar lipids in the cell membrane were extracted and analysed by the methods proposed by Minnikin *et al.* [57] and analysed by the one- and two-dimensional TLC techniques of Collins and Jones [58]. Menaquinones were extracted as described by Collins *et al.* [59] and were analysed by high-performance liquid chromatography with a Cosmosil 5C18 column (4.6×150 mm, Nacalai Tesque) [60]. To prepare the cells for cellular fatty acid analysis, RCPT1-4^T was cultured in ISP 2 broth on a rotary shaker (200 r.p.m.) at 30 °C for five days. Fatty acids were extracted, methylated and analysed using the Sherlock Microbial Identification (MIDI) system and the ACTIN version 6 database [61, 62].

Typical chemotaxonomic characteristics, e.g., LL-diaminopimelic acid (LL-DAP), acetyl type of muramic acids and absence of mycolic acids in the cells, were detected in RCPT1-4^T. These characteristics are commonly reported in all species of the genus *Streptomyces* [3, 63]. The strain was found to have MK-9(H_6) and MK-9(H_4) as the major menaquinones, while MK-9(H_2) and MK-9(H_8) were also detected. Cell hydrolysates contained glucose, mannose and ribose as whole-cell sugars. Diphosphatidylglycerol (DPG), glycolic acid (GA), hydroxyl-phosphatidylethanolamine (OH-PE), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), phosphatidylglycerol (PG), phosphatidylinositol (PI), ninhydrin-positive lipid (NPL), five unidentified phospholipids (PLs) and four unidentified lipids (Ls) were found in the membrane (Figs S5 and S6). The major fatty acids (>10%), anteiso-$C_{15:0}$ (25.4%), anteiso-$C_{17:0}$ (19.5%), and iso-$C_{16:0}$ (18.7%), were observed (Table S5). This major fatty acid pattern was also found in *S. fumigatiscleroticus* NBRC 12999^T and *S. spiralis* NBRC 14215^T with different proportions. In contrast, a minor fatty acid component, 3OH-$C_{18:0}$, was not detected in *S. fumigatiscleroticus* NBRC 12999^T. The chemotaxonomic data indicated that RCPT1-4^T represented a member of the genus *Streptomyces*.

RCPT1-4^T was a Gram-stain positive filamentous actinomycete that formed well-developed and nonfragmented branched substrate mycelia. The strain grew well on ISP 2, ISP 3 and ISP 7. Moderate growth was observed on ISP 4, ISP 5, ISP 6, glucose-asparagine and nutrient agar. In contrast, the growth on Czapek's sucrose agar was poor (Table S6). On ISP 2 medium, the substrate mycelia were light yellowish-brown. After 14 days of cultivation at 30 °C, a greyish-white aerial spore mass was developed. The strain could produce light greenish-yellow to light yellow diffusible pigments in ISP 2, ISP 5 and ISP 7 media. Spore chains were spiral, and the spore surface was hairy (Fig. S7). This spore morphology was consistent with the description of spore-chain morphology for the members of the genus *Streptomyces* [1, 64]. In contrast, spiral spore chains with smooth spore surfaces have been reported in *S. fumigatiscleroticus* NBRC 12999^T, the closest relative according to the results of 16S rRNA gene analysis [3]. Other cultural characteristics of RCPT1-4^T and closely related type strains are listed in Table 1 and the protologue. The strain could be distinguished from the closely related type strains, *S. fumigatiscleroticus* NBRC 12999^T and *S. spiralis* NBRC 14215^T, by their physiological and biochemical characteristics. Unlike RCPT1-4^T, *S. fumigatiscleroticus* NBRC 12999^T, and *S. spiralis* NBRC 14215^T produced spiral spore chains with the smooth surfaces of the spores. Other physiological and biochemical properties, i.e. the maximum NaCl tolerance, temperature range for growth, hydrolysis of urea, milk coagulation, nitrate reduction, utilization of dextran, D-melibiose, D-raffinose, L-rhamnose, D-ribose, D-trehalose, and sucrose as sole carbon sources, utilization of DL-2-aminobutyric acid, L-arginine, 4-hydroxyproline, L-methionine and L-phenylalanine as sole nitrogen sources, acid production from dextran, *myo*-inositol, inulin, D-mannitol, D-melibiose, D-raffinose, L-rhamnose, sucrose and D-xylose, decomposition of hypoxanthine and L-tyrosine and the production of lipase (C14), α-mannosidase, *N*-acetyl-β-glucosaminidase and trypsin, were crucial points for differentiating RCPT1-4^T from closely related species. It is evident from taxonomic data that RCPT1-4^T could be judged to represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces sennicomposti* sp. nov. is proposed.

DESCRIPTION OF *STREPTOMYCES SENNICOMPOSTI* SP. NOV.

Streptomyces sennicomposti (sen.ni.com.pos'ti. N.L. fem. n. *Senna*, the genus of a plant and also a botanical scientific name (*Senna siamea* Lam.); N.L. neut. n. *compostum*, compost; N.L. gen. n. *sennicomposti*, referring to the isolation of the type strain from *Senna siamea* (Lam.) compost).

Cells are Gram-stain-positive and aerobic. Grows well on ISP 2, ISP 3 and ISP 7. Grows moderately on ISP 4, ISP 5, ISP 6, glucose–asparagine and nutrient agar. Growth on Czapek's sucrose agar is poor. Yellowish-brown series substrate mycelium is observed on ISP 2 and ISP 3. White to grey series aerial spore masses are formed on ISP 2, ISP 3, ISP 4, ISP 6, ISP 7 and nutrient agar, which differentiate into spiral spore chains with hairy surfaces after 14 days of cultivation at 30 °C, and spores are non-motile. Light yellow, pale greenish-yellow, and light greenish-yellow diffusible pigments are detected on ISP 2, ISP 5 and ISP 7 agar, respectively. Hydrolysis of starch and urea, liquefaction of gelatin, coagulation and peptonization of milk, reduction of nitrate and production of catalase and oxidase are positive. Negative results are observed for hydrogen sulfide and melanin production. Decomposes adenine, hypoxanthine and L-tyrosine but not cellulose and xanthine. Utilizes L-arabinose, cellobiose, dextran, D-fructose, D-galactose, D-glucose, glycerol, lactose, D-mannose, melibiose, D-ribose, trehalose, xylitol and D-xylose; weakly utilizes *myo*-inositol, inulin, D-mannitol and sucrose; but does not utilize melezitose, raffinose or L-rhamnose, as sole carbon sources. Utilizes DL-2-aminobutyric acid, L-arginine, L-asparagine, L-cysteine, L-histidine, 4-hydroxyproline, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine and L-valine as sole nitrogen sources. Produces acid from L-arabinose, cellobiose, dextran, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, inulin, lactose,

Table 1. Differential characteristics of *Streptomyces sennicomposti* RCPT1-4^T and its close phylogenetic relatives

Strain: 1, *S. sennicomposti* RCPT1-4^T; 2, *S. fumigatiscleroticus* NBRC 12999^T; 3, *S. spiralis* NBRC 14215^T. All data were obtained during this study. +, Positive; –, negative; w, weakly positive.

Characteristics	1	2	3
Colour of aerial spore mass on ISP 2	Light grey	Pale greenish yellow	Greyish white
Spore surface ornamentation	Hairy	Smooth	Smooth
Maximum NaCl tolerance (%w/v)	6	5	7
Temperature range for growth (°C)	15–47	15–45	14–45
pH range for growth	5–10	6–10	6–10
Urea hydrolysis	+	+	–
Milk coagulation	+	+	–
Nitrate reduction	+	–	–
Carbon utilization from (1.0% w/v):			
Dextran	+	–	–
Raffinose	–	+	+
L-Rhamnose	–	+	+
D-Ribose	+	–	w
Sucrose	w	–	w
Trehalose	+	–	+
Acid production from (1.0% w/v):			
Dextran	+	–	–
myo-Inositol	+	–	–
Inulin	+	–	–
D-Mannitol	–	+	+
Melibiose	–	+	+
Raffinose	–	+	–
L-Rhamnose	–	+	+
Sucrose	–	+	+
D-Xylose	+	+	–
Nitrogen utilization from (1.0% w/v):			
DL-2-Aminobutyric acid	+	+	–
L-Arginine	+	–	+
4-Hydroxyproline	+	+	–
L-Methionine	+	+	–
L-Phenylalanine	+	+	–
Decomposition (1.0% w/v) of:			
Hypoxanthine	+	–	+
L-Tyrosine	+	–	+
Enzyme activity with API ZYM:			
N-acetyl-β-glucosaminidase	+	–	w

Continued

Table 1. Continued

Characteristics	1	2	3
Lipase (C14)	w	–	–
α-Mannosidase	+	–	w
Trypsin	w	–	–

D-mannose, D-ribose, trehalose, xylitol and D-xylose. The growth temperature is between 15 and 47 °C, while the optimum range is 25–30 °C. Maximum NaCl concentration for growth is 6% (w/v). The pH range for growth is 5–10. According to the API ZYM system, cells can produce acid phosphatase, alkaline phosphatase, esterase lipase (C8), β-glucosidase, leucine arylamidase, α-mannosidase, *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, and valine arylamidase Cells display weak activities of cystine arylamidase, esterase (C4), β-galactosidase, α-glucosidase, lipase (C14), and trypsin and no activities of cells on α-chymotrypsin, α-fucosidase, α-galactosidase and β-glucuronidase are observed. Cell wall peptidoglycan contains LL-diaminopimelic acid. The major menaquinones are MK-9(H_6) and MK-9(H_4), while MK-9(H_2) and MK-9(H_8) are minor components. Glucose, mannose and ribose are detected as whole-cell sugars. The phospholipid profile contains diphosphatidylglycerol, hydroxyphosphatidylethanolamine, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylinositol. The major fatty acids (>10%) are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$.

The type strain, RCPT1-4^T (=TBRC 11260^T=NBRC 114303^T), is an actinomycete isolated from a *Senna siamea* (Lam.) compost sample in Rayong province, Thailand. The DNA G+C content of the type strain is 73.1 mol%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RCPT1-4^T is OM661191. The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number JAIOAB000000000.

Funding information
The authors gratefully acknowledge the financial support from King Mongkut's Institute of Technology Ladkrabang Research Fund (RE-KRIS/005/64) to C. T.

Acknowledgements
The authors gratefully acknowledge Prof. Aharon Oren (The Hebrew University of Jerusalem, Jerusalem, Israel) for his valuable help on nomenclature aspects. We thank the Actinobacterial Research Unit (ARU), Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, for laboratory support.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Patent(s)/Petty Patent(s)

1. Application Number: 2103001471 "Actinomycete product for Fusarium wilt disease control in banana and plant growth promoting activity" Filed: 27 May 2021
2. Application Number: 2103001472 "The composition of *Streptomyces* culture solid media using red sorghum" Filed: 27 May 2021