IDENTIFICATION, ANTIFUNGAL AND PLANT GROWTH PROMOTING ACTIVITIES OF ENDOPHYTIC ACTINOMYCETES FROM ORCHIDS

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การพิสูจน์เอกลักษณ์ ฤทธิ์ต้านเชื้อราและส่งเสริมการเจริญเติบโตของพืชของแอคติโนมัยซีทที่เป็นเอนโดไฟต์จากกล้วยไม้

น.ส.นิสาชล เทศศรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Field of Study	Pharmaceutical Sciences and Technology
Thesis Advisor	Professor SOMBOON TANASUPAWAT, Ph.D.
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นิสาชล เทศศรี : การพิสูจน์เอกลักษณ์ ฤทธิ์ต้านเชื้อราและส่งเสริมการเจริญเติบโตของพืชของแอคติโนมัยซีทที่เป็นเอนโดไฟต์จากกล้วยไม้. (IDENTIFICATION, ANTIFUNGAL AND PLANT GROWTH PROMOTING ACTIVITIES OF ENDOPHYTIC ACTINOMYCETES FROM ORCHIDS) อ.ที่ปรึกษาหลัก : ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาร่วม : ศ. ภก. ดร.กิตติศักดิ์ ลิขิตวิทยาวุฒิ

แอคติโนมัยซีทเอนโดไฟต์ 62 สายพันธุ์คัดแยกได้จากกล้วยไม้ 15 ชนิดในประเทศไทย ทำการพิสูจน์เอกลักษณ์ได้เป็น *Streptomyces* (34 สายพันธุ์) *Micromonospora* (21 สายพันธุ์) *Streptosporangium* (2 สายพันธุ์) *Actinomadura* (1 สายพันธุ์) *Ammycolatopsis* (3 สายพันธุ์) และ *Pseudonocardia* (1 สายพันธุ์) โดยอาศัยลักษณะทางฟีโนไทป์ อนุกรมวิธานทางเคมี และลักษณะทางจีโนไทป์ ผลการคัดกรองฤทธิ์ต้านจุลินทรีย์พบว่า *Streptomyces* (18 สายพันธุ์) *Micromonospora* (12 สายพันธุ์) และ *Streptosporangium* (1 สายพันธุ์) สามารถต้าน *Bacillus subtilis* ATCC 6633 *Kocuria rhizophila* ATCC 9341 *Staphylococcus aureus* ATCC 25923 *Escherichia coli* ATCC 25922 *Pseudomonas aeruginosa* ATCC 27853 และ *Candida albicans* ATCC 10231 โดยสายพันธุ์ DR2-2 สามารถต้านการเจริญของจุลินทรีย์ทดสอบทุกสายพันธุ์ จากการวิเคราะห์ลำดับเบสบนยีน 16S rRNA ของสายพันธุ์ DR6-1 พบว่ามีความใกล้เคียงกับ *Amycolatopsis nivea* KCTC 39515^{T} เท่ากับ 99.06% มีค่าเฉลี่ยความเหมือนของลำดับเบสนิวคลิโอไทด์ของจีโนมเท่ากับ 93.8 % และค่าความคล้ายคลึงของ ดีเอ็นเอ-ดีเอ็นเอเท่ากับ 52.50 % ดังนั้นสายพันธุ์ DR6-1 เป็นแอคติโนมัยซีทสปีชีส์ใหม่ จึงเสนอตั้งชื่อว่า *Amycolatopsis dendrobii* สายพันธุ์ DR5-3 มีค่าความใกล้เคียงของลำดับเบสยีน 16S rRNA กับ *Micromonospora yasonensis* DSM 45980^{T} เท่ากับ 98.96% ดังนั้นสายพันธุ์ DR5-3 เป็นแอคติโนมัยซีทสปีชีส์ใหม่ จึงเสนอตั้งชื่อว่า *Micromonospora dendrobii* การทดสอบฤทธิ์ต้านเชื้อรา พบว่า สายพันธุ์ DR5-1 DR7-3 DR8-5 และ DR8-8 สามารถยับยั้งการเจริญของเส้นใยของราก่อโรคพืช *Fusarium oxysporum* SA01 *Fusarium solani* SA02 *Alternaria alternata* SA01 *Curcuria oryzae* SA04, และ *Colletotrichum gloeosporioides* SA03 โดยสายพันธุ์ DR7-3 แสดงฤทธิ์การต้าน *C. oryzae* SA04 ได้สูงสุด และเมื่อทำการวิเคราะห์สารเมแทบอไลต์ทุติยภูมิในสารสกัดหยาบโดยเทคนิคแก๊สโครมาโทกราฟี-แมสสเปกโตรเมทรี พบมีสารทุติยภูมิ 15 ชนิด eicosane, phenol-2,4-bis(1,1-dimethylethyl), hexadecane และ hexadecanoic acid-methyl ester มีฤทธิ์ในการยับยั้งการเจริญของเชื้อรา การคัดกรองการส่งเสริมการเจริญเติบโตของพืช พบว่าแอคติโนมัยซีทเอนโดไฟต์สามารถผลิตกรดอินโดลแอซีติกได้จาก 0.04±0.36 to 294.10±12.17 µg/mL โดยสายพันธุ์ DR1-2 สามารถผลิตกรดอินโดลแอซีติกได้สูงสุด เมื่อทำการหาภาวะที่เหมาะสมต่อการผลิตกรดอินโดลแอซีติกของ DR1-2 พบว่าเมื่อใช้ L-tryptophan ที่ความเข้มข้น 0.5% ระดับความเป็นกรด-ด่าง 6 และบ่มที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 7 วัน ทำให้ผลิต IAA เพิ่มขึ้นเป็น 489.73±8.90 µg/mL กรดอินโดลแอซีติกที่ได้จากสายพันธุ์ DR1-2 สามารถส่งเสริมความยาวราก ความยาวยอด จำนวนของราก และน้ำหนักสดของต้นอ่อนข้าวพันธุ์ กข49 ผลการศึกษานี้พบว่าแอคติโนมัยซีทจากกล้วยไม้ไทยเป็นแหล่งของสารต้านจุลินทรีย์และฮอร์โมนพืชสำหรับใช้ในการเกษตร

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KEYWORD: endophytic actinomycetes, antimicrobial activity, plant growth promoting bacteria, indole-3-acetic acid, Thai orchid

Nisachon Tedsree : IDENTIFICATION, ANTIFUNGAL AND PLANT GROWTH PROMOTING ACTIVITIES OF ENDOPHYTIC ACTINOMYCETES FROM ORCHIDS. Advisor: Prof. SOMBOON TANASUPAWAT, Ph.D. Co-advisor: Prof. KITTISAK LIKHITWITAYAWUID, Ph.D.

Sixty-two endophytic actinomycetes were isolated from 15 Thai orchids. They were identified as *Streptomyces* (34 isolates), *Micromonospora* (21 isolate), *Streptosporangium* (2 isolates), *Actinomadura* (1 isolates), *Amycolatopsis* (3 isolates) and *Pseudonocardia* (1 isolates) based on their phenotypic, chemotaxonomic and genotypic characteristics. The antimicrobial activity screening revealed that *Streptomyces* (18 isolates), *Micromonospora* (12 isolates) and *Streptosporangium* (1 isolate) exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231. Strain DR2-2 could inhibit all tested pathogens. Based on 16S rRNA gene sequencing, strain DR6-1 was closely related to *Amycolatopsis nivea* KCTC 39515^{T} with 99.06%, average nucleotide identity (ANI) with 93.8 % and digital DNA-DNA hybridization (dDDH) with 52.50 %. Therefore, strain DR6-1^{T} was a new actinomycete species and was proposed as *Amycolatopsis dendrobii*. Based on 16S rRNA gene sequencing, strain DR5-3 was closely related to *Micromonospora yasonensis* DSM 45980^{T} (98.96%), Therefore, strain DR5-3^{T} was a new actinomycete species and was proposed as *Micromonospora dendrobii*. Strains DR5-1, DR7-3, DR8-5, and DR8-8 showed inhibitory activity against five phytopathogenic fungi including *Fusarium oxysporum* SA01*, Fusarium solani* SA02*, Alternaria alternata* SA01*, Curcuria oryzae* SA04*, and Colletotrichum gloeosporioides* SA03*.* Strain DR7-3 exhibited a broad-spectrum antifungal activity against five fungi, especially *C. oryzae* SA04. The analysis of secondary metabolites from ethyl acetate extract of strain DR7-3 using the gas chromatography-mass spectrometry, fifteen identified compounds were found. Eicosane, phenol-2,4-bis(1,1-dimethylethyl), hexadecane, and hexadecanoic acid-methyl ester showed antifungal activity. Screening for plant-growth promoting activity, endophytic actinomycetes could produce Indole-3-acetic acid (IAA) ranged from 0.04±0.36 to 294.10±12.17 µg/mL. Strain DR1-2 showed maximum IAA of 489.73±8.90 µg/mL, when optimized using 0.5% L-tryptophan, pH 6, with incubation at 30°C for 7 days. The IAA of strain DR1-2 enhanced the root length, shoot length, the number of roots, and fresh weight of rice seedlings (*Oryza sativa* L. cv. RD49). Results indicated that actinomycetes from Thai orchids were promising sources of antimicrobial compounds and plant hormones for agricultural applications.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I

INTRODUCTION

Actinomycetes are filamentous Gram-positive bacteria belonging to the phylum *Actinobacteria* that have their morphology like fungi due to their elongated cells and branch into filaments or hyphae (Ventura et al., 2007). *Actinomyces, Arthrobacter, Bifdobacterium, Cellulomonas, Clavibacter, Corynebacterium, Frankia, Microbacterium, Micrococcus, Mycobacterium, Nocardia, Propionibacterium, Pseudonocardia, Rhodococcus, Sanguibacter* and *Streptomyces* in the phylum *Actinobacteria* demonstrated the various activity that could be used as biocontrol agents for crops protection under the stress conditions from phytopathogenic fungi for sustainable agriculture (Yadav & Yadav, 2019). Among all microorganisms, actinobacteria showed the most significant role in plant disease management. Nowadays, *actinomycetes* have been widely attracted because of their abilities to produce numerous biologically active compounds. Most members of *Actinobacteria*, especially *Streptomyces* strains, are the superior source for the production of biocontrol agents, including antifungal compounds and antibiotics for crop protection (Běhal, 2000). These bioactive compounds have been exploited to control infected diseases from bacteria, fungi, pests, and insects (Solanki et al., 2016) as agrochemicals in agricultural management (Berdy, 2005a). Bioactive compounds (75 % commercial) are produced by *Streptomyces* strains. More than 10000 bioactive compounds from actinomycetes, 7600 are from *Streptomyces* strains and 2500 are from rare actinomycetes species.

Actinomycetes are widely distributed in diverse habitats and conditions such as soils, compost, freshwater, seawater, plants, and unfavorable environmental conditions because of their filamentous and sporulating properties of them (Vurukonda et al., 2018). They have been interested as a source of bioactive compounds because of their low toxicity and ecofriendly. In addition, identifying the mode of action of biocontrol agents is important for developing actinomycetes based on degradation in nature while highly specific and less toxic to non-target

organisms that lead to the development of safer products for the environment and human health (Flores-Gallegos & Nava-Reyna, 2019). There are many reports that *Streptomyces* strains can produce secondary metabolites such as antibiotics and antifungal compounds (Dhanasekaran et al., 2012). For example, all of the significant antifungal activity against *Fusarium oxysporum* caused by *Fusarium* wilt disease in various economic crops was found by *Streptomyces* sp. 201 (Gajen N. Bordoloi et al., 2001), *Streptomyces* sp. TP-A0569 (Sasaki et al., 2002), *Streptomyces luozhongensis* (Zhang et al., 2017), *Streptomyces griseorubens* E44G (Al-Askar et al., 2015) and *Saccharothrix algeriensis* NRRL B-24137 (Merrouche et al., 2017). Due to an increase in the world population by 2050, more abilities of biocontrol agents should be exposed to improve crop yield (Olanrewaju & Babalola, 2019). *Streptomyces* strains were used as plant growth promoters by providing the plant hormones such as auxin, cytokinin and gibberellin (Yadav et al.). They have become one of the candidates to enhance the growth and yield in economic crops. *Streptomyces* strains provided a source of natural products that may have potential in agricultural management. Their activity has been evaluated against different plant pathogens for suppressing plant diseases and promoting plant growth in agricultural management.

The metabolites from actinomycetes indicated their future potential for use in pharmaceutical and agricultural applications. The study of these metabolites has linked with actinomycetes both the isolation and screening of their bioactive compounds. Isolation is the crucial step while obtaining pure cultures of actinomycete strain from the host plant. The host species, sampling, tissue type and ages of plant, geographic, climate, habitat, culture conditions, surface sterilization, and selective media also impact the species and number of endophytes (Zhang et al., 2017). The production of bioactive compounds of actinomycetes usually comes from genetics. However, it can be influenced by environmental management such as a nutrient in variation media, supplement of a stimulant in growth rate, and environmental condition changes (Harir et al., 2018). In this study, actinomycetes will be isolated and identified from selected orchids. Thailand has rich source of tropical plants. It offers the opportunity to study the genetic diversity of endophytic actinomycetes, leading to the discovery of potential actinomycetes. Their genetic

information, morphological characters, and biochemical properties will be determined. Their inhibitory activities against phytopathogenic fungi and plant growth promoting will be evaluated.

1.1 Research hypothesis

Endophytic actinobacteria are known as a source of many valuable bioactive secondary metabolites. Many researches showed that plants are potential resources of new actinobacteria and new bioactive compounds. However, there is no document for the endophytic actinobacteria associated with the orchid before. The hypothesis of this study is that the healthy orchids of Thailand may harbor the new actinobacteria. Therefore, if the isolation was carried out using a healthy orchid, several new actinobacteria should be obtained. Consequently, these new actinobacterial isolates may produce new bioactive compounds or plant growth hormones that can be used for further agricultural applications.

1.2 Research objectives

1. To isolate and identify endophytic actinomycetes based on phenotypic, chemotaxonomic and genotypic characteristics.

2. To screen antimicrobial activity and the plant growth promoting activity of endophytic actinomycete isolates.

3. To analyse chemical profiling of secondary metabolites produced by the selected endophytic actinomycete isolates.

1.3 Expected benefits

1. The genetic information, morphological characters, and biochemical properties of endophytic actinomycetes.

2. The inhibitory activities against phytopathogenic fungi and plant growth promoting of endophytic actinomycetes.

CHAPTER II

LITERATURE REVIEW

Actinomycetes are filamentous Gram-positive bacteria with high guanine (G) plus cytosine (C) content (> 55 mol%) in their genomic DNA. They belonged to the phylum *Actinobacteria*, one of the largest phyla within the domain bacteria. This phylum contains six classes (*Actinobacteria*, *Thermoleophilia*, *Nitriliruptoria*, *Rubrobacteria*, *Coriobacteriia Acidimicrobiia*), 29 orders, 67 families and 391 genera (Yadav & Yadav, 2019). The principal genera of *Actinobacteria* such as *Streptomyces, Streptosporangium, Pseudonocardia, Nocardia, Saccharopolyspora, Nocardioides, Nonomuraea, Kitasatospora, Micromonospora, Actinomadura, Actinomyces, Actinoplanes*, and *Amycolatopsis* (Yadav & Yadav, 2019). Among these genera, *Streptomyces* is the dominant genera. Actinomycetes can be divided into two major groups: *Streptomyces* and non-*Streptomyces* (rare-actinomycetes).

Actinobacteria produce elongated cells and branch into filaments or hyphae. However, actinobacteria also have many bacterial properties. Their hyphae are 1 μm in diameter within the bacterial size. They do not have a nuclear envelope, prokaryotic cytology, which is very different from eukaryotic fungi (Lechevalier & Lechevalier, 1967). The life cycle of actinomycetes is more complex than other bacteria. They produce spores, like conidia forming singly or in a chain of various lengths or enclosed in sporangia for reproduction. In the appropriate environment, the spore will germinate the germ tube and differentiate from substrate mycelium toward the solid surface. At the different stages, the aerial mycelia are formed and then developed into a chain of spores (Lechevalier & Lechevalier, 1967).

Actinomycetes inhabit various environments such as soil, aquatic sediment, marine sediment, extreme environment, sponges, insects, lichens, and plant tissue. They are generally believed to have an essential role as decomposers that recycle nutrients in the environment. Most are saprophytes, but some are parasitic or mutualistic associated with plants. They contribute to the degradation of lignin, organic matter and chitin, the formation and stabilization of humus, and the

production of valuable bioactive secondary metabolites. Most of the members of actinobacteria are candidates for use as sources of biocontrol agent production, particularly *Streptomyces* strains. Many studies revealed that they are usually harmless and particularly beneficial microorganisms in the pharmaceutical industry, agriculture and environment, such as antibiotics, antifungals, antimalarials, anti-cancers, enzymes, pesticides, and plant growth hormones (Berdy, 2005b) (Flores-Gallegos & Nava-Reyna, 2019).

2.1 Endophytic actinomycetes

Endophytic actinomycetes are bacteria that live in the tissues of living plants, mainly in the root system and xylem tissues. However, there is no effect on the morphology and physiology of host plants (Kumar et al., 2015). The major role of endophytic bacteria is to improve the health and growth of the host plant. The overall interaction between endophytic bacteria and host plants is mutually beneficial. The bacteria inside the plant usually get protection and nutrition from the host plant. On the other hand, they provide good health to the host plants by producing some beneficial metabolites (Shimizu, 2011). Endophytes could be promising agents for controlling plant infections from pathogens because they have the ability to the production of bioactive secondary metabolites in order to control other endophytes or plant pathogens (Patil & Chaudhari, 2011) and promote the gene expression in plant resistance via both biotic and abiotic stress (Schrempf, 2001) (Karthik et al., 2015).

2.1.1 Diversity of endophytic actinomycetes

Endophytic actinobacteria had been reported by various groups of actinomycetes. The most common genus is *Streptomyces*, along with other frequent isolated genera such as *Microbispora*, *Nocardia*, *Nocardioides*, *Micromonospora,* and *Streptosporangium* (Shimizu, 2011). Endophytic actinobacteria from various groups of plants have been reported especially from medicinal plants that are the richest source of novel species of actinobacteria (Singh & Dubey, 2018). Endophytic

actinomycetes have been isolated from various plant hosts such as *Streptomyces platensis* 3-10 from healthy rice (Shakeel et al., 2016), *Streptomyces* sp. KLBMP 5084 from healthy *Limonium sinense* (Qin et al., 2017), *Streptomyces* spp. from herbal products (Mohd-Fuat et al., 2010), 24 actinomycete strains from herbaceous and arbor plants (Igarashi et al., 2002)(Igarashi et al. 2002), 120 endophytic actinomycetes strains from root tissues of *Alpinia galangal* (Taechowisan et al., 2006). Actinomycetes were isolated from different parts of plants, such as *Streptomyces* sp. TP-A0569 from a leaf of *Allium tuberosum* (Sasaki et al., 2002), *Streptomyces hygroscopicus* OsiSh-2 from rice sheath (Xu et al., 2019), *Streptomyces* sp. AUR4 from roots, stems and leaves of chickpea (Vijayabharathi et al., 2018). Moreover, actinomycetes can be found in contaminated plants such as *Streptomyces* sp. UPMRS4 from blast infected rice (Awla et al., 2017), *Streptomyces rochei* UU07, *S. vinaceusdrappus* UU11 and *Streptomyces* sp. UU15 from rotten wheat straw (Singh et al., 2019).

2.2 Taxonomy of actinomycetes

Actinomycetes are members of the phylum Actinobacteria and class Actinobacteria. *Streptomyces* is the dominant genera. The genus *Streptomyces* belongs to the family *Streptomycetaceae*. *Streptomyces albus* is the type species for this genus. They develop on a highly branching substrate and aerial mycelia, where they produce smooth, warty, rugose, spiny, or hairy spore chains. They lack diagnostic sugars in whole-cell hydrolysates, but their chemotaxonomic characteristics contain *LL*-diaminopimelic acid in the peptidoglycan of the cell wall (Cell wall chemotype I) (Lechevalier et al., 1977). Predominant menaquinones are MK-9(H_6) and MK-9(H_8). The phospholipid profile contains phosphatidylethanolamine (PE) as a diagnostic phospholipid (Type PII phospholipid pattern) (Lechevalier et al., 1977). There is no mycolic acid. This genus now has 854 species and 38 subspecies with officially published names (www.bacterio.net/streptomyces.html) that are found in soil, marine sediment, freshwater sediment, animals, insects, lichens, and plants. The genus *Micromonospora* belong to the family *Micromonosporaceae*. In this

genus, *Micromonospora chalcea* is the type species. On substrate mycelium, they produce a single conidial spore but lack aerial mycelium. This genus presently has 110 species and 7 subspecies with validly published names (www.bacterio.net/micromonospora.html). This genus is found in mangrove, sandy, and rhizosphere soils, limestone quarries, nickel mining sites, sea water, volcanic sediments, insects, and plants.

2.3 Identification of endophytic actinomycetes

Actinomycetes were identified based on morphological and cultural characteristics of different media, including yeast extract-malt extract (ISP2), oat-meal agar (ISP3), inorganic salts-starch agar (ISP4), glycerol-asparagine agar (ISP5), peptone-yeast extract iron agar (ISP6), tyrosine agar (ISP7) and nutrient agar (NA) that related with the colour of substrate mycelium, aerial mycelium and soluble pigment (Shirling & Gottlieb, 1966). The chemotaxonomic characteristics of the cell composition including diaminopimelic acid (DAP), menaquinones, polar lipid composition, mycolic acids. Generally, *Streptomyces* strains contained the LL-DAP isomer and other actinomycetes contained *meso*-DAP. The morphological characteristics were observed in colonial appearances, colour, and sporulation, distinguished by scanning electron microscopy (SEM) (Abdallah et al., 2013). The phenotypic characteristics of *Actinomycetes* including cultural, physiological and biochemical properties such as carbon utilization, the temperature test, and pH tolerance test, are determined by the standard protocol of International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966). The genotypic characteristics were analyzed by amplification and 16S rRNA gene sequencing. The degree of DNA similarity was estimated using the bio-edit software and blast at www.ncbi.nlm.nih. gov/blast (Tamura et al., 2013). The phylogenetic tree was constructed using MEGA software for studying the relationship of evolution, including neighbor, maximum parsimony, and maximum likelihood (Felsenstein, 1985).

2.3.1 Phenotypic characteristics

Phenotypic characteristics comprise morphological, cultural, biochemical and physiological characteristics that use the characterization methods (Shirling & Gottlieb, 1966). The morphological characteristics are concerned with spore germination, which results in the formation of aerial mycelium, substrate mycelium, and spore. The shape, surface, and arrangement of the spore chain are the main characteristics of actinomycete identification at the genus level during spore development. The cultural, biochemical and physiological characteristics as the color of colony, both substrate and aerial mycelium, soluble pigment production, pH, temperature, and NaCl tolerance growth, carbon and nitrogen utilization, gelatin liquefaction, nitrate reduction, skim milk coagulation and peptonization, enzyme activities, acid production, and starch hydrolysis are various characteristics in the species level (Arai et al., 1975).

2.3.2 Chemotaxonomic characteristics

Chemotaxonomic characteristics, such as whole-cell hydrolysate analysis as diaminopimelic acid and whole-cell sugar (Staneck & Roberts, 1974), cellular fatty acid (Sasser, 1990), phospholipids (Minnikin et al., 1984), and menaquinone (Sasser, 1990), classify the genus level of actinomycetes that determine the components of cell wall and cell membrane (Collins et al., 1977). A polymer of N-acetylglucosamine acid (NAG), N-acetylmuramic acid (NAM), and peptide moiety linked chain called peptididoglycan is found in bacterial cell walls. Based on the variation of the peptide moiety, the types of diaminopimelic acid isomers, and the N-acyl types of muramic acid, these three components have been used for the classification of bacteria (Lechevalier et al., 1977). The majority of the peptide chain, 2,6-diaminopimelic acid (DAP; A2pm), has three stereoisomers (*LL*-, *DD*-, and *meso*-DAP), 3-OH-DAP, and 3,4-dihydroxyl diaminopimelic acid (3,4-OH-DAP). Streptomyces' hydrolyzed cell wall usually contains LL-DAP, but other rare actinomycetes may have meso-DAP, 3-OH-DAP, 3-4-OH-DAP, or a combination of isomers. Whole-cell sugar composition is used to classify the sporulated actinomycetes which have *meso*-DAP in the cell wall.

Menaquinone-containing isoprenoid quinones in the membranes of bacterial cells are involved in the electron transport mechanism of cell respiration and oxidative phosphorylation. Bacterial identification based on the number of isoprene units and the degree of hydrogenation of double bonds in the isoprenyl chain. Menaquinones constitute the majority of the isoprenoid quinones in the membranes of actinomycete cells (Collins et al., 1977). The lipid bilayer of bacterial cell membranes contains phospholipids, which are related to permeability and control at the membrane. The phospholipid that can be used to identify actinomycete include diphosphatidylglycerols (DPG), phosphatidylinositol (PI), phosphatidylinositol-mannosides (PIMs), acyl phosphatidylglycerol (APG), phosphatidylethanolamine (PE), methylphosphatidyl-ethanolamine (PME), glycophospholipids, phosphatidylcholine (PC), and unidentified phospholipids containing glucosamine. Long-chain fatty acids are found in the lipid bilayer of bacterial cell membranes. The 12–20 carbon atoms and the 20–80 carbon atoms of the fatty acids are separated. The key to bacterial characterization is based on the length of the carbon chain, where the methyl groups branch (iso- or anteiso-), and where the double bond is located (Minnikin et al., 1984).

2.3.3. Genotypic characteristics

Most bacteria's chromosomal DNA is a circular, double-helix strand made of four nucleotide bases that form hydrogen bonds, such as adenine (A) and thymine (T) and cytosine (C) and guanine (G). The genotypic characteristics are evaluated using traditional (wet-lab) and bioinformatics (in-silico genome-to-genome comparison) methods on the comparative microorganisms to show the distinctive phylogenetic and phylogenomic relationships based on DNA based composition, 16S rRNA gene sequence, multilocus sequence, and genomic sequence. In recent years, developments from PCR-based capillary sequencing to whole genome next-generation sequencing (WGS) have transformed how bacteria are identified, their putative genes for secondary metabolite genes or pathogenic genes are discovered, and their natural products are discovered.

2.4 Antifungal activity of actinomycetes

The secondary metabolites of actinomycetes are usually produced in the late stage of the growth phase. The production of these compounds is genetically made, but they can be influenced by environmental management. Thus, lack of a nutrient, stimulating and/or sedative supplement, and environmental changes are typical initiators of secondary metabolism. Endophytic actinomycetes, particularly from medicinal plants, can prevent or inhibit a various variety of pathogens, including bacteria, fungi, and viruses. Bioactive compounds from endophytic actinomycetes have been reported on antagonistic effects in phytopathogenic fungi. For instance, *Colletotrichum musae* and *Candida albicans* were inhibited by actinomycin D from endophytic *Streptomyces* sp. Tc022 derived from *Alpinia galangal* roots (Taechowisan et al., 2006). Chrestoxanthone A from *Streptomyces chrestomyceticus* BCC 24770 was active against *Curvularia lunata* and *Alternaria brassicicola* (Bunyapaiboonsri et al., 2016). 1-ethylthio-3-methyl-1, 3-butadiene and (chloromethyl)-2-cyclopropyloxirane, 2, 4- ditert-butylphenol from three different strains of *Streptomyces* sp., SS1, SS5, and SS8 can suppress the growth of fungal pathogen (Patel et al., 2018). For use in agricultural systems, these compounds indicated their future potential for use as antifungal agents.

Around 10-20% of the extracts from endophytic actinomycetes displayed antagonistic activity against fungal or bacterial causing plant diseases (Igarashi et al., 2002). For example, *Alternaria brassicicola* TP-F0423, a fungal pathogen in the sprouts of Chinese cabbage, was inhibited by several extracts from actinomycetes (Igarashi et al., 2003). The crude extracts from *Streptomyces* sp.201 (Gajen N Bordoloi et al., 2001), *Streptomyces* sp. TP-A0569 (Sasaki et al., 2002), *Streptomyces luozhongensis* (Zhang et al., 2017), *Streptomyces griseorubens* E44G (Al-Askar et al., 2015), and *Saccharothrix algeriensis* NRRL B-24137 (Merrouche et al., 2017) showed significant antifungal activity against *Fusarium oxysporum*, causing *Fusarium* wilt disease in economic crops. In addition, many reports showed that actinomycetes are commonly used as biocontrol agents against phytopathogens, including *Colletotrichum* (Boukaew et al., 2018), *Alternaria* (Khamna et al., 2009) (Kaur & Manhas, 2014) (Phuakjaiphaeo et al., 2016), *Fusarium* (Toumatia et al., 2016),

Rhizoctonia (Ahsan et al., 2017) and *Botrytis* (Cho et al., 2017; Wang et al., 2018). However, there are many factors in secondary metabolite production from these actinomycetes to inhibit pathogens, such as the strain of the organism, the type of medium, and growth conditions (Waksman et al., 2010). The active antifungal compounds from various actinomycete strains are shown in table 1.

Table 1 Antifungal bioactive compounds of actinomycete strains.

Strain	Bioactive compounds	Phytopathogenic fungi	References
Streptomyces lydicus No. AZ-55	Natamycin	*Fusarium oxysporum, Alternaria alternata, Rhizoctonia solani*	Atta *et al*., 2015
Streptomyces sp. X852460	Eicosane, Dibutyl phthalate	*R. solani*	Ahsan *et al*., 2017
S. sanglieri AUM 00500	Cycloheximide, Dctiphenol	*Ganoderma boninense*	Azura *et al*., 2016
Streptomyces sp. N2.	Antifungalmycin N2	*R. solani, Pythium italicum, Pyricularia grisea, F. oxysporum, C.gloeosporioides*	Xu *et al*., 2015
S. griseus H7602	1H-pyrrole-2-carboxylic acid	*Phytophthora capsici*	Nguyen *et al*., 2015
S. mutabilis strain IA1	Actinomycin D	*F. oxysporum*	Toumatia *et al*., 2015
Streptomyces sp. S4–7	Caryolan-1-ol	*Botrytis cinerea*	Cho *et al*., 2017
S. chrestomyceticus BCC 24770	Chrestoxanthone A	*Curvularia lunata, Alternaria brassicicola*	Bunyapaiboonsri *et al*., 2016
Streptomyces sp. SS1, SS5 and SS8	2(chloromethyl)-2-cyclopropyloxirane	*Magnaporthe oryzae, R. solani*	Patel, Madaan, and Archana, 2018
Streptomyces sp. KNF2047	Neopeptins	*C. lagenarium, A. mali, B. cinerea, Magnaporthe grisea. Didimella bryoniae*	Kim *et al*., 2007
Streptomyces sp.201	2-methyl- heptyl-isonicotinate	*F. semitectum F. moniliforme F. solani F. oxysporum*	Bordoloi *et al*., 2001
Streptomyces sp. TP-A0356	Yatakemycin	*Aspergillus fumigatus*	Igarashi *et al*., 2003
Streptomyces sp. TP-A0569	6-Prenylindole	*Alternaria brassicicola,*	Sasaki *et al*., 2002

Strain	Bioactive compounds	Phytopathogenic fungi	References
		F, oxysporum	
S. cavourensis NA4	Bafilomycins B1, C1	Soilborne fungal pathogens	Pan *et al*., 2015
S. malaysiensis	Malayamycin	*Stagonospora nodorum*	Li et al., 2008
Streptomyces sp. UPMRS4	Fungichromin, Amicomacin, Ergotamine, Rapamycin	*Pyricularia oryzae*	Awla *et al*., 2016
Streptomyces sp. SN194	Chloroxaloterpin A, B	*B. cinerea*	Bi and Yu, 2016
Streptomyces sp. CEN26	2,5-bis(hydroxymethyl) furan monoacetate	*A. brassicicola*	Phuakjaiphaeo *et al*., 2016
Streptomyces sp. FJAT-31547	n-Hexadecanoic acid	*F. oxysporum* *R. solanacearum*	Zheng *et al*., 2019
S. gancidicus HG29	Oligomycins A and E	*Fusarium* spp.	Khebizi *et al*., 2018
Streptomyces sp. CB-75	Benzenedicarboxylic acid diisooctyl ester	*Colletotrichum musae*	Chen *et al*., 2018
Streptomyces sp. No. T-7545	Validamycins A and B	*Pellicularia sasakii* *R. solani*	Iwasa, Yamamoto, and Shibata, 1970
S. humidus S5-55	Phenylacetic acid, sodium phenylacetate	*Pythium ultimum,* *Phytophthora capsici,*	Hwang *et al*., 2001
S. hydrogenans DH16	10-(2,2-Dimethyl-cyclohexyl)-6,9-dihydroxy-4,9-dimethyl-dec-2-enoic acid methyl ester	*A. brassicicola*	Kaur *et al*., 2016
S. padanus PMS-702	Fungichromin	*R. solani*	Shih *et al*., 2003

2.5 Plant growth promoting activity of actinomycetes

Plant hormones are chemical compounds produced from specific tissues in the plant and transported to target tissues for plant development at low concentrations. Indole acetic acid (IAA) is one type of plant hormone. It stimulates cell elongation by increasing the osmotic potential in the cell, increase the permeability of water into the cell, increase protein synthesis and cell wall synthesis, which play a significant role in inducing root formation, callus formation, and parthenocarpy (Zhao, 2010). IAA is a normal product of L-tryptophan metabolism

produced by various microorganisms. Tryptophan exudates from a root plant and induces the IAA synthesis by microorganisms. Many researches indicated that actinomycete strains could synthesize IAA through the indole-3-acetamide (IAM) pathway.

Actinomycetes produced an excellent source of indole acetic acid to plant growth promotion, which increased the yield of crops. For example, (Khucharoen et al., 2016) Khucharoenphaisan et al. (2016) reported the potential of *S. malaysiensis* LB35 to produce a biocontrol agent to inhibit *Phytophthora* sp. cause of root rot in cassava and produced IAA that stimulates the growth of cassava. *Streptomyces* sp. CMU-H009 showed the maximum IAA production at 300 µg/mL and a significant increase in the elongation of maize root and germination of cowpea seeds (Khamna et al., 2010). *Streptomyces* sp. MBRL 10 from limestone showed significant antagonism against *Rhizoctonia solani* and showed the ability to promote the germination of rice (Tamreihao et al., 2016). (Anwar et al., 2016) Anwar, Ali, and Sajid (2016) showed IAA production from *S. nobilis* WA-3 IAA that promoted the growth of wheat (*Triticum aestivum*) seeds, fresh weight, dry weight, root length, number of roots, and number of leaves. *Streptomyces* sp. AUR4 significantly enhanced seed numbers, seed weight, pod numbers, pod weight, and biomass in chickpea genotype JG11 (Vijayabharathi et al., 2018). (Tamreihao et al., 2016) Tamreihao et al. (2016) studied the potential in IAA production of *S. corchorusii* UCR3-16 to promote rice growth. Both pot trial and field experiments showed significant growth increase and grain yield production using talcum powder formulation. *S. rochei* UU07, *S. vinaceusdrappus* UU11 and *Streptomyces* sp. UU15 isolated from rotten wheat straw were screened and were found to have potential to plant growth promoting (PGP) traits (IAA, phosphate solubilization, HCN, ammonia, siderophore, and AAC deaminase (Singh et al., 2019). *Streptomyces sp.* NCIM 5533 showed the potential as a plant growth-promoting bacteria by producing IAA and solubilization of ammonia and phosphate. In the laboratory and greenhouse, NCIM 5533 also showed the ability to colonize roots and promote the growth of tomatoes (Puppala et al., 2019). Mohandas et al. (2013) (Mohandas et al., 2013) showed the abilities of *S. canus* on phosphate solubilization, siderophore production, IAA and GA3 production, and

antagonistic activity against pathogens, including chitinase activity and it also increases the yield of guava. *Streptomyces sp.* PM9 promoted the growth of *Eucalyptus globulus* and *E. grandis*, and this isolate showed the potential to use as a biocontrol agent in forestry (Salla et al., 2014). *Streptomyces sp.* DBT204 enhanced the growth of the seedling of chili and tomato by producing phytohormones (Passari et al., 2016). Actinomycetes as plant growth promoters on target plants are shown in Table 2.

Table 2 Plant growth promoting activity of actinomycete strains on target plants.

Strain	Target plants	References
Streptomyces sp. CMU-H009	Maize, Cow pea	Khamna *et al*., 2010
S. malaysiensis LB35	Cassava	Khucharoenphaisan *et al*., 2016
Streptomyces sp. VSMGT1014	Rice	Harikrishnan *et al*., 2014
Streptomyces sp. MBRL	Rice	Tamreihao *et al*., 2018
S. nobilis WA-3	Wheat	Anwar *et al*., 2016
S. variabilis (4NC), *S. fradiae* (8PK)	Stevia plant	Tolba *et al*., 2019
Streptomyces sp. AUR4	Chickpea	Vijayabharathi *et al*., 2018
S. corchorusii UCR3-16	Rice	Tamreihao *et al*., 2016
Streptomyces sp. NCIM 5533	Tomato	Puppala *et al*., 2019
Streptomyces sp. KLBMP 5084	*L. sinense*	Qin *et al*., 2017
Streptomyces sp. A1RT	Potato	Sarwar *et al*., 2018
Streptomyces sp. KLBMP S0051	Wheat	Gong *et al*., 2018
S. canus	Pomegranate	Poovarasan *et al*., 2013
S. canus	Guava	Mohandas *et al*., 2013
Streptomyces sp. DBT204	Chili & tomato	Passari *et al*., 2016
S. ramulosus EUSKR2S82	Eucalyptus	Himaman *et al*., 2016
S. rochei WZS1-1	Wheat	Han *et al*., 2018
S. sundarbansensis WZS2-1		
Streptomyces sp. PM9	Eucalyptus	Salla *et al*., 2014

Indole-3-acetic acid and antifungal compounds from actinomycetes are used as plant growth-promoting agents (help to produce plant growth hormone) and agrochemical compounds, respectively. It could be a promising candidate for utilization in the agriculture system to improve the growth of plants.

CHAPTER III

RESEARCH METHODOLOGY

In this study, the endophytic actinomycetes were isolated from orchids in Thailand and identified based on phenotypic, chemotaxonomic and genotypic characterization. All strains were screened for antimicrobial activity and plant growth promoting activity. The interesting strains, which show the effective bioactivities of secondary metabolites, will further be studied on their chemical structure using spectrometry.

3.1 Sample collection and Isolation

The Thai orchids samples including *Dendrobium christyanum*, *D. formosum*, *D. kentrophyllum*, *D. findlayanum*, *D. chrysanthum*, *Calanthe cardioglossa*, *D. friedericksianum*, *D. chrysotoxum*, *D. crumenatum*, *D. heterocarpum*, *Coelogyne lawrenceana*, *Eria ornate*, *Cleisostoma rostratum*, *Coelogyne assamica,* and *Pinalia globulifera.* were collected from Ubon Ratchathani, Suphanburi, Chiang Mai and Prachinburi Province. The whole plant was separated into three parts, which are leaves, stems and roots. The samples were cleaned with detergent and washed with tap water. Each part of the sample was cut about 2-3 cm in length pieces. The sample surface was sterilized with 70% ethanol for 5 minutes, followed by 3% $NaClO_4$ solution for 6 minutes, and rinsed with sterilized water three times. Plant samples were soaked in 10% $NaHCO_3$ for 10 minutes and dry for three hours. Then, the samples were crushed using a mortar with 4% glucose solution and making ten-fold serial dilution. Each dilution was spread on four different media, including gellen gum, starch casein (Küster and Williams 1964), glycerol arginine (Arai et al., 1975) and gause No.1 (Gause et al., 1983) supplemented with nalidixic acid (25 µg/ml) and cycloheximide (50 µg/ml). The plates were incubated for one month at 30°C. The selected actinobacterial colonies were inoculated to ISP 2 medium for purification and stock cultures. The purified isolates were preserved on ISP 2 medium at 4°C, freezing at -20°C in glycerol solution and dry by freeze-drying.

3.2 Identification of actinomycetes

The selected isolates were identified based on phenotypic, chemotaxonomic, and genotypic characteristics.

3.2.1 Phenotypic characteristics

All isolates were cultivated in ISP 2 broth by shaking at 180 rpm and incubated at 30 ℃ for five days. The cells were collected by centrifugation at 6,500 rpm for 5 minutes, then washed with 0.85% normal sterile saline solution three times. These inoculums were used for all phenotypic studies.

- Morphological characteristics

The spore and mycelial characteristics of selected strains cultivated on ISP 2 medium at 30 ℃ for 7-14 days were observed using light and scanning electron microscopes.

- Cultural characteristics

The substrate mycelia, aerial mycelia and diffusible pigment were observed on the isolates cultivated at 30 ℃ for 14 days on various media, including yeast extract-malt extract (ISP 2), oat-meal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract iron agar (ISP 6), tyrosine agar (ISP 7) and nutrient agar (Shirling & Gottlieb, 1966). The colour designation of mycelia was compared with the NBS/IBCC colour system (Kelly, 1964).

- Physiological characteristics

All isolates were evaluated by cultivating on ISP 2 agar at different temperatures (20-50 ℃) and NaCl concentrations (0-9 % w/v) while the effect of pH for growth, at pH range of 4-12 (at intervals of 1 pH unit) using the following buffer system: acetate buffer (pH 4-5), phosphate buffer (pH 6-8), and glycine-sodium hydroxide buffer (pH 9-12), was observed in ISP 2 broth at 30 ℃ for 14 days.

- Biochemical characteristics

All isolates were determined by carbon utilization, starch hydrolysis, nitrate reduction, milk peptonization, gelatin liquefaction and acid production that cultivated on various media at 30 ℃ for 14 days (Arai et al., 1975; Shirling & Gottlieb, 1966).

- The carbon utilization was determined on ISP9 medium supplemented with 1% (w/v) of carbon sources. The media containing glucose and no carbon sources is positive and negative controls, respectively.

- Starch hydrolysis was determined using an inorganic-salt starch medium (ISP4). The isolated will be flooded with 1% (v/v) of iodine solution. The positive result of starch hydrolysis is the clear zone around the colony.

- The ability to liquefy gelatin was observed using bouillon gelatin broth. After incubation for 14 days, the culture was placed at 4 °C for 1 hour. The positive result is the liquid solution of gelatin.

- The ability to peptonize skim milk was observed on the skim milk agar. The positive result of skim milk peptonization is the clear zone around the colony.

- The ability to reduce nitrate to nitrite was determined using peptone KNO_3 broth. After incubation for 14 days, the culture broth was added with 0.5 ml of sulfanilic acid and N, N-dimethyl-α-naphtylamine solutions. The pink to red color represented the presence of nitrite (positive). If the color not changes, zinc powder was added to detect the over nitrate reduction. After adding zinc powder, the red to pink color indicated the negative for nitrate reduction test while no color change indicated the over nitrate reduction (positive).

3.2.2 Chemotaxonomic characteristics

The selected strains were determined using freeze-dried cells obtained from the cultures grown in ISP 2 broth at 30 °C for three days in a shaking condition at 180 rpm. The cell culture was washed with sterile distilled water three times before freeze-drying. The freeze-dry cell was used for all chemotaxonomic studies. The isomers of diaminopimelic acid and whole-cell sugars were evaluated using the standard TLC method (Staneck & Roberts, 1974). Phospholipid profiles were identified by 2-dimensional TLC (Minnikin et al., 1984). The cellular fatty acids were prepared according to the MIDI Sherlock Microbial Identification System protocol and analyzed by gas chromatography (Sasser, 1990). Menaquinones were extracted following Collins et al. (1977)(Collins et al., 1977) and analyzed by HPLC.

3.2.3 Genotypic characteristics

- The extraction of genomic DNA

The genomic DNA was obtained from the cell grown in ISP 2 medium on a shaker at 180 rpm 30 ℃ for five days. Each strain was washed with sterile distilled water and collected cell mass using centrifugation. The cells were lysed using a micro-mixer by adding 300 µl of TE buffer and a small amount of aluminum oxide into the washed cells. Then, the phenol: chloroform (1:1) was added and mixed. The suspension was centrifuged for separation. The upper layer was added with 3 mM sodium acetate and cold ethanol for DNA sedimentation. The DNA was centrifuged and discarded the solvent. The DNA tube was cleaned with 70% and 95% ethanol, respectively, and air-dried. The DNA was dissolved with a small amount of sterile ultrapure and preserved at 4 ℃.

- The amplification of 16S rRNA gene

The amplification of 16S rRNA gene was operated using two universal primers 20F and 1500R. The master mix of PCR reaction (final volume 50 µl) contained 2 µl each of primers (10 pmol/µl), 1 µl of dNTP (10 mM), 5 µl of 10x Taq buffer, 4 µl of MgCl (25 mM), 0.25 µl of Taq DNA polymerase, 30.75 µl of dH_2O and 5 µl of template DNA. The PCR condition was operated following the method of (Suriyachadkun et al., 2009). The PCR purification kit (Gene aid) was used to purify the PCR product and sequence the nucleotides using universal primers 27F, 518F, 800R and 1492R (Lane, 1991). The purified PCR products were sequenced on a DNA sequencer (Macrogen) using universal primers (Lane, 1991). The 16S rRNA gene sequences were aligned using BioEdit software (Hall, 1999) and compared for sequence similarity with related strains via the EzBiocloud server (https://www. ezbiocloud. net/) (Yoon et al., 2017). The phylogenetic tree was reconstructed through neighbor-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981), and maximum-parsimony (Fitch, 1971) based on 1000 replicates by mega 7.0 software (Kumar et al., 2016).

- Whole genome sequences analysis

Whole-genome sequencing of the selected isolates was performed with an Illumina Miseq platform (Illumina, Inc., San Diego, US-CA) using 2 × 250 bp paired-end reads. The assembling of the reads to contigs was managed using SPAdes 3.12

(Bankevich et al., 2012). All genomes were annotated on Prokka software 1.13 (Seemann, 2014) in line with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Average nucleotide identity (ANI), ANI-Blast (ANIb), and ANI-MUMmer (ANIm) values between the closely related type strains were calculated pairwise using the JSpeciesWS web service (Richter et al., 2016). The digital DNA-DNA hybridization (dDDH) was evaluated using the Genome-to-Genome Distance Calculator (GGDC 2.1) with the BLAST+ method (Meier-Kolthoff et al., 2013), and the results are dependent on recommended formula 2 (identities/HSP length). The selected strains that exhibited the ANI and dDDH values less than 95-96% and 70% (Richter & Rosselló-Móra, 2009) are indicated as new species.

- The prediction of secondary metabolites

The draft genome of selected strains was used to identify secondary metabolite gene clusters (smBGCs), the annotating was antiSMASH (antibiotics and Secondary Metabolite Analysis Shell) algorithm to detect putative biosynthetic gene clusters (BGCs) (Blin et al., 2019). Gene prediction was accomplished using the Rapid Annotation Subsystem Technology (RAST) SEED viewer (Aziz et al., 2008). An assembled genome of selected strains was submitted to the comprehensive genome analysis service at PATRIC to identify all protein and RNA coding genes, and characterize their functions (Wattam et al., 2014).

3.3 Screening of antimicrobial activities

All isolates were cultured in ISP 2 broth for five days before transferring to the production media. The production media for studying the influence of media composition on antifungal activity of the isolates are ISP 2, ISP 2 plus 0.1% $CaCO_3$, 30 medium, and 57 medium. The incubation condition is shaking at 180 rpm 30 °C. After 14 days, the metabolites were extracted with 95% ethanol and centrifuged in the cell suspension. The supernatant was used for testing the antimicrobial activity. The medium without the culture is negative control.

3.3.1 Screening of antibacterial activity

Screening of antibacterial activity was operated using the agar disc diffusion method. The medium for testing is Mueller Hinton agar (MHA) plates. The paper disc was added with the supernatant amount of 50 µl and dried at room temperature. The discs were put on the surface of the agar plate containing tested bacteria. The plates were incubated for 24 hours at 37 °C. The inhibition zone will be measured using a vernier caliper. All isolates were repeated three times. The tested bacteria are *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, and *Kocuria rhizophila* ATCC 9341.

3.3.2 Screening of antifungal activity

Screening of antifungal activity was operated using the agar disc diffusion method. The medium for testing is potato dextrose agar (PDA) plates. The paper disc was added with the supernatant of 50 µl and dried at room temperature. The actinomycete discs and phytopathogenic fungal disc were placed at position 2.5 cm from the edge and center of the plate, respectively. The plates are incubated at 30 °C until fungal mycelium in the control plate grows to the edge of the plate. The inhibition zone measured the distance between actinomycete discs and fungal mycelium edge. The percentage of inhibition is $[(C - T)/T] \times 100$, while C is the fungal radius in the control plate, and T is the fungal radius in the treatment plate. All isolates were repeated three times. The tested filamentous fungi are *Fusarium oxysporum* SA01, *Fusarium solani* SA02, *Alternaria alternata* SA01, *Colletotrichum gloeosporioides* SA03 and *Curvularia oryzae* SA04. Unicellular fungi *Candida albicans* ATCC 10231 were tested using the same antibacterial testing method.

3.3.3 The antifungal activity on the mycelial growth

The crude extracts were determined the antifungal activity against phytopathogenic fungi, including *F. oxysporum* SA01, *F. solani* SA02, *A. alternata* SA01, *C. gloeosporioides* SA03 and *C. oryzae* SA04. The percentage of mycelial inhibition was performed using the poisoned food technique. The fungi were cultured on PDA medium. The crude extract was tested at different concentrations in each treatment by dissolved with DMSO. The warm PDA medium 45-50°C was

added with different crude extract concentrations and poured into sterilized Petri dishes. The control plate is PDA medium with DMSO. The phytopathogenic fungal disc was placed center of the plate, both control and treatment plates. The plates were incubated at 30°C until fungal mycelium in the control plate grew to the edge of the plate. The percentage of mycelial inhibition is [(C - T)/T] × 100, while C is colony diameter in the control plate, and T is colony diameter in the treatment plate. The result was compared with the antifungal activity of commercial biocontrol agents. Each treatment will be repeated three times.

3.4 Chemical profile analysis of secondary metabolite of selected strains

3.4.1 Fermentation and extraction of large scale

The inoculum of the selected actinomycete strains was cultured in ISP 2 broth in a shaking condition at 180 rpm, 30 ℃, for 7-14 days. Each inoculum strain was transferred into the production medium that shows antifungal activity from the previous step. The incubation condition is shaking at 180 rpm, 30 ℃. The incubation period was varied depending on the strain. The culture was gathered and extracted with ethyl acetate (EtOAc) three times. The ethyl acetate layer was evaporated to dryness using a rotary vacuum evaporator.

3.4.2 Chemical profile analysis

The chemical profile of the crude extract of the selected strain was investigated using gas chromatography (GC-MS). The analysis was carried out with an GC Agilent 6890/MS Hewlett 5973. The injection port temperature was maintained at 250°C, and the column oven temperature program was set at 40°C 2 min, then increased to 250°C (5°C /min), ending with a 20 min isothermal at 250°C. The carrier gas was Helium (1 ml/min), HP-5MS (30 m × 0.32 mm × 0.25 µm) as the stationary phase and an injection volume of 1 µL was used. The chemical components were identified by comparison of their mass fragmentation patterns to those of the standard reference data of NIST libraries.

3.5 Screening and optimization of IAA production

3.5.1 Screening of IAA production

All isolates were streaked on an ISP 2 agar medium and incubated at 30 °C. After 7 days, the agar discs containing actinomycete mycelia were transferred to ISP 2 broth (L-tryptophan 100 µg/ml), pH 7.0 and incubated for 7 days at 30 °C with shaking at 180 rpm. The culture was centrifuged at 6,500 rpm for 5 minutes and the supernatant collected was used to determine the amount of IAA production by each strain. One ml of supernatant was added to two ml of Salkowski reagent [0.5 M of $FeCl_3$ in 35% $HClO_4$ in a proportion of 1:50 (v/v)] and keep in the dark for 30 minutes. The absorbance was measured at 530 nm using a UV-Vis spectrophotometer. The uninoculated medium with a reagent as a control. The amount of IAA produced per milliliter culture broth was calculated based on the calibration curve of IAA obtained from standard IAA at different concentrations (0- 100 µg/ml). The amount of IAA in the culture was expressed as µg/ml.

3.5.2 Determination of IAA using Thin Layer Chromatography (TLC)

The strains with a maximum production of IAA were selected for IAA extraction. The supernatant was acidified to pH 2.5 with HCl and extracted three times using ethyl acetate (1:1). The extracted ethyl acetate was dried using a rotary vacuum evaporator. The dried extract was dissolved in methanol. The IAA production was determined by thin-layer chromatography (TLC). The ethyl acetate extract was spotted on a TLC plate and developed using the mobile phase propanol and distilled water in the ratio of 8:2 (v/v) (Sameera and Prakash 2018). After development, the TLC plate was dried and sprayed with Salkowski reagent. Spots with Rf values were compared with authentic IAA.

3.5.3 Optimization of IAA production

IAA production of the potency IAA production isolates were optimized on the effects of culture media, carbon sources, temperature, pH, and L-tryptophan concentration in triplicate. The selected strain was cultivated in 100 ml of ISP 2 medium in a 500 ml flask shaking at 180 rpm. IAA production was evaluated by Salkowski's method with Salkowski's reagent. The effects of concentration of L-

tryptophan (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 1.0 and 1.5%), pH (4, 5, 6, 7, 8, 9 and 10) and temperature (25, 30, 37 and 40 °C) was performed and incubated for 7days. IAA concentration was measured by fixing all culture parameters and then changing one parameter at a time.

3.6 Determination of plant growth promoting activities

A single colony of bacteria was inoculated onto ISP 2 broth and incubated at 30 °C for 3 days. The seed culture was subsequently used for plant growth-promoting assays.

3.6.1 Ammonia production

The seed culture of stain DR1-2 was cultured in peptone water and incubated at 30°C with shaking at 180 rpm for 7 days. The bacterial culture was centrifuged at 4°C for 15 min. The supernatant was mixed with 0.5 mL of Nessler's reagent. A positive test for ammonia production was the color changed from pale yellow to dark brown (Cappuccino & Sherman, 1992). A spectrophotometer was used to measure the absorbance at 450 nm, and the results were compared to the standard curve of $(NH_4)_2SO_4$ and expressed in µg/mL.

3.6.2 Phosphate solubilization

Qualitative phosphate solubilization activity of strain DR1-2 was determined using Pikovskaya medium (PVK agar). The 20 µl of seed culture of stain DR1-2 was spotted onto PKV agar and incubated at 30°C for 7 days. A clear halo zone around a bacterial colony indicated phosphate solubilization. The solubilization index (SI) was calculated as the ratio of the solubilization zone diameter to the colony diameter (Pande et al., 2017).

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter + clear zone diameter (mm)}}{\text{Colony diameter (mm)}}$$

3.6.3 Zinc solubilization

Qualitative zinc solubilization activity of strain DR1-2 was determined using Tris-mineral salts medium with glucose (1% w/v). The medium was separately supplemented with 0.1% insoluble zinc compounds, including zinc sulfate ($ZnSO_4$),

zinc chloride ($ZnCl_2$), and zinc oxide (ZnO). Subsequently, a 20 µL of seed culture was spotted on the agar plate and incubated at 30°C for 7 days. Following incubation, the diameter of the halo zone around the colony and bacterial colony was determined, and the values were used to calculate the SI. The clearing zone formed by the isolates was determined using the equation above.

3.6.4 Siderophore production

Siderophore production was assessed using the universal procedure modified by (Schwyn & Neilands, 1987). 20 µL of seed culture was dropped on Chrome azurol S (CAS) agar plates and incubated for seven days at room temperature. Siderophore production was determined by forming a yellow-orange halo zone around the colony spot.

3.7 Statistical analysis

All treatments were analyzed by ANOVA using the SPSS software package (SPSS 22 for Windows). Duncan's multiple range test was performed at $P<0.05$ on each of the significant variables measured.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Diversity of endophytic actinomycetes

Sixty-two endophytic actinomycetes were recovered from the roots of Thai orchids. All isolated actinomycetes revealed 4, 29, 17 and 12 isolates from gellan gum, starch casein, glycerol arginine and Gause No.1, respectively (Table 3). Based on 16S rRNA gene sequence analysis and phenotypic characteristics, the isolates were classified into four taxa (Figure 1, Table 4), including, *Streptomyces* (Group I, 34 isolates), *Micromonospora* (Group II, 21 isolates), *Streptosporangium* (Group III, 2 isolates), *Actinomadura* (Group IV, 1 isolate), *Amycolatopsis* (Group V, 3 isolates) and *Pseudonocardia* (Group VI, 1 isolates). Most of the Group I isolates (54.83%) had LL-DAP, whereas the remaining 28 isolates (45.16%) carried *meso*-DAP (Booth, 1971). Similarities of all isolates and closely related isolates ranged between 98.97 and 100%. The NJ-phylogenetic tree based on 16S rRNA gene sequences is shown in Figures 2 and 3. The 16S rRNA gene sequences of the isolates were deposited in the NCBI database, with accession numbers listed in Table 3.

Group I contained 34 isolates. The isolates produced spiral spore chains (Figure 4A) with pale blue to greenish gray on ISP 2 agar after 7 days of incubation (Table 3). The isolates, DR2-3, DR2-4, DR5-1, DR5-2 and DR7-6 were closely related to *Streptomyces parvulus* NBRC 13193^{T} (99.40–99.92% similarity); DR3-5 and CC1-3 were closely related to *S. tendae* ATCC 19812^{T} (99.93–99.70%); CC1-1 and DR9-7 were closely related to *S. ardesiacus* NRRL B-1773^{T} (99.92–99.93%); CL1-6 and EO1-13 were closely related to *S. heilongjiangensis* NEAU-W2T (99.78-99.93%); DR1-1, DR3-2, DR3-4 and DR8-5 were closely related to *S. daghestanicus* NRRL B-5418^{T} (99.32-99.93%); DR7-3 and DR8-9 5 were closely related to *S. malaysiensis* NBRC 16446^{T} (99.85-99.92%); DR10-1, DR10-2 and DR10-3 were closely related to *S. thermoviolaceus* subsp. *apingens* DSM 41392^{T} (98.97-99.16%). The isolates DR2-2, DR7-2, DR8-1, DR8-6, DR8-8, DR8-10, DR9-1, DR9-4, DR9-5, DR10-6, DR10-8, CL1-8, CR1-8 and EO1-10 were closely related to *S. antibioticus* NBRC 12838^{T} (99.70%), *S.*

thermocarboxydus DSM 44293^{T} (99.71%), *S. gelaticus* NRRL B-2928^{T} (99.05%), *S. fractus* MV32^{T} (99.25), *S. malaysiense* MUSC 136^{T} (99.85%), *S. badius* NRRL B-2567^{T} (99.71%), *S. prunicolor* NBRC 13075^{T} (99.48%), *S. deserti* C63^{T} (99.41%), *S. spiralis* NBRC 14215^{T} (99.98%), *S. globosus* LMG 19896^{T} (99.93%), *S. collinus* NBRC 12759^{T} (99.93%), *S. olivaceus* NRRL B-3009^{T} (99.85%), *S. similanensis* KC-106^{T} (99.93%) and *S. zaomyceticus* NBRC 13348^{T} (99.48%), respectively (Table 3).

Group II contained 21 isolates. They produced single spores on mycelium substrate (Figure 4B). Colonies on ISP 2 agar were dark purplish red to greenish black. Based on the 16S rRNA gene sequences, the three isolates, DR4-1, CA1-5 and CA1-6 were closely related to *Micromonospora humi* DSM 45647^{T} (99.33-99.49%); five isolates, CR1-1, CR1-2, CA1-8, CA1-9 and CA1-10 were closely related to *M. maritima* D10-9-5^{T} (99.93–100.00%); DR6-8 and DR6-7 were closely related to *M. tulbaghiae* DSM 45142^{T} (99.75-99.83%); CA1-1 and CA1-2 were closely related to *M. schwarzwaldensis* HKI0641^{T} (99.47-99.92%); YG1-8, YG1-9 and YG1-10 were closely related to *M. citrea* DSM 43903^{T} (99.70-99.78%) and isolates DR5-3, DR5-6, YG1-1, YG1-7, EO1-6 and EO1-8 were closely related to *M. yasonensis* DSM 45980^{T} (99.03%), *M. chersina* DSM 44151^{T} (99.85%), *M. chalcea* DSM 43026^{T} (99.85%), *M. fluminis* A38^{T} (99.26%) and *M. aurantiaca* ATCC 27029^{T} (99.49%), respectively (Table 3).

Group III consisted of two isolates, DR9-9 and YG1-5. They produced spherical sporangia in the aerial mycelium (Figure 4C). On ISP 2 agar, the isolates were pale purple-pink (Table 1). The isolates DR9-9 and YG1-5 were closely related to *S. sandarakinum* GW-12028^{T} and *S. pseudovulgare* DSM 4318^{T}, based on 16S rRNA gene sequence similarity (99.32 and 99.93%), respectively (Table 3).

Group IV contained one isolate, CL1-5. This isolate formed straight chain spores on the tip of aerial mycelium (Figure 4D) and colonies and was vivid reddish-orange on ISP 2 agar plate. Based on 16S rRNA gene sequence analysis, the isolate CL1-5 was 99.85% closely related to *A. hibisca* NBRC 15177^{T} (Table 3).

Group V consisted of three isolates, DR6-1, DR6-2 and DR6-4. They comprised straight spore chains of fragmented rod-shaped elements (Figure 4E) and aerial mycelia were greyish yellow on ISP 2 agar plate. All isolates were closely related to

A. nivea KCTC 39515^{T} based on 16S rRNA gene sequence similarity 99.06-99.16% (Table 3).

Group VI contained one isolate, DR1-2. The isolate revealed the aerial mycelium fragmented into rod-shaped spores (Figure 4F) and the spore surface was smooth. Isolate DR1-2 show yellowish white aerial mycelium and deep orange yellow substrate mycelium on ISP 2 agar after 7 days incubation. Based on 16S rRNA gene sequence analysis, the isolate DR1-2 was 100.00% closely related to *P. carboxydivorans* Y8^{T} (Table 3).

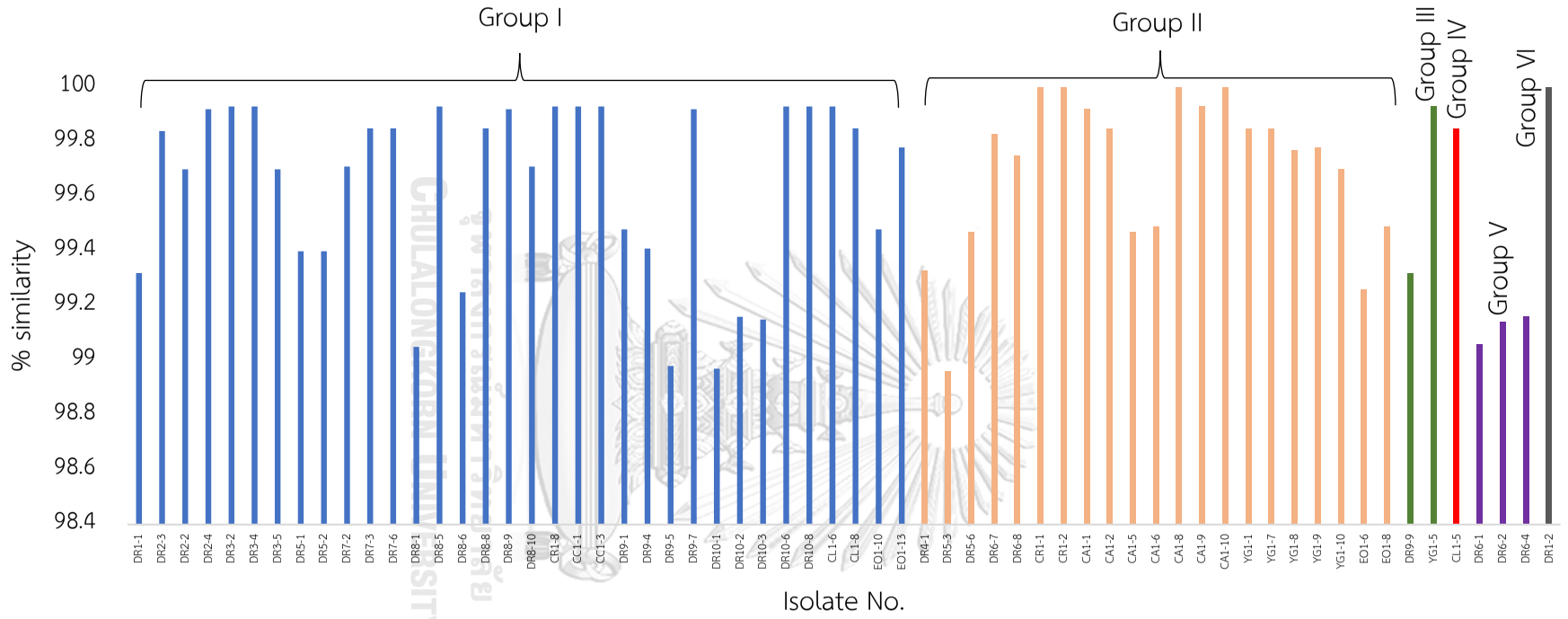


Figure 1 *Streptomyces* (Group I), *Micromonospora* (Group II), *Streptosporangium (Group III), Actinomadura* (Group IV), *Amycolatopsis* (Group V), and *Pseudonocardia* (Group VI) isolates based on 16S rRNA gene sequences.

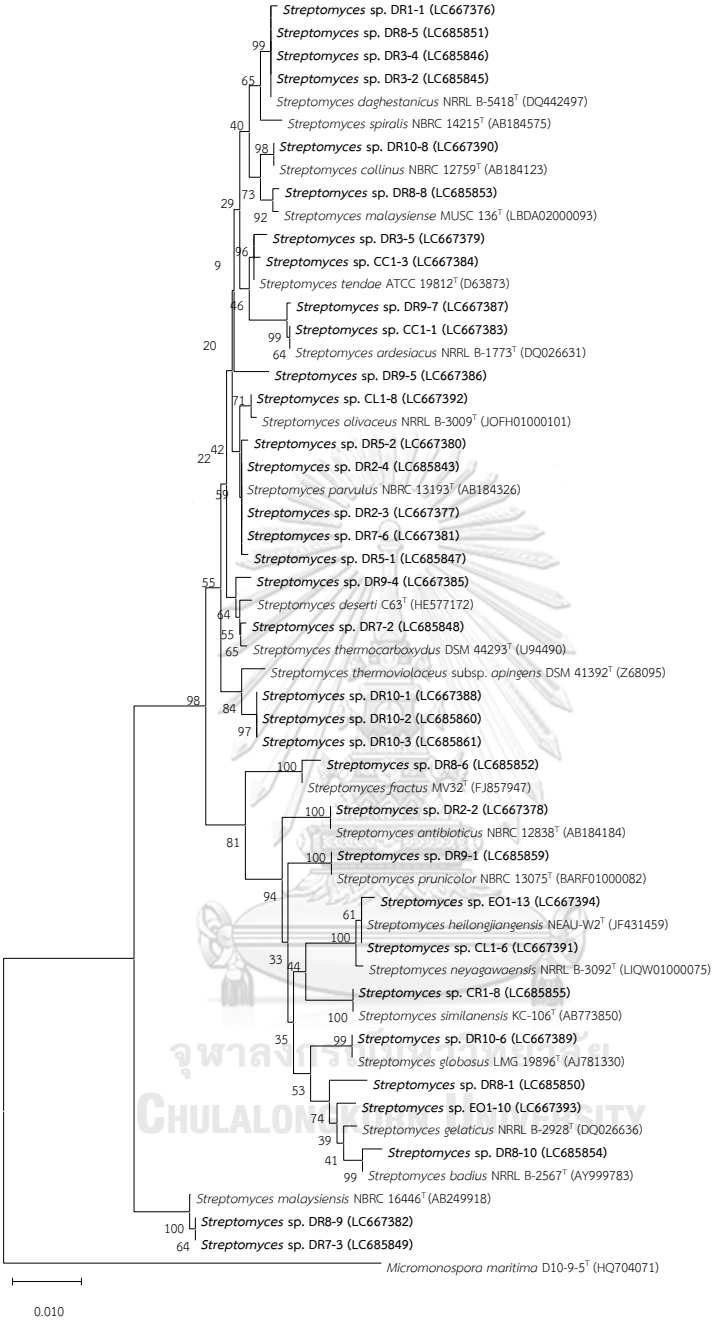


Figure 2 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between Group I and related type strains. Numbers at branch nodes indicate bootstrap percentages derived from 1000 replications. Bar, 0.01 substitutions per nucleotide position.

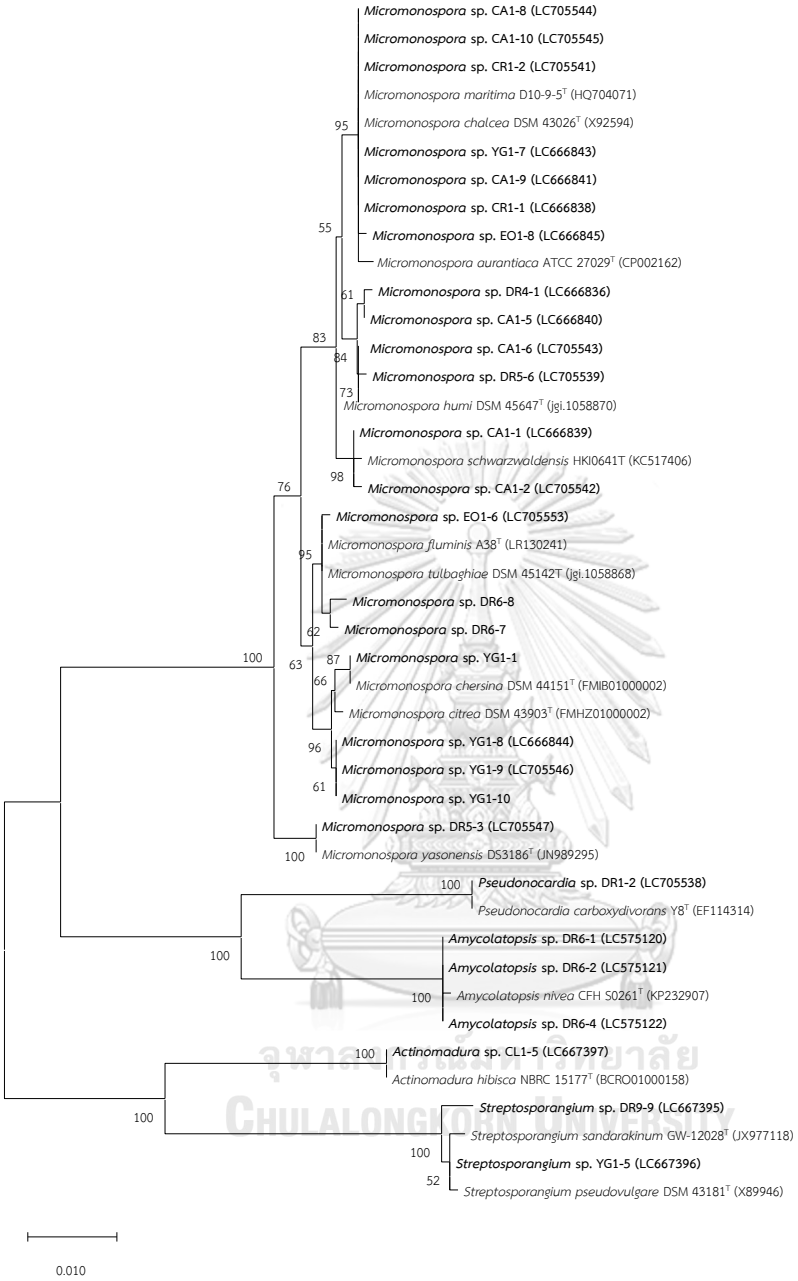


Figure 3 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between Group II, III, IV, V, VI and related type strains.

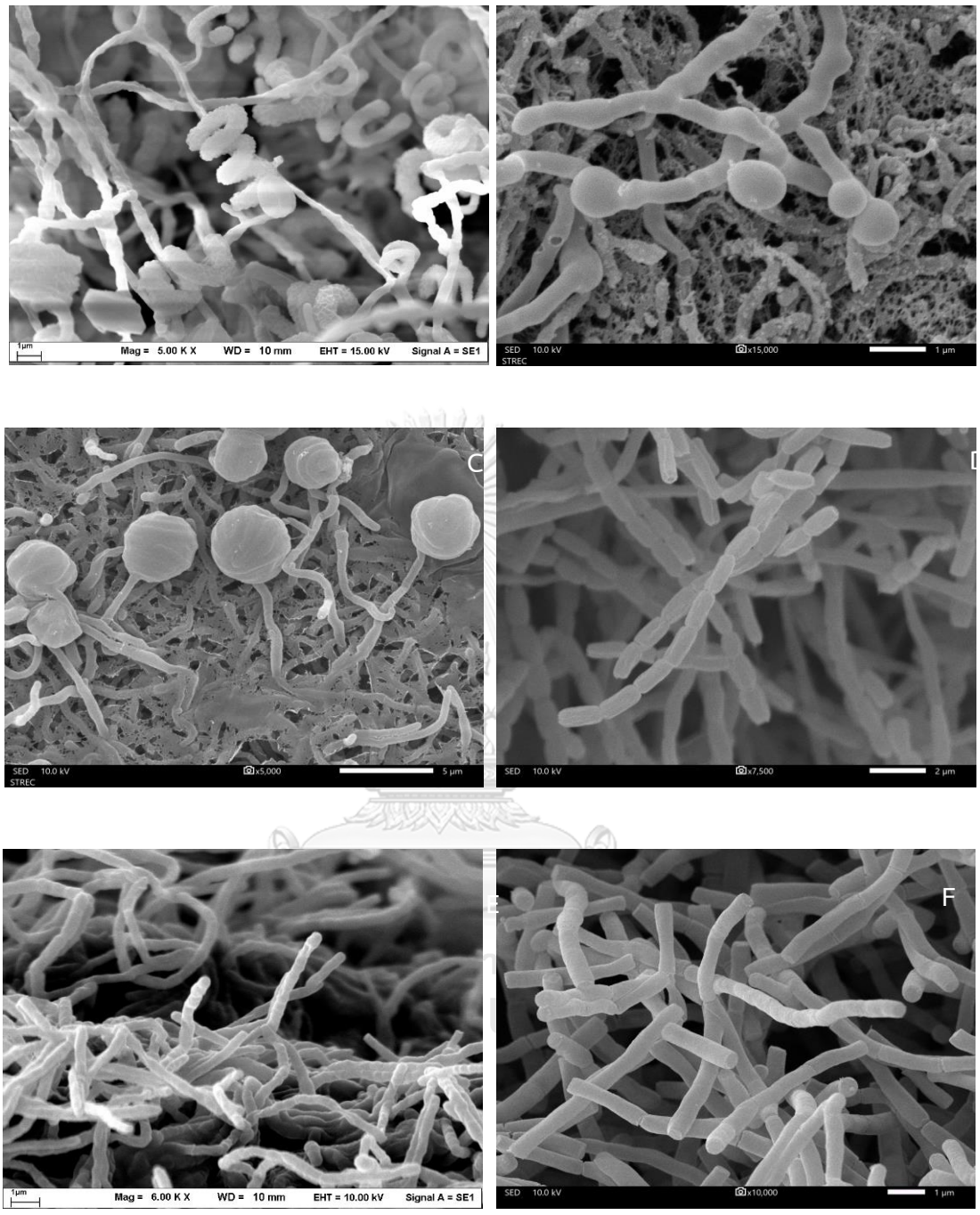


Figure 4 Scanning electron micrograph of A) GroupI, B) Group II, C) Group III, D) Group IV, E) Group V and F) Group VI grown on ISP 2 agar at 30 °C for 14 days.

Table 3 Source, isolate number, cultural characteristics and the nearest isolate species based on 16S rRNA gene sequence similarity.

Group	Plant	Isolate no.	Cultural characteristics		% Similarity	Accession No	Nearest species
			Upper color	Reverse color			
I	*Dendrobium christyanum*	DR1-1[b]	Greenish gray	Light yellow	99.32	LC667376	*S. daghestanicus* NRRL B-5418^T
	Dendrobium polyanthum	DR2-3[b]	Pale blue	Light yellow	99.84	LC667377	*S. parvulus* NBRC 13193^T
	Dendrobium polyanthum	DR2-2[b]	Brilliant greenish blue	Light yellow	99.70	LC667378	*S. antibioticus* NBRC 12838^T
	Dendrobium polyanthum	DR2-4[c]	Pale blue	Light yellow	99.92	LC685843	*S. parvulus* NBRC 13193^T
	Dendrobium formosum	DR3-2[b]	Greenish gray	Light yellow	99.93	LC685845	*S. daghestanicus* NRRL B-5418^T
	Dendrobium formosum	DR3-4[c]	Greenish gray	Light yellow	99.93	LC685846	*S. daghestanicus* NRRL B-5418^T
	Dendrobium formosum	DR3-5[b]	Greenish gray	Moderate olive brown	99.70	LC667379	*S. tendae* ATCC 19812^T
	Dendrobium kentrophyllum	DR5-1[c]	Pale blue	Light yellow	99.40	LC685847	*S. parvulus* NBRC 13193^T
	Dendrobium kentrophyllum	DR5-2[b]	Pale blue	Light yellow	99.40	LC667380	*S. parvulus* NBRC 13193^T
	Dendrobium findlayanum	DR7-2[b]	Greenish white	Light yellow	99.71	LC685848	*S. thermocarboxydus* DSM 44293^T
	Dendrobium findlayanum	DR7-3[d]	Bluish gray	Light yellow	99.85	LC685849	*S. malaysiensis* NBRC 16446 ^{T}
	Dendrobium findlayanum	DR7-6[b]	Pale blue	Light yellow	99.85	LC667381	*S. parvulus* NBRC 13193^T
	Dendrobium chrysanthum	DR8-1[b]	Bluish gray	Dark grayish brown	99.05	LC685850	*S. gelaticus* NRRL B-2928 ^{T}
	Dendrobium chrysanthum	DR8-5[b]	Brilliant greenish blue	Light yellow	99.93	LC685851	*S. daghestanicus* NRRL B-5418^T
	Dendrobium chrysanthum	DR8-6[b]	Greenish white	Pale yellow	99.25	LC685852	*S. fractus* MV32^T
	Dendrobium chrysanthum	DR8-8c	Bluish gray	Light yellow	99.85	LC685853	*S. malaysiense* MUSC 136^T
	Dendrobium chrysanthum	DR8-9[b]	Bluish gray	Light yellow	99.92	LC667382	*S. malaysiensis* NBRC 16446^T
	Dendrobium chrysanthum	DR8-10[d]	Greenish white	Yellowish white	99.71	LC685854	*S. badius* NRRL B-2567^T
	Cleisostoma rostratum	CR1-8[b]	Light olive gray	Grayish yellowish brown	99.93	LC685855	*S. similanensis* KC-106^T
	Calanthe cardioglossa	CC1-1[b]	Olive gray	Light olive brown	99.93	LC667383	*S. ardesiacus* NRRL B-1773^T
	Calanthe cardioglossa	CC1-3[b]	Greenish gray	Moderate olive brown	99.93	LC667384	*S. tendae* ATCC 19812^T
	Dendrobium friedericksianum	DR9-1[c]	Greenish white	Light olive brown	99.48	LC685859	*S. prunicolor* NBRC 13075 ^{T}

Group	Plant	Isolate no.	Cultural characteristics		% Similarity	Accession No	Nearest species
			Upper color	Reverse color			
	Dendrobium friedericksianum	DR9-4[b]	Greenish gray	Yellowish gray	99.41	LC667385	*S. deserti* C63^T
	Dendrobium friedericksianum	DR9-5[a]	Light olive gray	Yellowish gray	98.98	LC667386	*S. spiralis* NBRC 14215^T
	Dendrobium friedericksianum	DR9-7[a]	Olive gray	Light olive brown	99.92	LC667387	*S. ardesiacus* NRRL B-1773^T
	Dendrobium chrysotoxum	DR10-1[a]	Grayish olive green	Light olive gray	98.97	LC667388	*S. thermoviolaceus* subsp. *Apingens* DSM 41392^T
	Dendrobium chrysotoxum	DR10-2[b]	Grayish olive green	Light olive gray	99.16	LC685860	*S. thermoviolaceus* subsp. *Apingens* DSM 41392^T
	Dendrobium chrysotoxum	DR10-3[c]	Grayish olive green	Light olive gray	99.15	LC685861	*S. thermoviolaceus* subsp. *Apingens* DSM 41392^T
	Dendrobium chrysotoxum	DR10-6[d]	Moderate yellow	Light yellow	99.93	LC667389	*S. globosus* LMG 19896^T
	Dendrobium chrysotoxum	DR10-8[d]	Bluish white	Brownish gray	99.93	LC667390	*S. collinus* NBRC 12759^T
	Coelogyne lawrenceana	CL1-6[c]	Bluish gray	Dark bluish gray	99.93	LC667391	*S. heilongjiangensis* NEAU-W2^T
	Coelogyne lawrenceana	CL1-8[c]	Greenish gray	Moderate olive	99.85	LC667392	*S. olivaceus* NRRL B-3009^T
	Eria ornata	EO1-10[b]	Pale blue	Grayish greenish yellow	99.48	LC667393	*S. zaomyceticus* NBRC 13348^T
	Eria ornata	EO1-13[b]	Greenish gray	Moderate olive brown	99.78	LC667394	*S. heilongjiangensis* NEAU-W2^T
II	*Dendrobium crumenatum*	DR4-1[c]	Greenish black	Greenish black	99.33	LC666836	*M. humi* DSM 45647^T
	Dendrobium kentrophyllum	DR5-3[b]	Pale orange yellow	Pale orange yellow	98.96	LC705554	*M. yasonensis* DSM 45980^T
	Dendrobium kentrophyllum	DR5-6[b]	Dark olive brown	Dark olive brown	99.47	LC705539	*M. schwarzwaldensis* HKI0641^T
	Dendrobium heterocarpum	DR6-7[b]	Moderate olive brown	Moderate olive brown	99.83	LC705540	*M. tulbaghiae* DSM 45142^T
	Dendrobium heterocarpum	DR6-8[c]	Moderate olive brown	Moderate olive brown	99.75	LC666837	*M. tulbaghiae* DSM 45142^T
	Cleisostoma rostratum	CR1-1[c]	Grayish red	Grayish red	100	LC666838	*M. maritima* D10-9-5^T
	Cleisostoma rostratum	CR1-2[b]	Grayish red	Grayish red	100	LC705541	*M. maritima* D10-9-5^T
	Coelogyne assamica	CA1-1[c]	Dark olive brown	Dark olive brown	99.92	LC666839	*M. schwarzwaldensis* HKI0641^T

Group	Plant	Isolate no.	Cultural characteristics		% Similarity	Accession No	Nearest species
			Upper color	Reverse color			
	Coelogyne assamica	CA1-2[b]	Dark olive brown	Dark olive brown	99.85	LC705542	*M. schwarzwaldensis* HKI0641^T
	Coelogyne assamica	CA1-5[c]	Greenish black	Greenish black	99.47	LC666840	*M. humi* DSM 45647^T
	Coelogyne assamica	CA1-6[b]	Greenish black	Greenish black	99.49	LC705543	*M. humi* DSM 45647^T
	Coelogyne assamica	CA1-8[b]	Dark grayish red	Dark grayish red	100	LC705544	*M. maritima* D10-9-5^T
	Coelogyne assamica	CA1-9[a]	Dark grayish red	Dark grayish red	99.93	LC666841	*M. maritima* D10-9-5^T
	Coelogyne assamica	CA1-10[c]	Dark grayish red	Dark grayish red	100	LC705545	*M. maritima* D10-9-5^T
	Pinalia globulifera	YG1-1[d]	Greenish black	Greenish black	99.85	LC666842	*M. chersina* DSM 44151^T
	Pinalia globulifera	YG1-7[d]	Very dark purplish red	Very dark purplish red	99.85	LC666843	*M. chalcea* DSM 43026^T
	Pinalia globulifera	YG1-8[d]	Brownish black	Brownish black	99.77	LC666844	*M. citrea* DSM 43903^T
	Pinalia globulifera	YG1-9[b]	Brownish black	Brownish black	99.78	LC705546	*M. citrea* DSM 43903^T
	Pinalia globulifera	YG1-10[c]	Brownish black	Brownish black	99.70	LC705547	*M. citrea* DSM 43903^T
	Eria ornata	EO1-6[d]	Moderate olive brown	Moderate olive brown	99.26	LC705553	*M. fluminis* A38^T
	Eria ornata	EO1-8[d]	Deep reddish brown	Deep reddish brown	99.49	LC666845	*M. aurantiaca* ATCC 27029^T
III	*Dendrobium friedericksianum*	DR9-9[d]	Pale pink	Pinkish gray	99.32	LC667395	*S. sandarakinum* GW-12028^T
	Pinalia globulifera	YG1-5[d]	Pale purple pink	Pale purple pink	99.93	LC667396	*S. pseudovulgare* DSM 4318^T
IV	*Coelogyne lawrenceana*	CL1-5[c]	Very deep red	Very deep red	99.85	LC667397	*A. hibisca* NBRC 15177^T
V	*Dendrobium heterocarpum*	DR6-1[b]	greyish yellow	greyish yellow	99.06	LC575120	*A. nivea* KCTC 39515^T
	Dendrobium heterocarpum	DR6-2[c]	greyish yellow	greyish yellow	99.14	LC575121	*A. nivea* KCTC 39515^T
	Dendrobium heterocarpum	DR6-4[d]	greyish yellow	greyish yellow	99.16	LC575122	*A. nivea* KCTC 39515^T
VI	*Dendrobium christyanum*	DR1-2[b]	yellowish white	deep orange yellow	100	LC705538	*P. carboxydivorans* Y8^T

All isolates grew well on ISP 2 agar medium. a, gellan gum; b, starch casein gellan gum; c, glycerol arginine gellan gum; d gauze synthetic no.1.

Table 4 Differential phenotypic characteristics of the isolates.

Characteristic	Group					
	I	II	III	IV	V	VI
No. of isolates	34	21	2	1	3	1
Temp. range, °C	20-37	20-40	25-37	25-37	20-37	25-37
pH range	5-10	5-8	5-10	5-10	5-10	5-10
NaCl tolerance						
4%	+(-6)	-(+7)	w	+	+	+
6%	- (+15)	-(+5)	-	-	+	+
8%	-(+13)	-	-	-	-	+
Starch hydrolysis	+(-7)	+(-11)	+	+	-	-
Coagulation	-(+14)	-(+2)	-	-	+	-
Peptonization	-(+15)	-(+6)	-	-	+	-
Nitrate reduction	-(+8)	-(+9)	+	+	+	+
Gelatin liquefaction	-(+13)	-(+6)	-	-	-	-
Utilization of						
D-Glucose	+(w6)	+(-5)	+	+	+	+
Sucrose	+(w8)	+(-3, w5)	+	+	-	+
Lactose	+(w7)	+(-4, w3)	+	+	-	+
Dextrose	+(w3)	+(-5, w6)	+	+	+	+
Maltose	+	+(w8)	+	+	+	+
D-Mannitol	+(-, w2)	-(+4, w5)	+	+	+	+
D-Xylose	+(w5)	+(-2, w6)	+	w	+	+
Sorbitol	-(+8, w4)	+(-6)	w	+	+	+

+, positive reaction; w, weakly positive reaction; –, negative reaction. Numbers in parentheses indicate numbers of isolates showing positive, weak and negative reactions.

In this study, thirty-four *Streptomyces* isolates were associated with the twelve orchids *D. christyanum*, *D. polyanthum*, *D. formosum*, *D. kentrophyllum*, *D. findlayanum*, *D. chrysanthum*, *C. cardioglossa*, *D. friedericksianum*, *D. chrysotoxum*, *C. lawrenceana, C. rostratum* and *E. ornate*. Twenty-one *Micromonospora* isolates were found in the seven orchids, *D. crumenatum*, *D. kentrophyllum, D. heterocarpum*, *C. rostratum*, *C. assamica*, *P. globulifera* and *E. ornate*. Two *Streptosporangium* isolates were distributed in the two orchids *D. friedericksianum* and *P. globulifera*; one *Actinomadura* isolate was found in *Coelogyne lawrenceana*; three *Amycolatopsis* were isolates from *D. heterocarpum*, and one *Pseudonocardia* isolate was found in *D. christyanum.* The isolates DR2-3, DR5-2 and DR6-6, all closely related to *S. parvulus* NBRC 13193^{T}, were found in *D. polyanthum*, *D. kentrophyllum* and *D. findlayanum*, while the isolates DR3-5 and CC1-3 closely related to *S. tendae* ATCC 19812^{T} were presented in *Dendrobium* and *Calanthe. Streptomyces* and *Micromonospora* isolates were found in *E. ornate*, while *Streptomyces* and *Streptosporangium* isolates were found in *D. friedericksianum.* These results indicated that various associated bacteria were extensively distributed among the host plants.

Most of the isolates (54.83%) belonged to the genus *Streptomyces*, previously identified as a dominating organism in sugar cane roots (Sinma et al., 2015), *Citrus reticulata* L. (Shutsrirung et al., 2013) and *Acacia auriculiformis* (Bunyoo et al., 2009) in Thailand. Our result was similar to previous research that found *Streptomyces* strains in plant roots (Taechowisan & Lumyong, 2003) (Gangwar et al., 2012) (Shan et al., 2018). Actinobacteria were prominent in the roots and stems of *Neottia ovata* (50.02 and 48.47%, respectively) and the seeds of *Spiranthes spiralis* (48.95%) (Alibrandi et al., 2020). Endophytic actinomycetes were distributed in plant roots, where water and nutrients were absorbed (Passari et al., 2015). Many plant species had diverse strains of *Microbispora* (Bunyoo et al., 2009) and *Micromonospora* (Kuncharoen et al., 2019), including novel species of *Amycolatopsis dendrobii* from the root of *Dendrobium heterocarpum* Lindl. (Tedsree et al., 2021) and *Streptomyces radicis* from the roots of plants (Kuncharoen et al., 2022).

4.2 *Amycolatopsis dendrobii* and *Micromonospora dendrobii,* two novel species of endophytic actinomycetes

4.2.1 *Amycolatopsis dendrobii* sp.nov. an endophytic actinomycete isolated from *Dendrobium heterocarpum*

Strains DR6-1^T, DR6-2 and DR6-4 were isolated from SCG, glycerol asparagine gellan gum and gauze synthetic No.1 agar plates, respectively. The strains showed good growth on all media. They produced pale yellow aerial mycelia on all media, except on ISP 2, which resulted in the production of greyish yellow aerial mycelia. The strains showed white aerial mycelia on all media tested but did not produce diffusible pigments. The aerial mycelium comprised straight chains of fragmented rod-shaped elements (Figure 5). Growth was observed at pH 5-10 (optimum 7) and 20-37 °C (optimum 30 °C). All strains grew well with 0-5 % and weakly with 7% (w/v) NaCl but failed to grow above 8 % (w/v) NaCl. The physiological and biochemical characteristics of the strains compared with closely related type strains are given in Table 5.

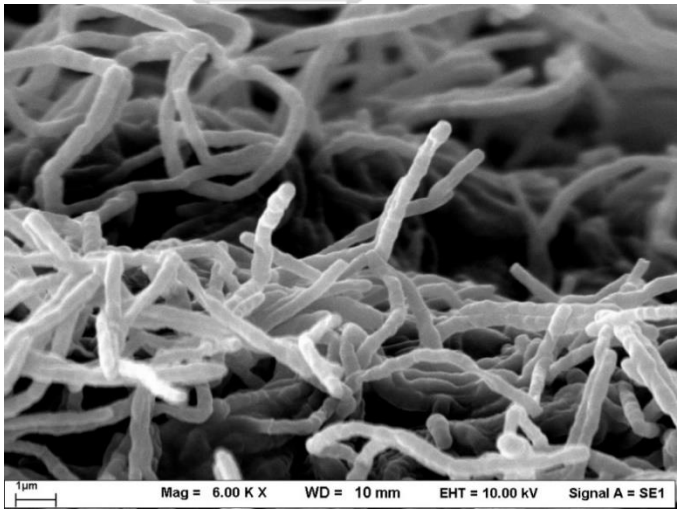


Figure 5 Scanning electron micrograph of strain DR6-1^T grown on ISP 2 agar at 30 °C for 14 days, showing the fragmenting aerial mycelium.

Strains DR6-1^T, DR6-2 and DR6-4 contained meso-diaminopimelic acid, and arabinose and galactose were the major sugars in the cell-wall peptidoglycan (type IV cell wall, according to (Lechevalier et al., 1986)). The phospholipids in strain DR6-1^T included diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), hydroxyphosphatidyethanolamine (OH-PE), phosphatidylinositol (PI), three unknown lipids (L) and four unidentified glycolipids (GL), a phospholipid type II profile. Strains DR6-1^T, DR6-2 and DR6-4 contained MK-9(H_4) (90.2, 96.4, 91.9 %) and MK-8(H_4) (9.8, 3.7, 7.8 %). The major fatty acids were iso-$C_{16:0}$ (26.1, 21.5, 19.7 %), iso-$C_{15:0}$ (25.5, 29.4, 20.3 %) and $C_{16:0}$ (19.8, 13.7, 21.8 %); nonetheless, there were some differences among the closely related type strains. Based on a comparison of the chemotaxonomic characteristics of strain DR6-1^T and its related type strains, strains DR6-1^T, DR6-2 and DR6-4 were classified as members of the genus *Amycolatopsis*.

Table 5 Differential characteristics of strains DR6-1^T, DR6-2, DR6-4 and related type strains. Strains: 1, DR6-1^T; 2, DR6-2; 3, DR6-4; 4, *A. echigonensis* JCM 21831^T; 5, *A. rubida* JCM 10871^T; 6, *A. nivea* KCTC 39515^T.

Data are from this study. +, positive; -, negative; w, weak. All are positive for milk peptonization and coagulation; utilization of arabinose, glucose, glycerol (weak), mannitol, mannose, *myo*-inositol, and xylose; acid production from adonitol, arabinose, cellobiose, erythritol, galactose, *myo*-inositol, mannitol, and xylose but are negative for starch hydrolysis and gelatin liquefaction; utilization of amygdalin, cellulose, galactose, dulcitol, lactose, raffinose, rhamnose, salicin and sucrose; acid production from dextrin, lactose, maltose, melezitose, melibiose, methyl α-D-glucoside, raffinose, rhamnose, salicin, sorbital, sucrose, and trehalose.

Characteristics	1	2	3	4	5	6
Aerial mycelial colour	Grayish yellow	Grayish yellow	Grayish yellow	Moderate yellow	Light yellow	Grayish yellow
Growth on ISP 4	Good	Good	Good	Good	Poor	Good
Growth at 45 °C	-	-	-	w	-	-
Growth at 7 % NaCl	w	w	w	-	w	w

Characteristics	1	2	3	4	5	6
Growth at 8 % NaCl	-	-	-	-	w	w
Carbon source utilization:						
Cellulose	-	-	-	-	-	+
Glycerol	w	w	w	w	w	+
Lactose	-	-	-	+	-	+
Raffinose	-	-	-	-	-	+
Rhamnose	-	-	-	-	w	+
Sucrose	-	-	-	w	+	+
Acid production from:						
Dextrin	-	-	-	+	-	+
Lactose	-	-	-	+	-	-
Mannitol	+	+	+	+	-	+
Maltose	-	-	-	+	-	-
Melezitose	-	-	-	+	-	-
Melibiose	-	-	-	+	-	-
Methyl α-D-glucoside	-	-	-	+	-	-
Raffinose	-	-	-	+	-	-
Rhamnose	-	-	-	-	+	-
Salicin	-	-	-	+	+	+
Trehalose	-	-	-	+	+	-
API ZYM						
Alkaline phosphatase	+	+	+	w	w	w
Esterase (C 4)	w	w	w	-	-	+
Lipase (C 14)	-	w	w	-	-	+
Valine	w	w	w	w	w	+

Characteristics	1	2	3	4	5	6
arylamidase						
Naphthol-AS-BI-phosphohydrolase	+	+	+	w	+	+
β-Galactosidase	+	+	+	w	w	-
α -Glucosidase	+	+	+	+	w	+
β -Glucosidase	w	-	-	+	-	w
α-Mannosidase	+	+	+	-	+	+

Pairwise alignment of the 16S rRNA gene sequence among the three novel strains, DR6-1^T, DR6-2 and DR6-4, revealed 99.9-100 % similarity and clearly confirmed that all strains were members of the genus *Amycolatopsis*. Strains DR6-1^T, DR6-2 and DR6-4 showed relatively high 16S rRNA gene sequence similaritiy values of 98.7, 98.8 and 98.8 % to *Amycolatopsis echigonensis* LC2^T, respectively. Although the 16S rRNA gene sequence similarity showed that the three strains were mostly related to *A. echigonensis* LC2^T, they shared a clade with *Amycolatopsis nivea* CFH S0261^T in the neighbor-joining phylogenetic tree (Figure 6). However, this clade could not be recovered with the maximum-likelihood and the maximum-parsimony phylogenetic analysis. In addition, the phylogenetic analysis based on whole-genome sequences (Figure 7) indicated that strain DR6-1^T was phylogenetically closest to *Amycolatopsis rubida* 13.4^T and *A. nivea* CFH S0261^T. Hence, based on integration of the whole genome sequence and 16S rRNA gene sequence similarity, including phylogenetic analysis, *A. echigonensis* LC2^T (=JCM 21831^T), *A. rubida* 13.4^T (=JCM 10871^T) and *A. nivea* CFH S0261^T (=KCTC 39515^T) were considered for comparative taxonomic purposes.

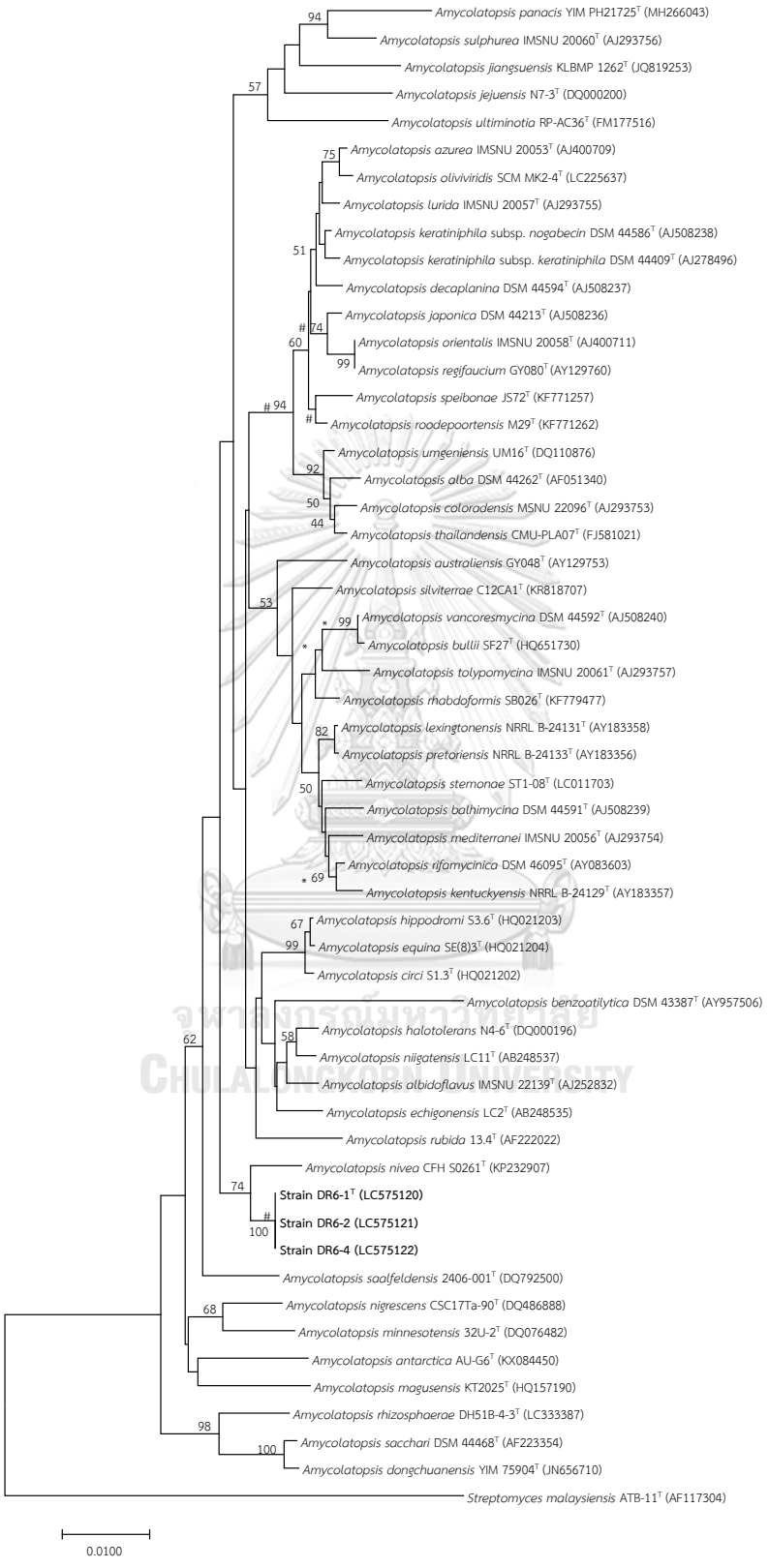

Amycolatopsis panacis YIM PH21725T (MH266043)
Amycolatopsis sulphurea IMSNU 20060T (AJ293756)
Amycolatopsis jiangsuensis KLBMP 1262T (JQ819253)
Amycolatopsis jejuensis N7-3T (DQ000200)
Amycolatopsis ultiminotia RP-AC36T (FM177516)
Amycolatopsis azurea IMSNU 20053T (AJ400709)
Amycolatopsis oliviviridis SCM MK2-4T (LC225637)
Amycolatopsis lurida IMSNU 20057T (AJ293755)
Amycolatopsis keratiniphila subsp. nogabecin DSM 44586T (AJ508238)
Amycolatopsis keratiniphila subsp. keratiniphila DSM 44409T (AJ278496)
Amycolatopsis decaplanina DSM 44594T (AJ508237)
Amycolatopsis japonica DSM 44213T (AJ508236)
Amycolatopsis orientalis IMSNU 20058T (AJ400711)
Amycolatopsis regifaucium GY080T (AY129760)
Amycolatopsis speibonae JS72T (KF771257)
Amycolatopsis roodepoortensis M29T (KF771262)
Amycolatopsis umgeniensis UM16T (DQ110876)
Amycolatopsis alba DSM 44262T (AF051340)
Amycolatopsis coloradensis MSNU 22096T (AJ293753)
Amycolatopsis thailandensis CMU-PLA07T (FJ581021)
Amycolatopsis australiensis GY048T (AY129753)
Amycolatopsis silviterrae C12CA1T (KR818707)
Amycolatopsis vancoresmycina DSM 44592T (AJ508240)
Amycolatopsis bullii SF27T (HQ651730)
Amycolatopsis tolypomycina IMSNU 20061T (AJ293757)
Amycolatopsis rhabdoformis SB026T (KF779477)
Amycolatopsis lexingtonensis NRRL B-24131T (AY183358)
Amycolatopsis pretoriensis NRRL B-24133T (AY183356)
Amycolatopsis stemonae ST1-08T (LC011703)
Amycolatopsis balhimycina DSM 44591T (AJ508239)
Amycolatopsis mediterranei IMSNU 20056T (AJ293754)
Amycolatopsis rifamycinica DSM 46095T (AY083603)
Amycolatopsis kentuckyensis NRRL B-24129T (AY183357)
Amycolatopsis hippodromi S3.6T (HQ021203)
Amycolatopsis equina SE(8)3T (HQ021204)
Amycolatopsis circi S1.3T (HQ021202)
Amycolatopsis benzoatilytica DSM 43387T (AY957506)
Amycolatopsis halotolerans N4-6T (DQ000196)
Amycolatopsis niigatensis LC11T (AB248537)
Amycolatopsis albidoflavus IMSNU 22139T (AJ252832)
Amycolatopsis echigonensis LC2T (AB248535)
Amycolatopsis rubida 13.4T (AF222022)
Amycolatopsis nivea CFH S0261T (KP232907)
Strain DR6-1T (LC575120)
Strain DR6-2 (LC575121)
Strain DR6-4 (LC575122)
Amycolatopsis saalfeldensis 2406-001T (DQ792500)
Amycolatopsis nigrescens CSC17Ta-90T (DQ486888)
Amycolatopsis minnesotensis 32U-2T (DQ076482)
Amycolatopsis antarctica AU-G6T (KX084450)
Amycolatopsis magusensis KT2025T (HQ157190)
Amycolatopsis rhizosphaerae DH51B-4-3T (LC333387)
Amycolatopsis sacchari DSM 44468T (AF223354)
Amycolatopsis dongchuanensis YIM 75904T (JN656710)
Streptomyces malaysiensis ATB-11T (AF117304)
0.0100

Figure 6 Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strains DR6-1^{T}, DR6-2 and DR6-4 within the radiation of *Amycolatopsis* species.

Symbols indicate branches recovered in the maximum-likelihood (*) and maximum-parsimony (#) trees. Numbers at nodes indicate percentage levels of bootstrap support based on a neighbour-joining analysis of 1000 replications; only values over 50 % are shown. Bar, 0.01 nt substitutions per nucleotide position.

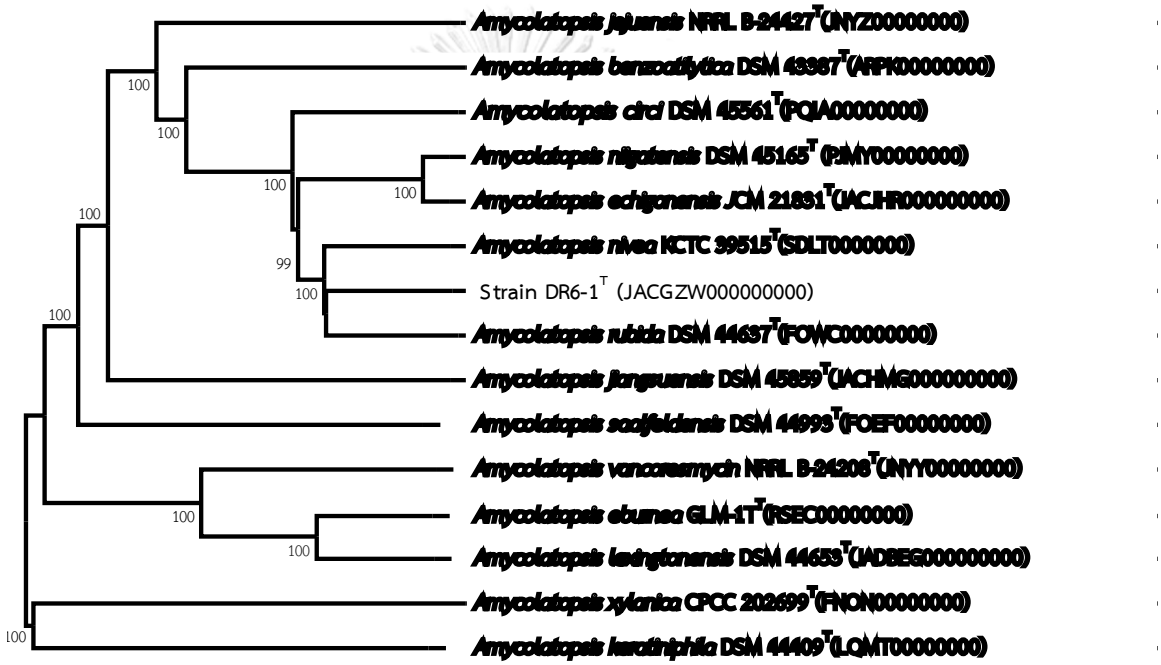


Figure 7 Phylogenomic tree of strain DR6-1^{T} and related *Amycolatopsis* species obtained from TYGS. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications.

The draft genome sequences of strain DR6-1^T, *A. echigonensis* JCM 21831^T, *A. rubida* JCM 10871^T and *A. nivea* KCTC 39515^T were 9.6, 9.6, 9.8 and 9.5 Mb in size, respectively, and the DNA G+C contents were 69.6, 69.5, 69.8 and 69.5 mol%, respectively (Table 6). The ANIb and ANIm values of the draft genomes between strain DR6-1^T and *A. echigonensis* JCM 21831^T, *A. rubida* JCM 10871^T and *A. nivea* KCTC 39515^T were 90.55 and 92.65 %, 92.25 and 93.88 %, and 92.60 and 93.85 %, respectively. Both ANI values were lower than the recommended 95-96 % for delineation of species (Lechevalier et al., 1977). The dDDH values between strain DR6-1^T and its closest strains, *A. echigonensis* JCM 21831^T, A. *rubida* JCM 10871^T and *A. nivea* KCTC 39515^T, were 47.20, 52.10 and 52.50 %, respectively, distinctly below the 70 % threshold used to confirm the strain as representing a novel species (Richter & Rosselló-Móra, 2009). Therefore, strain DR6-1^T could be recognized as representing a novel species within the genus *Amycolatopsis*.

Table 6 Genomic statistics of strain DR6-1^T (JACGZW000000000), *A. echigonensis* JCM 21831^T (JACJHR000000000), *A. rubida* JCM 10871^T (FOWC00000000), and *A. nivea* KCTC 39515^T (SDLT00000000).

Attribute	DR6-1^T	JCM 21831^T	JCM 10871^T	KCTC 39515^T
Genome sizes (bp)	9690793	9663932	9873592	9571567
G+C content (%)	69.6	69.5	69.8	69.5
N50	530751	92691	540869	181068
No. of contigs	59	302	45	135
Total genes	9015	9377	9254	9365
No. of coding sequences	8856	9088	9950	9283
RNA genes	65	65	61	59

The draft genomes of strain DR6-1^T, *A. echigonensis* JCM 21831^T, *A. rubida* JCM 10871^T and *A. nivea* KCTC 39515^T were determined using the antiSMASH server (Goris et al., 2007) to detect putative biosynthetic gene clusters (BGCs). More than 20 gene clusters were observed on the DR6-1^T, *A. echigonensis* JCM 21831^T, *A. rubida*

JCM 10871^T and *A. nivea* KCTC 39515^T genomes related to various BGCs, mainly type I polyketide synthase (T1PKS), non-ribosomal peptide synthetase (NRPS), terpene, ectoine and siderophore. The type of lassopeptide similar to anantin C was only found in strain DR6-1^T. The secondary metabolite biosynthetic gene clusters (smBGCs) of strain DR6-1^T and their type strains exhibited genetic relatedness to known BGCs producing macrotermycin (76–100 %), ectoine (100 %), geosmin (100 %), mirubactin (78 %), hopene (38 %), macrotetrolide (33 %) and isorenieratene (28-42 %). However, the BGCs of apoptolidin (76 %), anantin C (75 %), stambomycin A-D (36 %), cenerubin B (28 %) and mediomycin A (28 %) were found only in the genome of DR6-1^T compared to other related type strains. Interestingly, strain DR6-1^T contained smBGCs of apoptolidin, a promising therapeutic which exhibited extraordinary selectivity against cancer cells (Blin et al., 2019). In addition, the genome of strain DR6-1^T was found to contain several smBGCs showing no relatedness to any known smBGCs in antiSMASH. These data indicated that strain DR6-1^T could produce new secondary metabolites as a source of valuable bioactive compounds.

On the basis of phenotypic characteristics, including morphological characteristics, peptonization, coagulation, utilization of cellulose, glycerol, lactose, raffinose, rhamnose and sucrose, and acid production from dextrin, lactose, maltose, mannitol, melezitose, melibiose, methyl α-D-glucoside, raffinose, rhamnose, salicin and trehalose, strains DR6-1^T, DR6-2 and DR6-2 could be differentiated from *A. echigonensis* JCM 21832^T, *A. rubida* JCM 10871^T and *A. nivea* KCTC 39515^T. In addition, the genotypic characteristics and genomic data indicated that strains DR6-1^T, DR6-2 and DR6-4 represent a novel *Amycolatopsis* species for which the name *Amycolatopsis dendrobii* sp. nov. is proposed.

Description of *Amycolatopsis dendrobii* sp. nov.

Amycolatopsis dendrobii (den. dro'bi.i. N.L. gen. n. *dendrobii* of *Dendrobium heterocarpum*, the isolation source of the type strain).

Cells are Gram-positive, aerobic, non-motile, filamentous actinomycetes. The aerial mycelium is white and the substrate mycelium is greyish yellow (ISP 2). No diffusible pigment is produced. Growth occurs at 20-37 °C (optimum 30 °C), at pH 5-

10 (optimum 7.0) and with 5% (w/v) NaCl. Grows weakly with 7% NaCl (w/v). Peptonization, coagulation of skimmed milk and nitrate reduction are positive, but gelatin liquefaction and starch hydrolysis are negative. Positive for utilization of arabinose, glucose, glycerol, mannitol, mannose, myo-inositol and xylose as sole carbon sources, but not for amygdalin, cellulose, galactose, dulcitol, lactose, raffinose, rhamnose, salicin or sucrose. Acid is produced from adonitol, arabinose, cellobiose, erythritol, galactose, myo-inositol, mannitol and xylose, but not from dextrin, lactose, maltose, melezitose, melibiose, methyl α-D- glucoside, raffinose, rhamnose, salicin, sorbital, sucrose or trehalose. Positive for activities of alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and α-mannosidase but negative for activities of esterase (C4), lipase (C14), valine arylamidase and β-glucosidase. The cell-wall peptidoglycan contains meso-diaminopimelic acid. Whole-cell sugars are arabinose and galactose. The polar lipid profile comprises diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), hydroxyphosphatidyethanolamine (OH-PE) and phosphatidylinositol (PI). Predominant cellular fatty acids are iso-$C_{16:0}$ and iso-$C_{15:0}$. MK-9(H_4) is a major component of the isoprenoid quinone.

The type strain is DR6-1^T (=JCM 33742^T=KCTC 49546^T=TISTR 2840^T), isolated from the roots of *Dendrobium heterocarpum* Lindl. collected from Ubon Ratchathani Province, Thailand. The DNA G+C content of the type strain is 69.6 mol%. The GenBank accession numbers for the 16S rRNA gene sequence and the draft genome sequence of the type strain are LC575120 and JACGZW000000000, respectively.

4.2.2 *Micromonospora dendrobii* sp. nov., an endophytic actinomycete isolated from *Dendrobium kentrophyllum*

Strain DR5-3^T was aerobic actinomycete and was classified as the members of the genus *Micromonospora* that belonged to the family *Micromonosporaceae* based on morphology and 16S rRNA gene sequence. They produced monomeric spores on the non-fragmented branched substrate mycelia but not produced aerial mycelia on

various agar media (Figure 8). The strains showed good growth on ISP 2. The strain produced a pale orange yellow color in colonies, but no diffusible pigments.

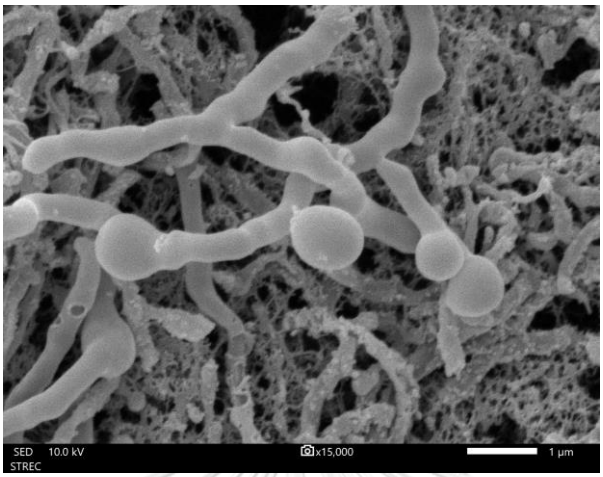


Figure 8 Scanning electron micrograph of strain DR5-3^T grown on ISP 2 agar at 30 ℃ for 14 days.

Strains DR5-3^T contained *meso*-diaminopimelic acid, and glucose, arabinose and xylose were the major sugars in the cell-wall peptidoglycan. The phospholipids in strain DR5-3^T included diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) a phospholipid type II profile, according to Lechevalier (1977) (Lechevalier et al., 1977). Based on a comparison of the chemotaxonomic characteristics of strain DR6-1^T and its related type strains, strains DR5-3^T were classified as members of the genus *Micromonospora*.

The cultural characteristics of strain DR5-3^T as well as the closely related type strains are described in Table 7. Growth was observed at pH 5-10 (optimum 7) and 20-40 ℃ (optimum 30 ℃). strain DR5-3^T grew well with 0-5 % and weakly with 6% (w/v) NaCl but failed to grow above 7 % (w/v) NaCl. Strain DR5-3^T are positive for nitrate reduction, starch hydrolysis, skim milk coagulation and gelatin liquefaction were positive, while skim milk peptonization was negative. It used glucose, lactose, rhamnose, and melibiose as sole carbon sources. The physiological and biochemical characteristics of the strains compared with closely related type strains are given in Table 8.

Table 7 Cultural characteristics of strains DR5-3^T and related type strains. Strains: 1, DR5-3^T; 2, *M. eburnea* JCM12345^T; 3, *M. narathiwatensis* JCM12394^T; 4, *M. yasonensis* DSM45980^T; 5, *M. viridifaciens* JCM3267^T; 6, *M. deserti* JCM32583^T.

Medium	1	2	3	4	5	6
ISP medium 2						
Growth	Very good	Very good	Very good	Very good	Very good	Very good
Colonial colour	Pale orange yellow	Light Grayish brown	Dark orange yellow	Moderate orange yellow	Dark yellowish brown	Strong orange yellow
Soluble pigment	None	None	None	None	None	None
ISP medium 3						
Growth	Weak	Weak	Good	Weak	Weak	Good
Colonial colour	Pale orange yellow	Yellowish white	Dark orange yellow	Moderate orange yellow	Moderate orange yellow	Strong orange yellow
Soluble pigment	None	None	None	None	None	None
ISP medium 4						
Growth	Very good	Very good	Very good	Very good	Very good	Very good
Colonial colour	Deep yellowish brown	Yellowish white	Light orange yellow	Moderate orange yellow	Deep yellowish brown	Strong orange yellow
Soluble pigment	None	None	None	None	None	None
ISP medium 5						
Growth	Weak	Good	Good	Weak	Weak	Good
Colonial colour	Pale orange yellow	Pale yellow	Light orange yellow	Pale orange yellow	Dark orange yellow	Pale orange yellow
Soluble pigment	None	None	None	None	None	None
ISP medium 6						
Growth	Very good	Very good	Very good	Very good	Very good	Very good
Colonial colour	Dark yellowish brown	Dark grayish yellowish brown	Strong yellowish brown	Dark orange yellow	Brownish black	Moderate orange yellow
Soluble pigment	None	None	None	None	None	None
ISP medium 7						
Growth	Good	Good	Good	Good	Good	Good
Colonial colour	Dark yellowish brown	Pale orange yellow	Moderate orange yellow	Moderate orange yellow	Dark yellowish brown	Moderate orange yellow
Soluble pigment	None	None	None	None	None	None
Nutrient agar						
Growth	Good	Very good	Very good	Good	Weak	Very good
Colonial colour	Pale orange	Light orange	Moderate	Light orange	Light yellowish	Moderate

Medium	1	2	3	4	5	6
	yellow	yellow	orange yellow	yellow	brown	orange yellow
Soluble pigment	None	None	None	None	None	None

Table 8 Differential characteristics of strains DR5-3^T and related type strains.
Strains: 1, DR5-3^T; 2, *M. eburnea* JCM12345^T; 3, *M. narathiwatensis* JCM12394^T; 4, *M. yasonensis* DSM45980^T; 5, *M. viridifaciens* JCM3267^T; 6, *M. deserti* JCM32583^T. +, positive; -, negative; w, weak.

Characteristics	1	2	3	4	5	6
Aerial mycelial colour	Pale orange yellow	Light Grayish brown	Dark orange yellow	Moderate orange yellow	Dark yellowish brown	Strong orange yellow
Growth on ISP 3	W	W	+	w	w	+
Growth on ISP 5	w	+	+	w	w	+
Growth at 40 °C	+	+	-	+	+	+
Growth at 5 % NaCl	+	-	-	w	w	-
pH 10	w	+	+	w	w	w
Peptonization	-	++	++	+	+	-
Coagulation	+	+	+	+	+	+
Starch hydrolysis	++	-	++	++	+	-
Gelatin liquefaction	+	+	-	-	-	-
Nitrate reduction	++	++	++	++	+	++
Carbon source utilization:						
Raffinose	-	-	+	-	-	++
Mannose	-	-	-	-	-	++
Manital	-	-	-	-	-	+
Cellobiose	-	-	-	-	+	++
Cellulose	-	-	-	-	-	+
Galactose	-	-	-	-	-	+
Maltose	-	-	+	-	-	++

Characteristics	1	2	3	4	5	6
Melezitose	-	-	-	-	-	++
Lactose	+	-	+	-	+	++
myo-inositol	-	-	-	-	+	++
Rhamnose	+	-	+	-	-	+
Sucrose	-	-	-	-	+	++
Melibiose	+	-	-	-	-	++
Nitrogen source utilization:						
L-Arginine	+	-	-	-	+	+
L-Asparagine	+	-	-	-	-	+
L-Proline	+	-	-	-	-	+
L-Valine	-	-	-	-	-	-
L-Cysteine	+	-	-	-	-	+
L-Tyrosine	-	-	-	-	-	+

The approximate genome size of strain DR5-3^T was 9,205,261 bp with a G+C content of 71.90 mol%. Phylogenetic analysis of 16S rRNA gene sequences of DR5-3^T showed 98.96 % similarity with *Micromonospora yasonensis* DSM 45980^T. The phylogenetic tree based on the neighbour-joining algorithm, DR5-3^T shared a clade with *Micromonospora deserti* JCM32583^T (Figure 9). In addition, the phylogenetic analysis based on whole-genome sequences (Figure 10) indicated that strain DR5-3^T was phylogenetically closest to *Micromonospora eburnean* JCM12345^T, *Micromonospora narathiwatensis* JCM12394^T and *Micromonospora viridifaciens* JCM3267^T. Hence, based on integration of the whole genome sequence and 16S rRNA gene sequence similarity, including phylogenetic analysis, *M. eburnean* JCM12345^T, *M. narathiwatensis* JCM12394^T, *M. yasonensis* DSM45980^T, *M. viridifaciens* JCM3267^T, and *M. deserti* JCM32583^T were considered for comparative taxonomic purposes.

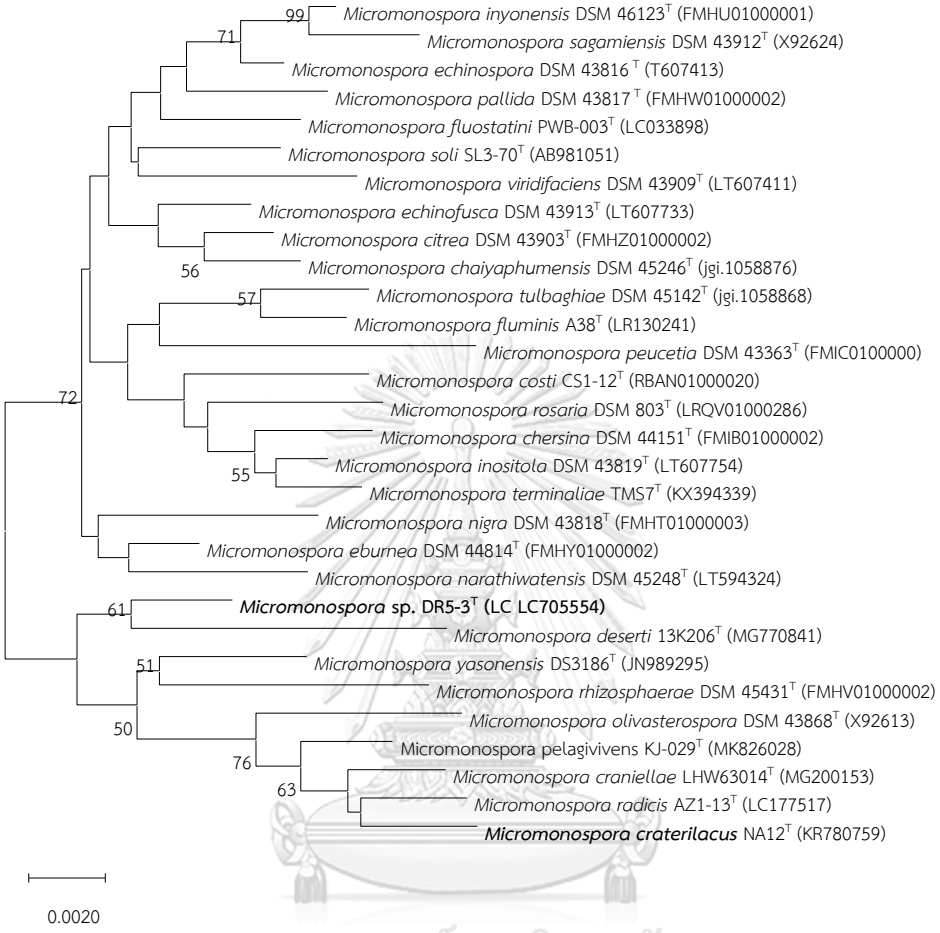


Figure 9 Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strains DR5-3^T within the radiation of *Micromonospora* species.

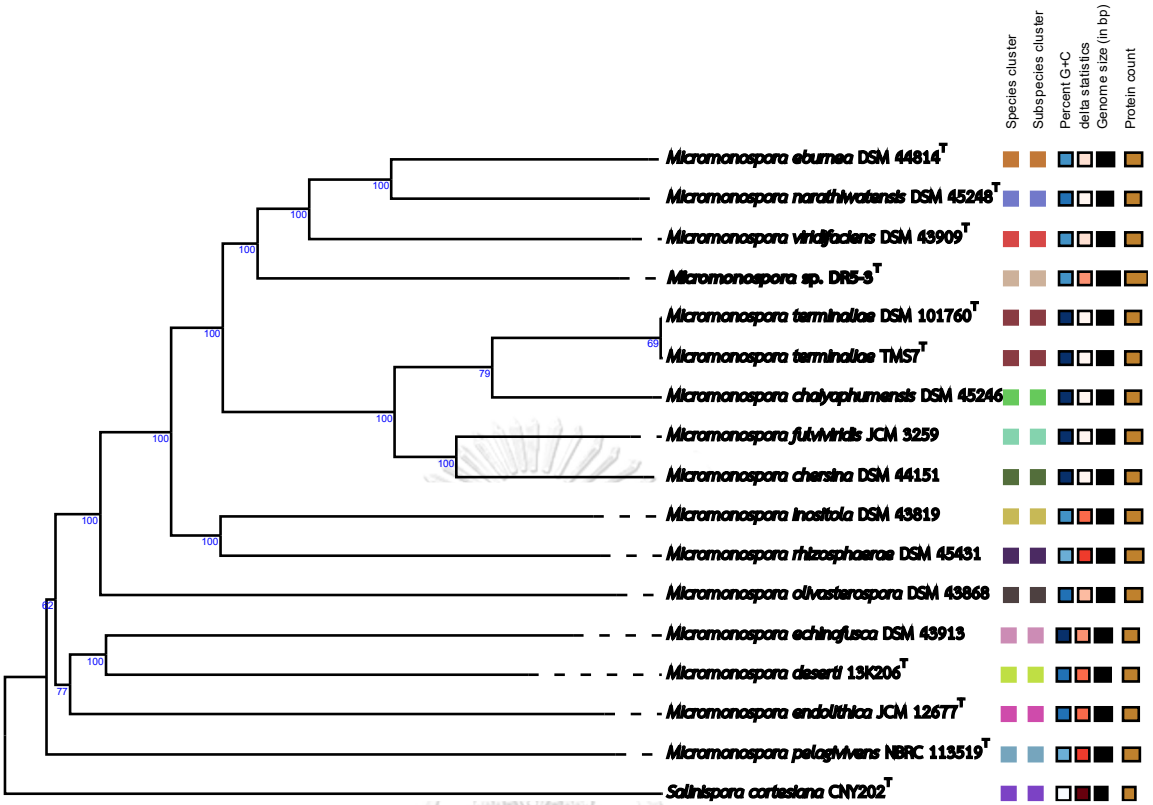


Figure 10 Phylogenomic tree of strain DR5-3^{T} and related Micromonospora species obtained from TYGS. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications.

Description of *Micromonospora dendrobii* sp. nov.

Micromonospora dendrobii (den. Dro'bi.i. N.L. gen. n. *dendrobii* of *Dendrobium kentrophyllum*, the isolation source of the type strain).

Cells are Gram-positive, aerobic, non-motile, filamentous actinomycetes. Cells grow well on ISP 2, ISP 4, ISP 6, ISP 7 and nutrient agar. The colonial colour is pale orange yellow on ISP 2. There is no production of a diffusible pigment. Growth occurs at 20-40 °C (optimum 30 °C), at pH 5-10 (optimum 7.0) and with 5% (w/v) NaCl and weakly with 7% NaCl (w/v). Coagulation of skimmed milk, nitrate reduction, gelatin liquefaction and starch hydrolysis are positive, but peptonization is negative. Positive for utilization of glucose, lactose, rhamnose and melibiose as sole carbon sources but do not utilize fructose, glycerol, raffinose, mannose, mannitol, xylose, cellobiose,

cellulose, galactose, maltose, *myo*-inositol, melezitose, sucrose. Utilizes L-arginine, L-asparagine, L-proline and L-cysteine. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. Whole-cell sugars are glucose, arabinose and xylose. The polar lipid profile comprises diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI).

The type strain is DR5-3^T (=JCM 35413^T) isolated from the roots of *Dendrobium kentrophyllum* collected from Suphanburi Province, Thailand. The DNA G+C content of the type strain is 71.9 mol%. The GenBank accession numbers for the 16S rRNA gene sequence are LC705554.

4.3 Antimicrobial activity of endophytic actinomycetes

Thirty-six isolates (58.06%) showed antimicrobial activity against at least one of the six tested bacteria (Table 9-13). Seven isolates of *Streptomyces* and seven isolates of *Micromonospora* inhibited *S. aureus* ATCC 25923 when cultivated in all production media. The isolate DR8-9 presented the highest antimicrobial activity against *S. aureus* ATCC 25923 (24.32±1.01 mm) when cultivated in 57 media. Nine isolates of *Streptomyces*, eleven isolates of *Micromonospora* and one isolate of *Streptosporangium* inhibited *K. rhizophila* ATCC 9341. Isolate DR6-8 showed the highest activity (26.24±0.99 mm), while isolates DR2-2 and DR4-1 showed 22.76±0.58 and 19.65±0.58 mm, respectively. Nine isolates of *Streptomyces* and five *Micromonospora* were active against *B. subtilis* ATCC 6633 when grown in all media. Isolate DR7-6, which was closely related to *S. parvulus* NBRC 13193^T, showed the highest activity (20.19±0.59 mm) in medium ISP 2 plus $CaCO_3$. Isolates DR1-1, DR3-2, DR3-4, DR7-3 and DR8-9 inhibited *C. albicans* ATCC 10231 in all production media, including DR2-2 in ISP 2 medium. The isolate DR3-4 was closely related to *S. daghestanicus* NRRL B-5418^T and showed the highest activity against *C. albicans* ATCC 10231 (26.63±0.20 mm) on ISP 2. Ten isolates of *Streptomyces* and six of *Micromonospora* were active against the Gram-negative bacteria *P. aeruginosa* ATCC 27853, while isolate DR2-2 inhibited this bacterium when cultivated on all production media. Interestingly, DR2-2 was closely related to *S. antibioticus* NBRC 12838^T

exhibited antimicrobial activity to *E. coli* ATCC 25922 at 16.41±0.58, 17.26±0.53 and 18.52±0.52 when cultivated in ISP 2, 30 and 57 media, respectively. Group IV *Actinomad*ura, Group V *Amycolatopsis* and Group VI *Pseudonocardia* did not exhibit antimicrobial activity.

Table 9 Antimicrobial activitiy on *S. aureus* ATCC 25923 (inhibition zone, mm in diameter) of Group I *Streptomyces* and Group II *Micromonospora* isolates cultivated in ISP 2, ISP 2+$CaCO_3$, 30 and 57 media.

Isolate no.	Medium			
	ISP 2	ISP 2+$CaCO_3$	30	57
Group I				
DR2-2	16.98±0.57^{c}	16.56± 0.58^{d}	16.43±1.00^{d}	17.98±0.58^{b}
DR5-1	14.64±0.19^{d}	13.87± 0.10^{e}	13.89± 0.13^{e}	9.76±0.27^{e}
DR5-2	15.24±1.00^{d}	14.16± 0.95^{e}	14.98±1.00^{e}	9.84±0.50^{e}
DR7-3	21.35±0.20^{b}	16.13± 0.49^{d}	14.30± 0.06^{e}	10.20±0.23^{de}
DR7-6	21.22±1.00^{b}	20.68± 1.00^{b}	19.11±1.00^{b}	18.01±1.00^{b}
DR8-9	22.64±0.75^{a}	22.57± 0.59^{a}	21.79±0.57^{a}	24.32±1.01^{a}
DR9-5	11.56±1.00^{e}	11.24± 1.00^{f}	12.44±0.61^{f}	12.46±1.01^{c}
Group II				
DR4-1	8.39±0.56^{ghi}	8.89±0.58^{hi}	9.54±0.58^{hi}	9.79±1.00^{e}
DR6-8	9.67±0.58^{f}	9.56±1.01^{gh}	9.90±0.58^{h}	10.42±0.62^{de}
CR1-1	9.46±0.57^{fg}	9.41±0.55^{hi}	9.47±0.53^{hi}	11.20±0.50^{8}
CA1-5	7.88±0.99^{i}	8.22±1.01^{i}	8.63±0.75^{ij}	9.39±0.60^{e}
CA1-6	8.21±0.65^{hi}	8.48±0.14^{hi}	8.28±0.16^{j}	9.52±0.13^{e}
CR1-2	9.37±0.07^{fgh}	10.71±0.22^{fg}	10.41±0.32^{g}	10.65±0.26^{de}
YG1-1	17.91±0.58^{c}	18.19±0.57^{c}	17.92±0.58^{c}	18.87±0.59^{b}

Data are expressed as mean ± standard deviation (SD), including the disc diameter (6 mm).

Different superscripts in the same row indicate significant differences ($P<0.05$).

Table 10 Antimicrobial activitiy on *K. rhizophila* ATCC 9341 (inhibition zone, mm in diameter) of Group I *Streptomyces*, Group II *Micromonospora* and Group III *Streptosporangium* isolates cultivated in ISP 2, ISP 2+$CaCO_3$, 30 and 57 media.

Isolate no.	Medium			
	ISP 2	ISP 2+$CaCO_3$	30	57
Group I				
DR2-2	21.43±1.01^{c}	21.17±1.00^{b}	21.43±1.01^{b}	22.76±0.58^{b}
DR3-4	8.21±0.21^{i}	10.33±0.21^{i}	10.54±0.31^{fg}	11.26±0.16^{hij}
DR5-1	10.53±0.09^{gh}	17.45±0.16^{cd}	15.56±0.17^{d}	16.35±0.16^{f}
DR5-2	15.89±1.00^{g}	16.78±0.57^{d}	15.89±1.00^{d}	16.95±1.01^{ef}
DR7-3	22.49±0.13^{b}	18.22±0.18^{c}	11.48±0.12^{f}	10.26±0.17^{jk}
DR8-9	15.66±0.57^{e}	16.88±0.64^{d}	15.35±1.00^{d}	18.52±1.00^{c}
DR10-1	8.07±0.58^{i}	8.39±0.58^{i}	8.17±0.81^{i}	7.97±0.84^{m}
DR10-2	11.42±0.15^{g}	11.33±0.12^{gh}	11.71±0.23^{f}	11.65±0.18^{hi}
DR10-3	8.13±0.11^{i}	8.54±0.08^{i}	8.52±0.35^{i}	9.41±0.28^{kl}
Group II				
DR4-1	19.65±0.58^{d}	18.40±0.58^{c}	19.11±0.57^{c}	17.77±0.58^{cd}
DR5-6	10.49±0.08^{gh}	10.43±0.26^{hi}	11.34±0.37^{fg}	11.07±0.06^{ij}
DR6-8	24.91±0.94^{a}	26.24±0.99^{a}	24.91±1.53^{a}	24.24±0.99^{a}
CR1-1	10.45±0.88^{gh}	12.26±1.00^{g}	11.36±1.00^{fg}	12.34±1.00^{h}
CR1-2	9.58±0.21^{h}	11.43±0.07^{g}	10.44±0.35^{fg}	11.73±0.26^{hi}
CA1-5	15.79±0.99^{e}	15.44±0.71^{e}	16.60±0.79^{d}	16.75±0.73^{ef}
CA1-6	15.46±0.27^{e}	15.61±0.22^{e}	16.40±0.15^{d}	16.38±0.15^{f}
YG1-1	13.86±1.00^{f}	13.86±1.00^{f}	13.91±0.58^{e}	14.68±0.57^{g}
YG1-8	7.85±0.50^{i}	7.58±0.58^{ij}	7.46±1.00^{i}	0.00±0.00
YG1-9	7.32±0.22^{i}	7.30±0.25^{j}	7.50±0.11^{i}	8.56±0.19^{lm}
YG1-10	7.74±0.15^{i}	7.52±0.10^{ij}	7.36±0.05^{i}	8.14±0.09^{m}
Group III				
DR9-9	10.32±0.57^{gh}	9.90±0.59^{i}	10.15±0.58^{h}	9.85±1.01^{k}

Data are expressed as mean ± standard deviation (SD), including the disc diameter (6 mm).

Different superscripts in the same row indicate significant differences ($P<0.05$).

Table 11 Antimicrobial activity on *B. subtilis* ATCC 6633 (inhibition zone, mm in diameter) of Group I *Streptomyces* and Group II *Micromonospora* isolates cultivated in ISP 2, ISP 2+$CaCO_3$, 30 and 57 media.

Isolate no.	Medium			
	ISP 2	ISP 2+$CaCO_3$	30	57
Group I				
DR2-2	16.66 ± 1.00^{b}	17.76 ± 0.57^{b}	18.47 ± 0.57^{b}	18.25 ± 0.58^{a}
DR5-1	16.54 ± 0.34^{b}	15.40 ± 1.00^{c}	17.43 ± 0.14^{b}	13.62 ± 0.28^{c}
DR5-2	16.51 ± 0.79^{b}	15.58 ± 1.00^{c}	17.84 ± 0.86^{b}	13.85 ± 1.00^{c}
DR7-3	17.39 ± 0.10^{b}	14.61 ± 0.27^{c}	8.27 ± 0.17^{f}	6.68 ± 0.29^{j}
DR7-6	19.87 ± 1.01^{a}	20.19 ± 0.59^{a}	20.00 ± 0.58^{a}	18.80 ± 0.57^{a}
DR8-9	14.53 ± 0.60^{c}	15.02 ± 0.57^{c}	14.35 ± 0.76^{c}	15.95 ± 0.99^{b}
DR10-1	9.55 ± 0.61^{efg}	9.54 ± 0.99^{ef}	9.62 ± 1.00^{e}	10.74 ± 1.00^{ef}
DR10-2	11.35 ± 0.20^{d}	11.41 ± 0.11^{d}	11.3 ± 0.139^{d}	12.59 ± 0.23^{cd}
DR10-3	9.64 ± 0.25^{efg}	9.54 ± 0.01^{ef}	9.44 ± 0.46^{ef}	10.42 ± 0.36^{fg}
Group II				
DR4-1	9.90 ± 0.57^{ef}	10.86 ± 1.00^{d}	11.68 ± 1.01^{d}	11.94 ± 0.99^{de}
DR6-8	10.36 ± 0.58^{de}	10.53 ± 0.76^{de}	10.78 ± 0.85^{d}	11.29 ± 0.72^{ef}
CA1-5	9.06 ± 0.58^{fg}	8.62 ± 0.77^{fg}	8.84 ± 0.56^{ef}	9.06 ± 0.99^{hi}
CA1-6	8.57 ± 0.30^{g}	8.58 ± 0.10^{fg}	8.36 ± 0.22^{f}	9.36 ± 0.19^{gh}
YG1-1	7.32 ± 1.00^{h}	7.43 ± 0.58^{g}	8.23 ± 1.00^{f}	7.84 ± 1.00^{ij}

Data are expressed as mean ± standard deviation (SD), including the disc diameter (6 mm).

Different superscripts in the same row indicate significant differences ($P<0.05$).

Table 12 Antimicrobial activity on *P. aeruginosa* ATCC 27853 (inhibition zone, mm in diameter) of Group I *Streptomyces* and Group II *Micromonospora* isolates cultivated in ISP 2, ISP 2+$CaCO_3$, 30 and 57 media.

Isolate no.	Medium			
	ISP 2	ISP 2+$CaCO_3$	30	57
Group I				
DR1-1	18.79±0.56^{a}	0.00±0.00	0.00±0.00	0.00±0.00
DR2-2	9.13±1.00^{g}	9.79±0.55	9.57±0.67	10.08±0.66
DR2-4	11.21±0.15^{e}	0.00±0.00	0.00±0.00	0.00±0.00
DR3-2	16.41± 0.31^{b}	0.00±0.00	0.00±0.00	0.00±0.00
DR7-2	10.26±0.12^{f}	0.00±0.00	0.00±0.00	0.00±0.00
DR8-8	9.27±0.10^{g}	0.00±0.00	0.00±0.00	0.00±0.00
DR8-9	0.00±0.00	0.00±0.00	0.00±0.00	9.36±0.58
DR8-10	9.47±0.13^{fg}	0.00±0.00	0.00±0.00	0.00±0.00
DR10-2	12.58±0.16^{d}	0.00±0.00	0.00±0.00	0.00±0.00
DR10-3	11.46±0.34^{e}	0.00±0.00	0.00±0.00	0.00±0.00
Group II	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
DR4-1	8.28±0.60^{h}	0.00±0.00	0.00±0.00	0.00±0.00
CR1-1	14.55±1.02^{c}	0.00±0.00	0.00±0.00	0.00±0.00
CR1-2	6.89±0.48^{i}	0.00±0.00	0.00±0.00	0.00±0.00
CA1-1	9.72±0.53^{fg}	0.00±0.00	0.00±0.00	0.00±0.00
YG1-9	9.56±0.32^{fg}	0.00±0.00	0.00±0.00	0.00±0.00
YG1-10	9.26±0.16^{g}	0.00±0.00	0.00±0.00	0.00±0.00

Data are expressed as mean ± standard deviation (SD), including the disc diameter (6 mm).

Different superscripts in the same row indicate significant differences ($P<0.05$).

Table 13 Antimicrobial activity on *C. albicans* ATCC 10231 (inhibition zone, mm in diameter) of Group I *Streptomyces* isolates cultivated in ISP 2, ISP 2+$CaCO_3$, 30 and 57 media.

Isolate no.	Medium			
	ISP 2	ISP 2+$CaCO_3$	30	57
Group I				
DR1-1	20.60±0.58^{b}	21.90± 0.77^{a}	21.90±0.77^{c}	20.37±1.00^{b}
DR2-2	17.23±0.61^{d}	0.00±0.00	0.00±0.00	0.00±0.00
DR3-2	19.34±0.22^{c}	20.31± 0.17^{b}	22.41±0.34^{b}	17.16±0.16^{c}
DR3-4	26.63±0.20^{a}	20.37± 0.33^{b}	23.54±0.47^{a}	23.62±0.16^{a}
DR7-3	13.28±0.16^{e}	16.46± 0.26^{c}	16.87±0.11^{d}	16.70±0.31^{c}
DR8-5	12.57±0.28^{e}	13.29± 0.15^{d}	0.00±0.00	14.72±0.14^{d}
DR8-9	12.76±0.59^{e}	13.01± 0.57^{d}	13.01±0.57^{e}	12.37±0.57^{e}

Data are expressed as mean ± standard deviation (SD), including the disc diameter (6 mm).

Different superscripts in the same row indicate significant differences ($P<0.05$).

Endophytic actinomycetes have been reported for their antibacterial activity against pathogenic bacteria (Taechowisan & Lumyong, 2003) (Passari et al., 2015). In this study, both *Streptomyces* and *Micromonospora* isolates inhibited *S. aureus, B. subtilis* and *K. rhizophila*. Our results concurred with Musa et al. (2020)(Musa et al., 2020) who discovered that 54 of 126 endophytic actinobacteria strains were resistant to at least one indicator species. Notably, the majority of *Streptomyces* strains exhibited antagonistic activities. (Rao et al., 2015) Rao et al. (2015) found that all *Streptomyces* strains from *Combretum latifolium* showed significant antimicrobial activity against both bacterial and fungal pathogens. This study reported on the incidence of possible endophytic actinomycetes that suppress pathogenic bacteria.

4.4 Antifungal activity of endophytic *Streptomyces* strains from *Dendrobium* orchids and the secondary metabolites of strain DR7-3 with its genome analysis

4.4.1 Isolation and identification of isolates

Four endophytic actinomycetes were recovered from the roots of the Thai orchid. Strains DR5-1 and DR7-3 were isolated from *D. kentrophyllum* and *D. findlayanum*, respectively, whereas strains DR8-5 and DR8-8 were obtained from *D. chrysanthum.* Results from 16S rRNA gene sequence and phenotypic characteristic data indicated that all the isolates were *Streptomyces* spp. The pairwise alignment of the 16S rRNA gene sequence among all was 98.15-99.93 % similarity. Strains DR5-1, DR7-3, DR8-5, and DR8-8 were closely related to *S. pravulus* NBRC 13193^{T} (99.40%), *S. solisilvae* HNM0141^{T} (99.15%), *S. daghestanicus* NRRL B-5418^{T} (99.93%), and *S. malaysiensis* MUSC 136^{T} (99.85%), based on 16S rRNA gene sequence similarity. The 16S rRNA gene sequences of these strains have been deposited at NCBI database, and their accession numbers are listed in Table 14. The strains produced spiral spore chains with pale blue to gray color on ISP 2 agar after 7-day incubation. Scanning electron micrograph of strain DR7-3 revealed spiral spore chains on aerial hyphae (Figure 11). All strains contained LL-diaminopimelic acid (Williams and Cross, 1971). The optimum temperature of all strains was 25-30°C and pH range 5-10. All strains utilized various sugars for growth and grew in a range of 5-10% NaCl concentrations. The cultural, physiological, and biochemical characteristics of the isolates are shown Table 14.

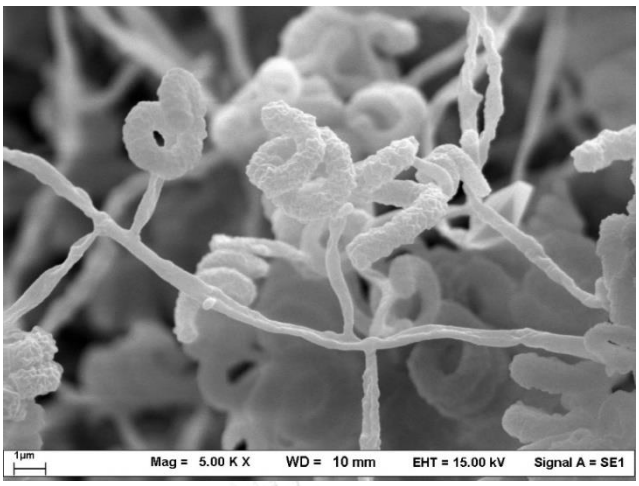


Figure 11 Scanning electron micrograph of strain DR7-3 grown on ISP 2 agar at 30 °C for 14 days.

Orchids are known to be a rich source of endophytic microorganisms. Studies on the tissues of several terrestrial orchids indicated that the number and type of endophytes follow the seasonal rhythm of the year (Chutima et al., 2011) In this study of *Dendrobium* orchids, *Streptomyces* was found to be the main genus, similar to a previous report (Tsavkelova et al., 2007). In addition, *Streptomyces* sp. viji10 was reported from the velamen roots of *Vanda spathulata* orchid (Senthilmurugan et al., 2013), whilst *Actinomycetospora endophytica* was identified as a novel species from the wild orchid *Podochilus microphyllus* (Sakdapetsiri et al., 2018).

Table 14 Phenotypic characteristics of isolates.

Characteristic	Isolate no.			
	DR5-1	DR7-3	DR8-5	DR8-8
Accession	LC685847	LC685849	LC685851	LC685853
Upper colour	Pale blue	Bluish gray	Greenish white	Bluish gray
Reverse colour	Light yellow	Light yellow	Light yellow	Light yellow
Diffusible pigment	-	-	-	-
Diaminopimelic acid	LL-DAP	LL-DAP	LL-DAP	LL-DAP
Temp (C)	25-30	25-30	25-30	25-30
pH range	5-10	5-10	5-7	5-10
NaCl (%)	8	6	10	6
Starch hydrolysis	+	+	+	+
Coagulation	+	-	-	-
Nitrate reduction	-	+	-	+
Gelatin liquefaction	+	+	+	+
Utilization of				
Glucose	+	+	+	+
Sucrose	+	+	w	+
Lactose	+	+	+	+
Dextrose	+	+	+	+
Maltose	+	+	+	+
Mannitol	+	+	+	+
Xylose	+	+	+	+
Sorbitol	-	w	w	w

4.4.2 Evaluation of antifungal activity

The antagonistic activity of the endophytic *Streptomyces* in this study was investigated in vitro against a wide range of phytopathogens using the dual culture technique. The inhibitory activities of strains DR5-1, DR7-3, DR8-5, and DR8-8 against five phytopathogenic fungi including *Fusarium oxysporum*, *Fusarium solani, Alternaria alternata, Colletotrichum oryzae*, and *C. gloeosporioides* are shown in Table 15.

Table 15 Antifungal activity of isolates.

Isolate no.	% Inhibition				
	F. oxysporum SA01	*F. solani* SA02	*A. alternata* SA01	*C. gloeosporioides* SA03	*C. oryzae* SA04
DR5-1	21.85±1.67^{h}	29.17±0.96^{cd}	24.29±0.86^{g}	27.33±0.72^{cdef}	29.55±0.83^{cd}
DR7-3	13.70±1.12^{k}	25.37±1.40^{fg}	19.24±1.00^{j}	26.57±1.31^{efg}	35.05±0.95^{a}
DR8-5	24.81±1.97^{g}	28.70±2.89^{cde}	11.90±0.44^{k}	21.52±0.72^{hi}	29.64±1.22^{c}
DR8-8	19.54±1.53^{ij}	27.22±1.21^{def}	17.33±1.15^{j}	25.24±0.87^{fg}	32.52±0.95^{b}

The different alphabets mean significant difference ($P<0.05$) mean ± SD.

Among the four *Streptomyces* isolates in this study, DR7-3 presented the most interesting result, showing the highest antifungal potential with 35.05±0.95 % inhibition against *C. oryzae* SA04 and thus was selected for further detailed studies (see below). Strain DR5-1 also exhibited remarkable antifungal activity with highest effects observed in three fungi, including against *A. alternata* SA01, *F. solani* SA02 and *C. gloeosporioides* SA03, whereas DR8-5 was the strongest when tested against *F. oxysporum* SA01.

Endophytic actinomycetes from several sources have been earlier studied for their inhibitory activity against phytopathogenic fungi. For example, *Streptomyces* sp. CMUAc130 isolated from *Zingiber officinale* could inhibit *Colletotrichum musae* and *Fusarium oxysporum* (Taechowisan & Lumyong, 2003) *Streptomyces* sp. S12-10 from Rice showed high percentages of inhibition against *Fusarium moniliforme, Helminthosporium oryzae* and *Rhizoctonia solani*, whereas *Streptomyces* strain CEN26 isolated from *Centella asiatica* (L.) displayed significant antifungal activity

against *Alternaria brassicicola* (Phuakjaiphaeo & Kunasakdakul, 2015). In a more recent study, wetland-derived *Streptomyces* sp. ActiF450 exhibited a broad-spectrum antifungal activity against *Aspergillus niger* MA2, *Fusarium oxysporum* F15, *Penicillium chrysogenum* ICF59, and *Scodapulariopsis candida* ICF53 (Benhadj et al., 2020). The inhibitory potential of a soil-borne *Streptomyces hygroscopicus* against the fungus *Colletotrichum gloeosporioides* was earlier described (Prapagdee et al., 2008). This study constitutes the first report of the antifungal activity of endophytic actinomycetes isolated from *Dendrobium* orchids.

4.4.3 Antifungal activity on the mycelial growth

The extract from the strain DR7-3 inhibited the mycelial growth of *C. oryzae* SA04 in a dose-dependent manner (Table 16). The colony growth was suppressed (100% inhibition) when the concentration of the extract reached 3000 ppm. The IC_{50} value of the extract against *C. oryzae* SA04 was 25.75 ppm, significantly lower than that of the chemical fungicide benomyl (178.5 ppm). Benomyl has been widely used against a variety of phytopathogenic fungi (Tobih et al., 2015), and its fungicide action was related to its capacity to be absorbed by phytopathogen cells (Summerbell, 1993).

Table 16 Antifungal activity of ethyl acetate extract against *C. oryzae* of strain DR7-3.

Treatments	% Inhibition								
	0 ppm	100 ppm	200 ppm	500 ppm	800 ppm	1000 ppm	2000 ppm	3000 ppm	IC_{50}
Crude DR7-3	0.00	69.40	75.40	81.80	82.60	83.20	95.00	100.00	25.75
Benomyl	0.00	37.60	43.40	44.00	50.00	60.60	71.00	80.00	178.5

Scanning micrograph analyses revealed that the *C. oryzae* SA04 mycelia taken from the colony's edge differed from the control. The control has a typical structural feature, such as a smooth outer surface on the cylindrically formed mycelium (Figure 12A), whereas mycelia treated with DR7-3 extract were severely deformed, with

uneven shrinkages, roughness, loss of smoothness, and a swollen mycelium surface (Figure 12B). Unusual morphology of fungal hyphae can be taken as evidence for antifungal activity of the test sample (Hashem et al., 2016). In a previous report, the fungicidal activity of a butanol extract of *Streptomyces blastmyceticus* 12-6 on *Colletotrichum acutatum* and *Fusarium oxysporum* was studied using the SEM technique, which showed the abnormal morphology of hyphae, such as swelling and a reduction in cytoplasmic content, with apparent separation of cytoplasm from cell wall (Kim et al., 2019). These phenomena were also observed in *Curvularia oryzae* PSUNK1012 when treated with the culture filtrate of *S. angustmyceticus* (PITHAKKIT et al., 2015). Similar observations were also reported for *F. oxysporum* Race 4 upon the addition of the extract of *Streptomyces* sp. CB-75 (Chen et al., 2018).

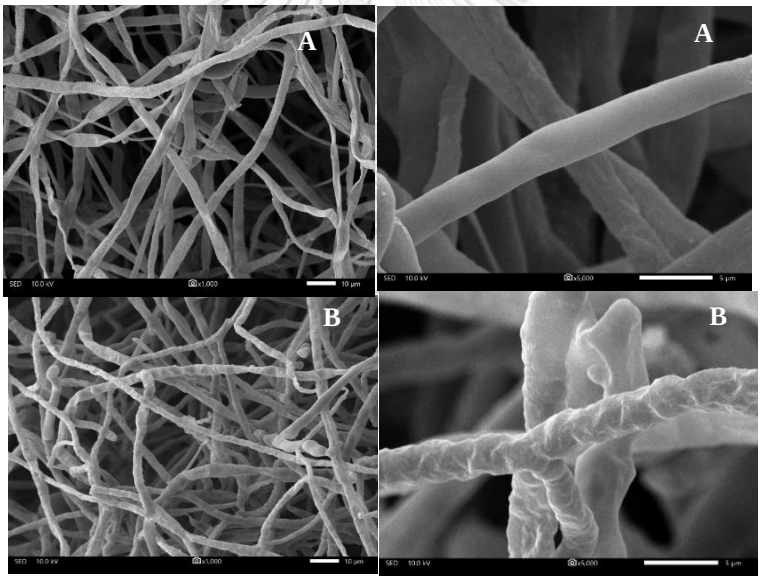


Figure 12 Scanning electron micrograph of mycelia of *Curvularia oryzae* SA04 without treatment (A and magnified A); treatment with ethyl acetate extract from strain DR7-3 (B and magnified B).

4.4.4 Genomic sequencing analysis

Genome analysis of strain DR7-3 revealed the size of 11,331,527 bp distributed in 159 contigs with G+C content of 71.12% and 9,582 protein coding sequences (CDSs). The phylogenetic analysis based on whole-genome sequences (Figure 13) indicated that strain DR7-3 was phylogenetically closed to *S. solisilvae* HNM0141^{T}. The average nucleotide identity-Blast (ANIb) and average nucleotide identity-MUMmer (ANIm) of the genomes DR7-3 and *S. solisilvae* HNM0141^{T} were 98.49 and 98.71%, respectively. The digital DNA-DNA hybridization (dDDH) values were the highest, 88.40% with *S. solisilvae* HNM0141^{T}. For genome comparison, ANI and dDDH values are considered well correlated when the values were ≥95 % (ANI) and ≥70% (dDDH), respectively (Fitch, 1971) (Seemann, 2014). Since the dDDH (90.90%) and the ANI (98.59-99.03%) values between strain DR7-3 and *S. solisilvae* HNM0141^{T} were higher than the species cut off, DR7-3 was identified as *S. solisilvae.*

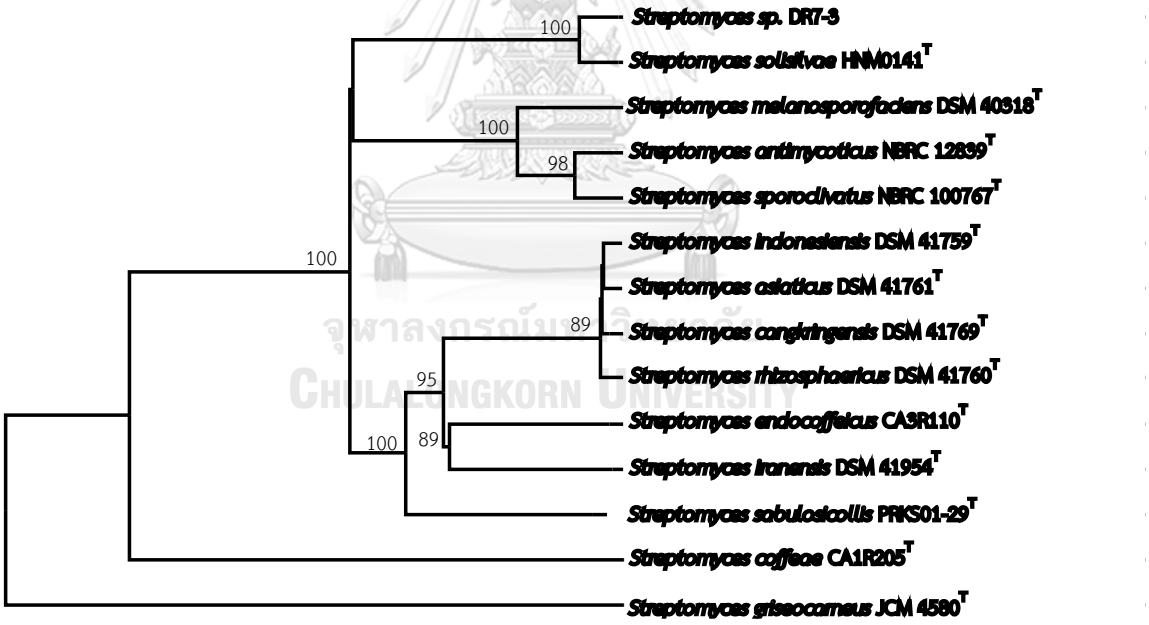


Figure 13 Phylogenomic tree of strain DR7-3 and related Streptomyces species obtained from TYGS. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications.

4.4.5 Gene function annotation and secondary metabolism gene clusters

The draft genome of strain DR7-3 was determined using the antiSMASH server to detect putative biosynthetic gene clusters (BGCs). More than 70 gene clusters were observed on DR7-3 genomes related to various BGCs, mainly type I polyketide synthase (T1PKS), non-ribosomal peptide synthetase (NRPS), terpene, and siderophore (Table 19). The secondary metabolite biosynthetic gene clusters (smBGCs) exhibited 100 % similarity genetic relatedness to the known clusters producing geosmin, desferrioxamin B, ectoine, coelichelin, pristinol and echoside. Interestingly, desferrioxamin B, echoside A and echoside B have a potential as SARS-CoV-2 inhibitors (Bellotti & Remelli, 2021) (Melinda et al., 2021). Thus, strain DR7-3 might be one of the sources of natural anti-COVID-19 compounds. Strain DR7-3 are predicted to produce anti-cancer agents such as geldanamycin (Fukuyo et al., 2010), salinomycin (Antoszczak & Huczyński, 2015), and hygrocin A / hygrocin B (Yin et al., 2017). The predicted secondary metabolites meilingmycin and herboxidiene can be used as an insecticide (Sun et al., 2003) and a herbicide (Pokhrel et al., 2015), respectively in agricultural farming.

Comparison of the BGCs of DR7-3 to those of the closely strain *S. solisilvae* HNM0141^{T} revealed similar main gene clusters. However, the numbers of some gene clusters, such as T1PKS and NRPS-like, were different. The T1PKS and NRPS-like in DR7-3 predicted secondary metabolites not found in *S. solisilvae* HNM0141^{T} (Table 17). The strain DR7-3 exhibited genetic relatedness to known 5 secondary metabolite biosynthetic gene clusters (smBGCs) that are associated with antifungal activity, rustmicin, elaiophylin, coelichelin, cyphomycin and rapamycin and found in *S. solisilvae* HNM0141^{T}. However, eight smBGCs, such as heronamide, niphimycins, fluvirucin B2, primycin, sceliphrolactam, niphimycins, pentamycin, and mediomycin A, were present only in strain DR7-3. In addition, the genome of strain DR7-3 contained nine smBGCs that displayed no similarity to any known smBGCs in antiSMASH (Table 17). These results suggested that strain DR7-3 might be a source of novel secondary metabolites with antifungal activity.

Table 17 Comparison of BGCs composition of strain DR7-3 and S. solisilvae HNM0141^{T}.

Type	Strain	
	DR7-3	*S. solisilvae* HNM0141^{T}
NRPS-like	5	2
Terpene	5(1)	5(1)
NRPS	3(1)	4
T1PKS	25(2)	9(1)
Siderophore	1(2)	1(2)
Butyrolactone	1(1)	1(1)
Redox-cofactor	1	1
Hserlactone	1	1
NAPAA	1	1
RiPP-like	(1)	(1)
Ectoine	1	1
Ladderane	1	1
Indole	1	1
RRE-containing	1	1
Hybrid	15(1)	14(1)

NRPS-Nonribosomal peptide synthetases; NRPS-like-NRPS-like fragment; T1PKS-type 1 polyketide synthetases; NAPAA-non-alpha poly-amino acids like e-Polylysin; RiPP-like-Other unspecified ribosomally synthesised and post-translationally modified peptide product; RRE-containing-RRE-element containing. Values in brackets indicate the number of clusters nonsimilar to those in the database.

4.4.6 Identification of bioactive compounds of strain DR7-3 by GC-MS

The secondary metabolites of strain DR7-3 extract were analyzed by GC-MS. A total of 15 chemical compounds were identified by alignment of the NIST library based on retention time, molecular mass, molecular formula, and their biological activity (Table 20). These compounds were identified as 1) 2,3-butanediol, 2) 4-hydroxy-4-methyl-2-pentanone, 3) phenyl ethanol, 4) phenyl propanoic acid, 5) 2-phenylethyl ester of acetic acid, 6) *N*-tetradecane, 7) 2,4-*bis*(1,1-dimethylethyl) phenol, 8) hexadecane, 9) 1-heptadecane, 10) *n*-octadecane, 11) 2-phenylethyl ester of phenyl-acetic acid, 12) methyl ester of hexadecanoic acid, 13) 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo, 14) eicosane and 15) *bis*(2-ethylhexyl) ester of hexanedioic acid. Among these compounds, eicosane, 2,4-*bis*(1,1-dimethylethyl) phenol, hexadecane, and methyl ester of hexadecanoic acid have been earlier reported to possess antifungal activity (Table 18).

Eicosane, a long-chain fatty acid, was detected in the crude extract of *Streptomyces* sp. KX852460 and showed antifungal activity against *Rhizoctonia solani* AG-3 KX852461, cause of leaf spot disease (Ahsan et al., 2017). This compound also presented in the flower of *Allium atroviolaceum*, contributing to the antimicrobial activity of the plant extract (Dehpour et al., 2012). 2,4-*Bis*(1,1-dimethylethyl)-phenol from the ethyl acetate extract of *Kutzneria* sp. TSII inhibited the pathogenic fungus *Pithomyces atroolivaceous* (Devi et al., 2021). This compound was produced by *Pseudomonas fluorescens* TL-1 and showed antifungal activity against *Curvularia lunata* (Ren et al., 2019). The long chain hydrocarbon, hexadecane from *Jatropha curcas* leaf extracts exhibited against groundnut late leaf spot disease caused by *Phaeosariopsis personata* (Francis et al., 2021). Hexadecanoic acid-methyl ester, a long chain fatty ester produced from *Streptomyces galbus* TP2 and *Streptomyces humidus*, has been identified as an antifungal constituent (Kawuri & Darmayasa, 2019). It should be noted that *bis*(2-ethylhexyl)-hexanedioic acid, the major compound in DR7-3 extract, possessed antimicrobial, antioxidant, and antiproliferative activities (Kadhim et al., 2017) (Paramanantham & Murugesan, 2014).

The endophytic actinomycetes DR5-1, DR7-3, DR8-5, and DR8-8 isolated from three *Dendrobium* orchids were belonged to the genus *Streptomyces.* They showed

inhibitory activity against several phytopathogenic fungi. Strain DR7-3 was identified as *Streptomyces solisilvae* and exhibited a broad-spectrum antifungal activity against five fungi that are causal agents of plant diseases. The ethyl acetate extract from this strain showed a high level of inhibition against *C. oryzae* SA04, compared with a standard chemical fungicide. Moreover, it suppressed mycelial growth and damaged the cell structure of the fungi. Four chemical components with antifungal activity were identified from the extract using the gas chromatography-mass spectrometric (GC-MS) technique. The draft genome sequence analysis of strain DR7-3 indicated that thirteen gene clusters are involved in the biosynthesis of these antifungal metabolites. In our investigation, *Streptomyces solisilvae* DR7-3 appears to be a promising source for developing new antifungal agents against phytopathogenic fungi.

Table 18 Chemical profile and bioactivity of ethyl acetate extract of strain DR7-3.

No.	RT	Compound name	MW	MF	Similarity (%)	Bioactivity
1	5.48	2,3-butanediol	90.07	$C_4H_{10}O_2$	91	Antibacterial activity (Wu et al., 2019)
2	6.85	4-hydroxy-4-methyl-2-pentanone	116.08	$C_6H_{12}O_2$	83	No activity reported
3	14.61	phenyl ethanol	122.07	$C_8H_{10}O$	94	Antibacterial activity (Corre et al., 1990)
4	16.89	phenyl propanoic acid	150.07	$C_9H_{10}O_2$	50	Anti-neuroinflammatory activities (Li et al., 2019)
5	19.17	2-phenylethyl ester of acetic acid	164.08	$C_{10}H_{12}O2$	90	Antimicrobial activity (Tayade & Jadhao, 2012; Valsalam et al., 2019)
6	23.10	*N*-tetradecane	198.24	$C_{14}H_{30}$	98	Antimicrobial activity, anti-tuberculosis (Girija et al., 2014)
7	25.78	2,4-*bis*(1,1-dimethylethyl) phenol	206.17	$C_{14}H_{22}O$	94	Anti-QS and antibiofilm activities (Padmavathi et al., 2014) Antifungal activity (Rangel-Sánchez et al., 2013) (Ren et al., 2019)
8	28.04	Hexadecane	226.27	$C_{16}H_{34}$	98	Antifungal, antibacterial, antioxidant (Yogeswari et al., 2012)
9	32.33	1-heptadecane	238.27	$C_{17}H_{34}$	91	No activity reported
10	32.48	*n*-octadecane	254.30	$C_{18}H_{38}$	98	No activity reported
11	34.82	2-phenylethyl ester of phenyl-acetic acid	240.12	$C_{16}H_{16}O_2$	91	No activity reported
12	35.03	methyl ester of hexadecanoic acid	270.26	$C_{17}H_{34}O_2$	99	Antifungal (Kawuri & Darmayasa, 2019) Antibacterial, antioxidant, antitumor, immunostimulant, and lipoxygenase inhibitor (Rahbar et al., 2012)
13	35.07	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo	210.14	$C_{11}H_{18}N_2O_2$	64	Cytotoxic activity (Narendhran et al., 2014) Antifungal (Hanif et al., 2017)
14	36.50	Eicosane	282.33	$C_{20}H_{42}$	99	Antifungal activity (Karanja et al., 2010) (Nandhini et al., 2015)
15	43.37	*bis*(2-ethylhexyl) ester of hexanedioic acid	370.31	$C_{22}H_{42}O_4$	91	Antimicrobial activity, Antioxidant, antiproliferative (Kadhim et al., 2017; Paramanantham & Murugesan, 2014)

4.5 Plant growth promoting endophytic actinomycetes

4.5.1 Screening of Indole-3-acetic acid production

Thirty-nine isolates (62.90%) were able to produce indole-3-acetic acid (IAA) more than 10.00 µg/mL of IAA (Table 19). In Group I *Streptomyces*, 24 isolates produced IAA ranging from 10.56±3.92 to 67.30±1.00 µg/mL. Isolate DR10-1 was closely related to *S. thermoviolaceus* subsp. *apingens* DSM 41392^{T} and produced the highest IAA (67.30±1.00 µg/mL), followed by DR9-7 closely related to *S. ardesiacus* NRRL B-1773^{T} and DR2-2 closely related to *S. antibioticus* NBRC 12838^{T} at 58.03±0.16 and 52.39±0.89 µg/mL, respectively. Eleven from twenty-one isolates of Group II *Micromonospora* produced between 18.22±0.84 and 58.53±15.75 µg/mL of IAA. Isolates CA1-5 and CA1-6 closely related to *M. humi* DSM 45647^{T} showed the highest IAA production (44.77±0.54-58.53±15.75 µg/mL) followed by isolate YG1-7 closely related to *M. chalcea* DSM 43026^{T} (30.91±0.80 µg/mL). Only one Group III *Streptosporangium*, DR9-9, closely related to *S. sandarakinum* GW-12028^{T} produced maximum IAA of 43.97±0.30 µg/mL (Table 10). Interestingly, Group VI *Pseudonocardia* exhibited the highest IAA production at 294.10±12.17 µg/mL. In this study, *Pseudonocardia* and *Streptomyces* showed the highest IAA production. Several previous studies demonstrated that IAA production of actinomycetes from different crops differed by species and strains. *Streptomyces* sp. En-1 from *Taxus chinensis* (Lin & Xu, 2013) and *S. rochei* ERY1 from *Eryngium foetidum* L. showed the ability to produce IAA (Suwitchayanon et al., 2018).

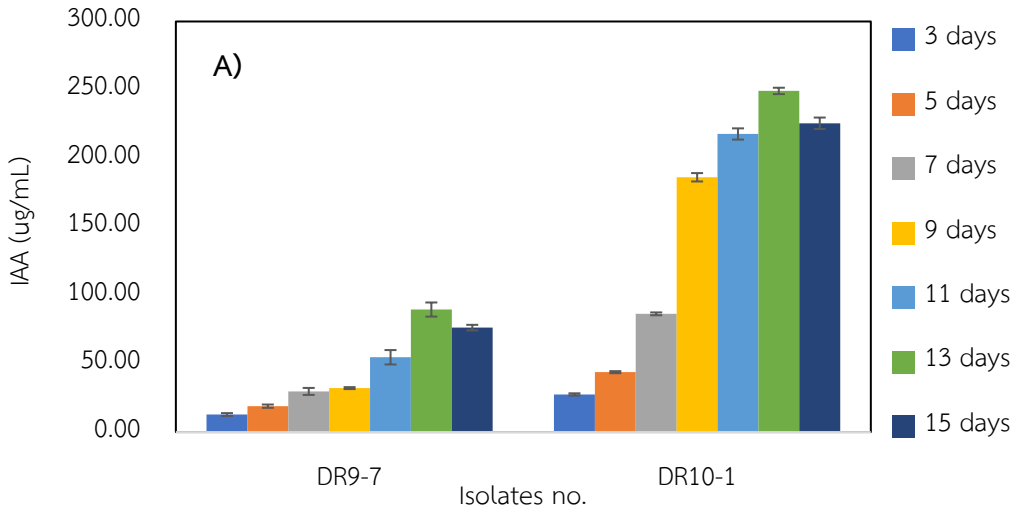
Table 19 IAA production of Group I *Streptomyces*, Group II *Micromonospora*, Group III *Streptosporangium* and IV *Actinomadura* isolates.

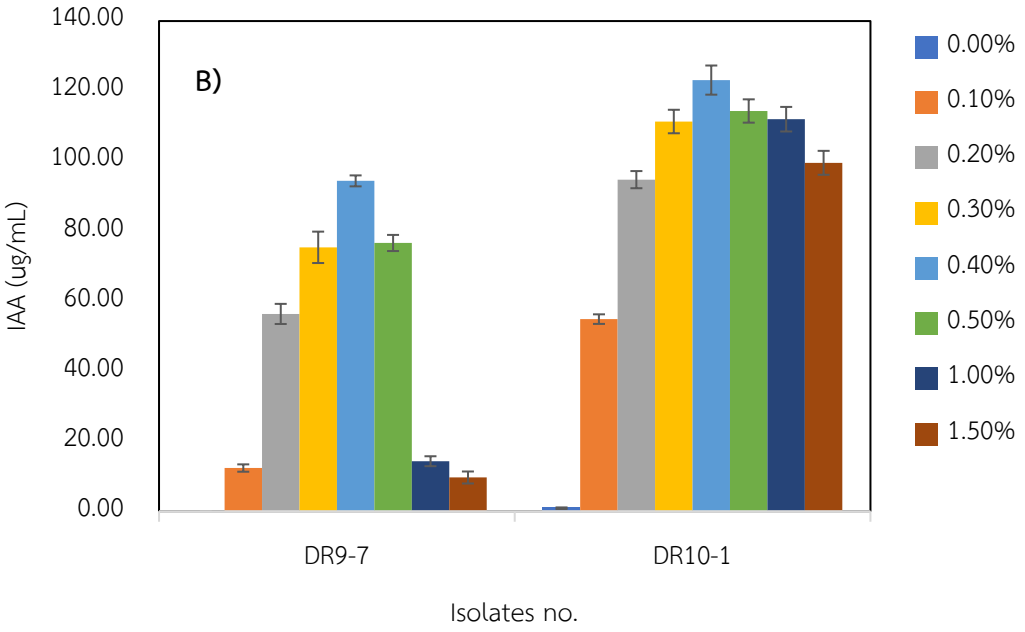
Group	Isolate no.	IAA (µg/mL)	Group	Isolate no.	IAA (µg/mL)
I	DR1-1	22.04 ± 0.89^{qr}	I	CL1-8	0.78 ± 0.24^{CD}
	DR2-3	52.36 ± 0.86^{de}		EO1-10	14.17 ± 0.33^{stuvw}
	DR2-2	52.39 ± 0.89^{de}		EO1-13	18.45 ± 0.37^{rst}
	DR2-4	41.19 ± 2.05^{jk}	II	DR4-1	29.13 ± 0.47^{mno}
	DR3-2	4.63 ± 0.61^{zABCD}		DR5-3	13.25 ± 1.86^{stuvw}
	DR3-4	12.14 ± 0.90^{tuvwx}		DR5-6	9.86 ± 1.45^{vwxy}
	DR3-5	19.67 ± 1.00^{rs}		DR6-7	38.82 ± 0.71^{lm}
	DR5-1	19.16 ± 3.10^{rs}		DR6-8	0.04±0.36E
	DR5-2	51.54 ± 0.84^{fg}		CR1-1	18.22 ± 0.84^{rst}
	DR7-2	6.09 ± 4.20^{yzABCD}		CR1-2	1.18 ± 0.40^{E}
	DR7-3	8.75 ± 4.02^{wxyz}		CA1-1	7.33 ± 0.84^{xyzAB}
	DR7-6	45.94 ± 0.67^{ghi}		CA1-2	6.16 ± 5.95^{xyzABCD}
	DR8-1	7.08 ± 5.60^{wxyz}		CA1-5	44.77 ± 0.54^{ijk}
	DR8-5	16.35 ± 4.74^{rstuv}		CA1-6	58.53 ± 15.75^{c}
	DR8-6	47.80 ± 1.12^{fgh}		CA1-8	25.97 ± 2.21^{pq}
	DR8-8	16.57 ± 7.58^{rstu}		CA1-9	21.54 ± 0.78^{qr}
	DR8-9	6.95 ± 0.65^{xyzABC}		CA1-10	26.60 ± 6.02^{pq}
	DR8-10	8.18 ± 1.03^{wxyzA}		YG1-1	1.63 ± 0.70^{BCD}
	CR1-8	10.56 ± 3.92^{uvwxy}		YG1-7	30.91 ± 0.80^{mn}
	CC1-1	10.59 ± 0.45^{uvwxy}		YG1-8	2.84 ± 0.73^{ABCD}
	CC1-3	11.92 ± 0.79^{tuvwx}		YG1-9	28.12 ± 4.97^{op}
	DR9-1	40.87 ± 11.90^{kl}		YG1-10	16.22 ± 0.84^{rstuv}
	DR9-4	32.61 ± 0.68^{mn}		EO1-6	2.77 ± 1.59^{DE}
	DR9-5	15.62 ± 0.72^{rstuv}		EO1-8	4.80 ± 0.54^{zABCD}
	DR9-7	58.03 ± 0.16^{cd}	III	DR9-9	43.97 ± 0.30^{ijk}
	DR10-1	67.30 ± 1.00^{b}		YG1-5	1.13 ± 0.90^{CD}
	DR10-2	47.84 ± 3.45^{fgh}	IV	CL1-5	14.48 ± 0.83^{stuvw}
	DR10-3	37.58 ± 13.34^{mn}	V	DR6-1	1.91 ± 0.50^{E}
	DR10-6	2.30 ± 0.16^{ABCD}		DR6-2	1.26 ± 0.12^{CD}
	DR10-8	6.47 ± 0.16^{yzABCD}		DR6-4	0.36 ± 0.10^{E}
	CL1-6	8.72 ± 0.28^{wxyz}	VI	DR1-2	294.10 ± 12.17^{a}

Different superscripts indicate significantly different ($P<0.05$) mean ± SD.

4.5.2 Optimization of IAA production of selected isolates

The two isolates DR9-7 and DR10-1 showed the highest IAA production after the screening was optimized at different factors. IAA production of isolates DR9-7 and DR10-1 started after 3 days and peaked at 106.32±2.04 and 298.12±4.19 µg/mL after 13 days (Figure 14A). *S. atrovirens* ASU14 exhibited the maximum IAA value when optimization at 30°C for 13 days (Abd-Alla et al., 2013). Maximum IAA production of the two isolates was observed in medium containing 0.4% L-tryptophan (Figure 14B). When the concentration of L-tryptophan increased from 0.1 to 0.4%, IAA production of DR9-7 and DR10-1 increased to maximum levels at 94.35±1.56 and 123.14±4.17%, respectively. Results indicated that different amounts of L-tryptophan had variable influence on IAA production, with tryptophan as an important element in increasing IAA production.

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IAA (ug/mL)
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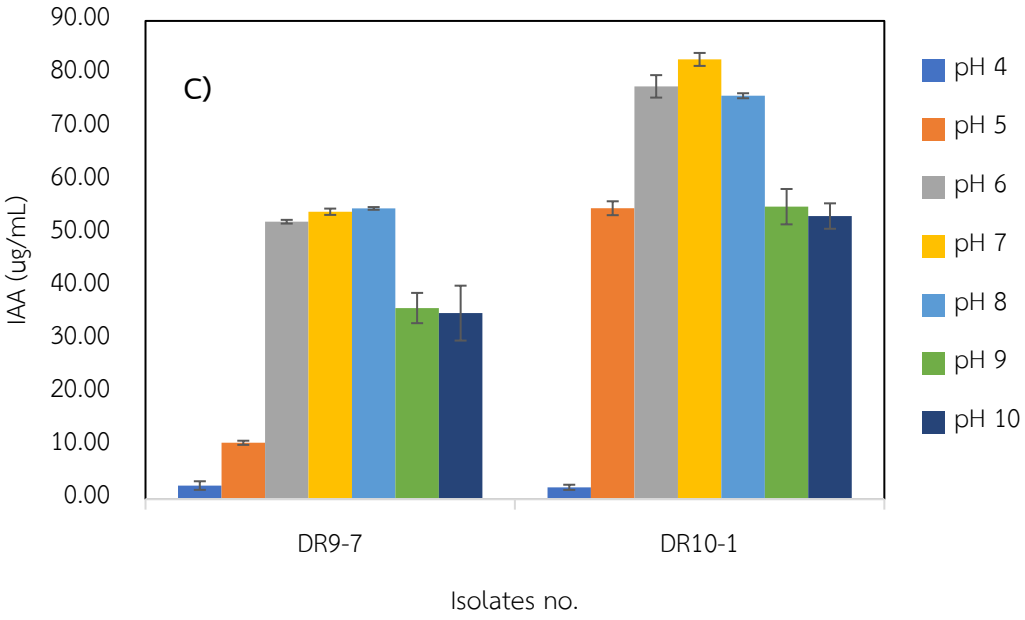
1.00%
1.50%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IAA (ug/mL)
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DR10-1
Isolates no.
pH 4
pH 5
pH 6
pH 7
pH 8
pH 9
pH 10

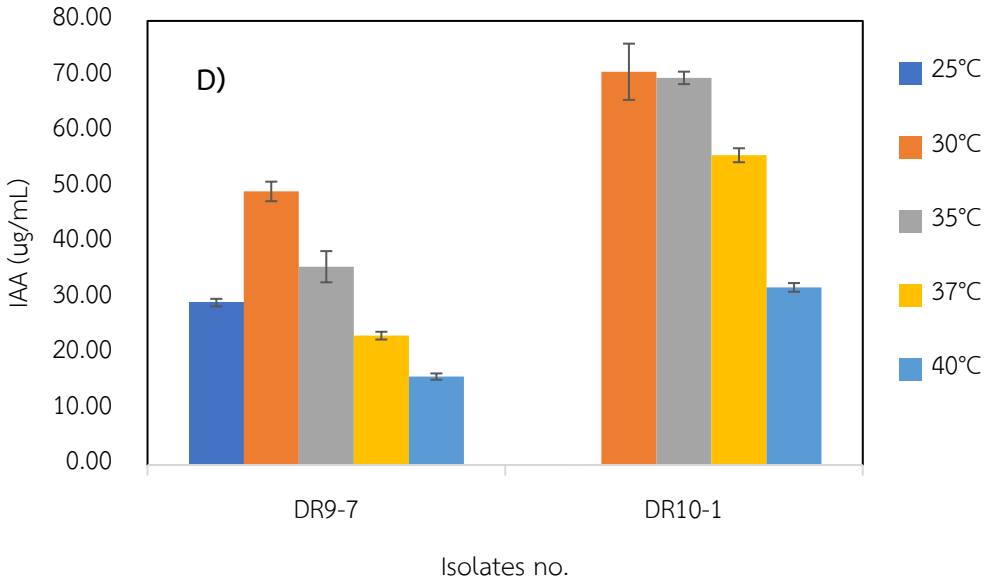


Figure 14 A) Effect of incubation time (day), B) Effect of L-tryptophan concentrations, C) Effect of pH, and D) Effect of temperature on IAA production by the selected isolates.
Vertical bars represent standard deviation from triplicate experiments.

Highest concentration of IAA was obtained from isolate DR10-1 at pH 7 (82.76 ±1.22 µg/mL) and DR9-7 cultivated at pH 7 and 8 at 54.08±0.59 and 54.71±0.25 µg/mL, respectively. IAA levels of DR9-1 and DR10-1 decreased when the pH value was less than 6 and greater than 8, (Figure 14C). *Streptomyces* and other actinomycete strains grew slowly in acidic or basic environments; therefore, pH levels are important for IAA synthesis (Shirokikh et al., 2007). Our findings concurred with Goudjal *et al*. (2013) (Goudjal et al., 2013), who showed that pH 7 was optimal for IAA production of *Streptomyces* sp. PT2 . Isolates DR9-7 and DR10-1 produced the highest IAA when grown at 30°C (Figure 14D). However, there was no significant change in IAA generation of DR10-1 at 35°C compared to 30°C. When temperature exceeded 30°C, IAA production of DR9-7 and DR10-1 decreased. A temperature of 30°C was found to be optimal for this investigation. *Streptomyces* sp. CMU H009 produced the largest IAA when cultivated at 30°C (Khamna et al., 2010). Accordingly, OFAT optimization experiments showed that the highest IAA production required

cultivation in ISP 2 broth with 0.4% L-tryptophan, pH 7 at 30°C for 13 days. Maximum IAA values of DR10-1 and DR9-7 were 284.87±8.24 and 132.35±9.39 µg/ mL, respectively.

4.5.3 Plant growth-promoting activity of the isolates

The effects of IAA in DR9-7 and DR10-1 supernatant cultures on rice seed germination, root length and shoot length were determined. Rice seeds soaked under various conditions exhibited significant differences in root lengths and quantity of roots compared to the controls (Table 20). Treatments with supernatant DR9-7 had the greatest influence on seedling root length, with no significant differences identified between supernatant DR10-1 and standard IAA. Supernatant of isolate DR10-1 showed the highest number of roots, whereas other treatments showed no significant differences. Fresh and dry weight of seedlings after treatment with DR9-7, DR10-1 and standard IAA were significantly different compared to the control. However, all treatments had no effect on seed germination. Our study results related to root growth of the host plants, as reported by Etesami *et al*. (2015) (Etesami et al., 2015). IAA-producing bacteria isolated from orchid rhizoplanes of *Dendrobium moschatum* (Tsavkelova et al., 2007) and *Cymbidium eburneum* (Faria et al., 2013) improved symbiotic seed germination. Our isolates indicated the presence of IAA production as a good option for use as plant growth enhancement in both economic and agricultural systems.

Table 20 Effect of isolates DR9-7 and DR10-1 on the growth of Rice (*Oryza sativa*).

Isolate	Growth parameters of actinomycetes treated rice					
	Root length (cm)	Shoot length (cm)	Number of roots	Seedling fresh weight (g)	Seedling dry weight (g)	% Seed germination
DR9-7	6.90±0.52^{a}	3.55±0.96^{c}	3.00±1.05^{b}	0.93±0.07^{a}	0.33±0.03^{a}	100
DR10-1	4.85±0.34^{b}	6.25±0.81^{a}	4.30±0.82^{a}	0.81±0.07^{a}	0.30±0.05^{ab}	100
IAA	4.85±0. 24^{b}	3.85±0.94^{c}	3.40±0.52^{b}	0.78±0.08^{a}	0.31±0.06^{ab}	100
Control	3.30±1.27^{c}	4.95±0.65^{b}	3.20±0.63^{b}	0.56±0.07^{b}	0.27±0.04^{b}	100

Different superscripts indicate significantly different ($P<0.05$) mean ± SD.

Endophytic actinomycetes are mainly distributed in the root system and xylem tissues of host plants. The major role of these bacteria is to improve the health and growth of the host plant by producing beneficial metabolites to control plant infections from pathogens and promote the growth of host plants. This study concluded that 34 *Streptomyces* isolates were associated with the roots of the Thai orchid species *D. christyanum*, *D. polyanthum*, *D. formosum*, *D. kentrophyllum*, *D. findlayanum*, *D. chrysanthum*, *C. cardioglossa*, *D. friedericksianum*, *D. chrysotoxum*, *C. lawrenceana, C. rostratum* and *E. ornate*. Twenty-one *Micromonospora* isolates were found in the seven orchids, *D. crumenatum*, *D. kentrophyllum, D. heterocarpum*, *C. rostratum*, *C. assamica*, *P. globulifera* and *E. ornate*. Two *Streptosporangium* isolates were distributed in the two orchids *D. friedericksianum* and *P. globulifera*; one *Actinomadura* isolate was found in *Coelogyne lawrenceana*; three *Amycolatopsis* were isolates from *D. heterocarpum*, and one *Pseudonocardia* isolate was found in *D. christyanum*. Our *Streptomyces, Micromonospora* and *Streptosporangium* isolates exhibited significant antimicrobial activities against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231. Only *Streptomyces antibioticus* DR2-2 inhibited all tested pathogens, while two endophyte isolates DR10-1 and DR9-7 showed high IAA activity that promoted the number of roots, shoot length, root length and fresh weight of rice seedlings. This is the first report on the diversity, antimicrobial and plant-growth-promoting properties of endophytic actinomycetes associated with the root of Thai orchids.

4.6 Genome analysis and plant growth promoting activity of *Pseudonocardia* strain DR1-2 from the root of *Dendrobium christyanum*

4.6.1 Isolation and identification of isolates

Strain DR1-2 was recovered from the roots of Thai orchid, *Dendrobium christyanum*. The strain was aerobic and stained Gram-positive, with yellowish white aerial mycelium and deep orange yellow substrate mycelium on ISP 2 agar after 7 days incubation. The strain grew very well on all media. Scanning electron micrograph of strain DR1-2 revealed the aerial mycelium fragmented into rod-shaped spores and the spore surface was smooth (Figure 15). The optimum temperature of all strains was 30°C and pH range 5-10. The strain utilized various sugars and nitrogen for growth and grew in a range of 5-10 % NaCl concentrations. The cultural, physiological, and biochemical characteristics of the strain are shown Table 21.

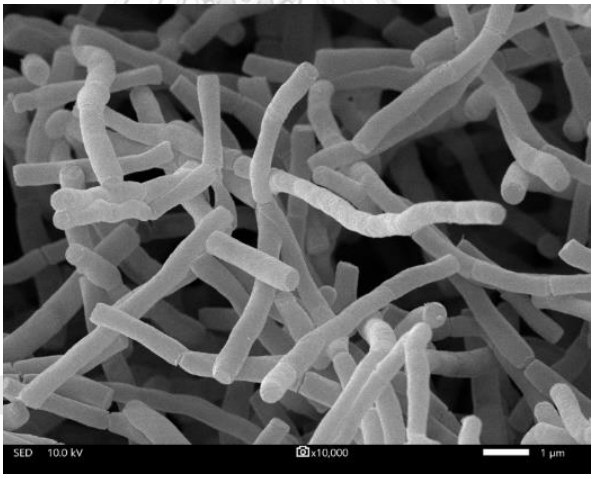


Figure 15 Scanning electron micrograph of strain DR1-2.

Table 21 Phenotypic characteristics of the strain DR1-2.

Characteristics	Results	Characteristics	Results
Growth	Filamentous aerial	**Carbon source utilization:**	
Diffusible pigment production	-	Fructose	++
Growth temperature range	25 °C to 37 °C	Glycerol	++
Growth optimal temperature	30 °C	Raffinose	++
Growth pH range	5-10	Mannose	++
Optimal pH for growth	7.0	Mannitol	++
Production of H_2S	-	Xylose	++
NaCl tolerant	2% to 10%	Cellobiose	++
Peptonization	-	Galactose	++
Coagulation	Negative	Maltose	++
Starch hydrolysis	Negative	Melezitose	++
Gelatin liquefaction	-	Lactose	++
Nitrate reduction	Positive	*myo*-inositol	++
Colour of aerial mycelium	**Colour of substrate mycelium**	Rhamnose	++
Yellowish white on ISP 2	Deep orange yellow on ISP 2	Sucrose	++
Yellowish white on ISP 3	Moderate yellow ISP 3	Melibiose	++
Pale yellow on ISP 4	Light olive brown ISP 4	**Nitrogen source utilization**:	
Yellowish white on ISP 5	Moderate orange yellow ISP 5	L-Arginine	+
Yellowish white on ISP 6	Strong yellowish brown ISP 6	l-Asparagine	+
Yellowish white on ISP 7	Deep orange yellow ISP 7	l-Proline	++
Yellowish white on NA	Deep orange yellow on NA	l-Valine	+
		l-Cysteine	+
		l-Tyrosine	++
		l-Arabinose	++

All grew very good on all media.

The pairwise alignment of DR1-2 showed relatively high 16S rRNA gene similarity to strains of *P. alni* DSM 44104^T (99.93%), *P. antarctica* DSM 44749^T (99.93%) and *P. carboxydivorans* Y8^T (100%). The phylogenetic tree based on the neighbour-joining algorithm, the strain DR1-2, and the three closest relatives were placed in the same position (Figure 16). The 16S rRNA gene sequences of this strain has been deposited in NCBI database, with accession numbers LC705538.

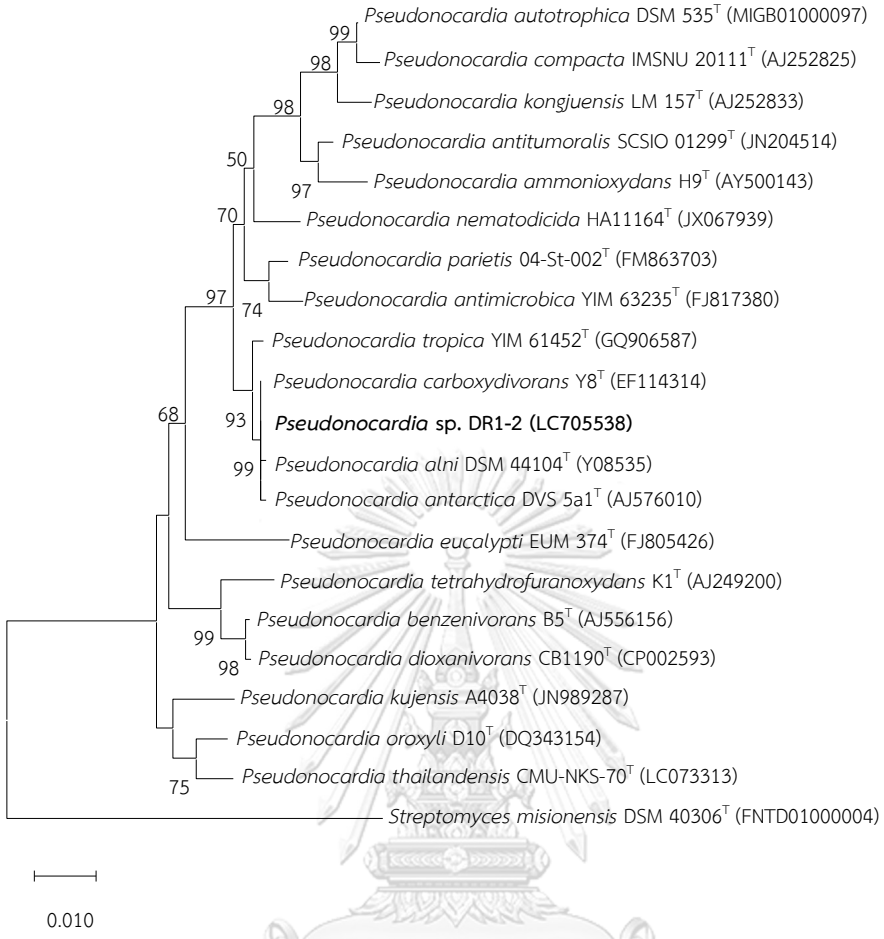


Figure 16 Phylogenetic tree of strain DR1-2 based on 16S rRNA sequences using the neighbor-joining method. Bootstrap percentages at nodes were calculated with 1,000 replicates.

4.6.2 Determination of plant growth promoting activities

Strain DR1-2 was evaluated for plant growth promoting activities, including phytohormone, siderophore, ammonia production, zinc, and phosphate solubilization. IAA production by DR1-2 was determined on ISP 2 broth 0.2% L-tryptophan, pH 7.0 at 30°C with 180 rpm shaking for 7 days. IAA production obtained was 294.10±12.17 µg/mL. The strain was able to dissolve phosphate in plates by producing a clear halo around the colony on Pikovskaya medium (2.20±0.08) but lacked zinc solubilizing ability in all of the sources of insoluble zinc. Strain DR1-2 was

positive for ammonia production at levels 36.99±2.24 µg/mL. Siderophore production was detected on CAS agar media by forming a clear orange halo zone around the colonies. Our results concurred with Borah and Thakur (2020), who discovered *P. carboxydivorans* T1LA3, an endophytic actinobacteria isolated from *Camellia sinensis* L. showed IAA, ammonia and siderophore production (Borah & Thakur, 2020).

4.6.3 Extraction, purification, and detection of IAA

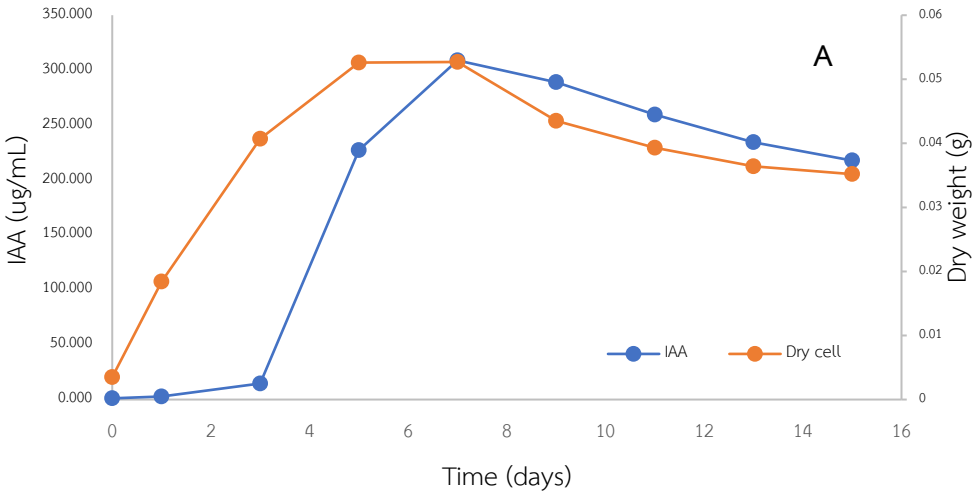
The crude extract of IAA of strain DR1-2 was extracted using ethyl acetate, and partial purification of IAA by C18 SPE column concentrated and then was detected on the TLC plate. The chromatograms were examined in both visible and ultraviolet light (254 nm). Pink-colored spots were observed after spraying with Salkowski's reagent, with an R_f value of 0.52, identical to the standard IAA (Figure 17). The results show that the purified compound was identified as IAA using thin-layer chromatography and compared to the R_f value of the standard IAA.

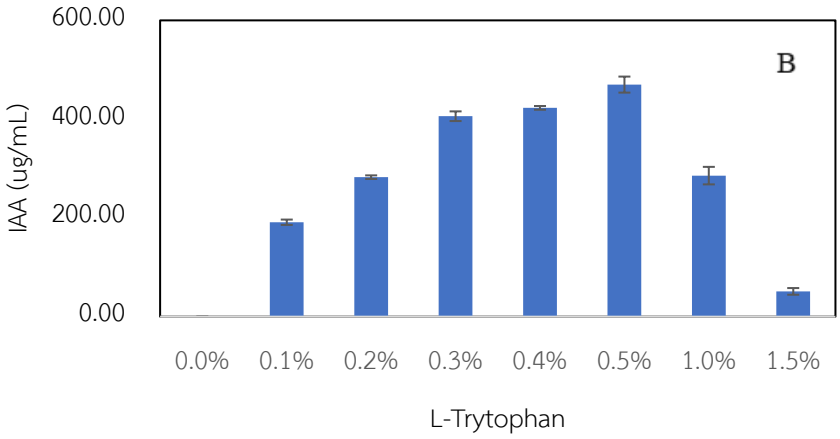
Figure 17 Confirmation of IAA production by strain DR1-2 using thin-layer chromatography.

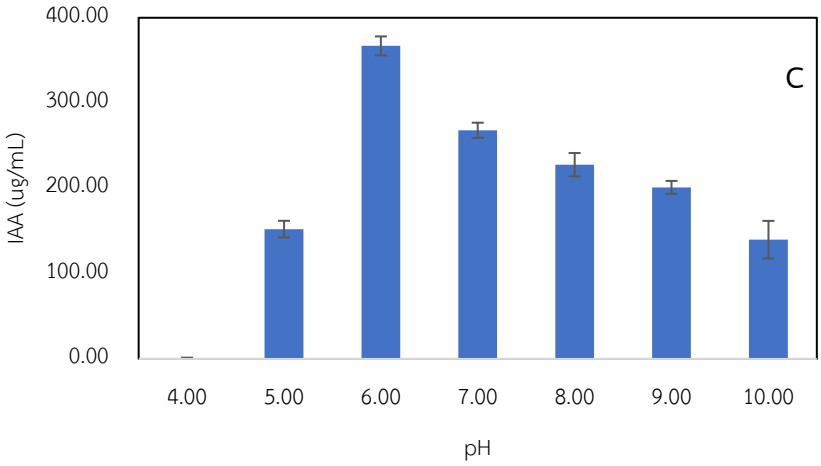
4.6.4 Optimization of IAA production

The mycelial growth and IAA formation by strain DR1-2 were estimated at 24 h intervals during the incubation period of 15 days. The mycelial growth was increased gradually with the incubation period reaching a maximum of 5-7 days. Also, IAA formation increased, reaching a maximum at 7 days of incubation and then decreasing slowly (Figure 18A). The reduction of IAA might be due to the release of IAA degrading enzymes such IAA oxidase and peroxidase, which degrade IAA (Datta & Basu, 2000). Maximum IAA production of the DR1-2 was observed in a medium containing 0.5% L-tryptophan (Figure 18B). When the concentration of L-tryptophan increased from 0.1 to 0.5%, IAA production increased to maximum levels at 469.98±16.25% µg/mL. A higher concentration of L-tryptophane above 0.5% decreased IAA production. Results indicated that different amounts of L-tryptophan had a variable influence on IAA production, with tryptophan as an essential element in increasing IAA production.

Strain DR1-2 had the highest concentration of IAA at pH 6, (367.06 ±11.09 g/mL). IAA levels of DR1-2 decreased when the pH value was less than 6 and greater than 7, (Figure 18C). The pH has an impact on the function of enzyme systems as well as the solubility of a variety of chemicals required for bacterial growth. *Streptomyces* and other actinomycete strains grew slowly in acidic or basic conditions because pH levels are important for IAA synthesis (Shirokikh et al., 2007). Different temperatures for IAA production are shown in Figure 18D. The optimum temperature for IAA production by DR1-2 was at 30°C at 278.84±3.76 µg/mL; when the temperature exceeded 30°C, IAA production decreased. A temperature of 30°C was found to be optimal for this investigation. This result is related to *Streptomyces* sp. CMU H009 produced the largest IAA when cultivated at 30°C (Khamna et al., 2010). Accordingly, OFAT optimization experiments showed that the highest IAA production required cultivation in ISP 2 broth with 0.5% L-tryptophan, pH 6 at 30°C for 7 days. The maximum IAA value of DR1-2 was 489.73±8.90 µg/ mL, which increased almost equal IAA production after optimization.


A
IAA (ug/mL)
Dry weight (g)
Time (days)
IAA
Dry cell
350.000
300.000
250.000
200.000
150.000
100.000
50.000
0.000
0.06
0.05
0.04
0.03
0.02
0.01
0
0
2
4
6
8
10
12
14
16


B
IAA (ug/mL)
600.00
400.00
200.00
0.00
0.0%
0.1%
0.2%
0.3%
0.4%
0.5%
1.0%
1.5%
L-Trytophan


C
IAA (ug/mL)
400.00
300.00
200.00
100.00
0.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
pH

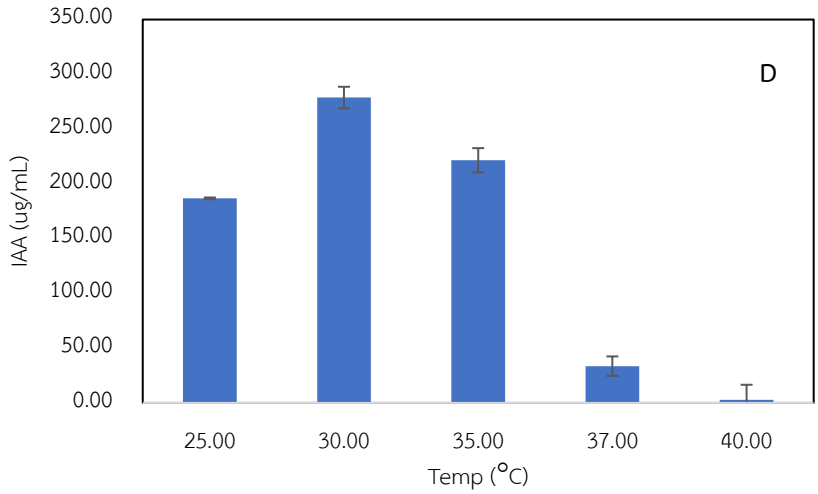


Figure 18 Effect of incubation time (day), A; effect of L-tryptophan concentrations, B; effect of pH, C and effect of temperature, D on IAA production by the selected isolates.

Vertical bars represent standard deviation from triplicate experiments.

4.6.5 Growth-promoting activity of DR1-2 on Rice

The effects of IAA purified from DR1-2 on rice seed germination, root length, and shoot length were determined. Rice seeds soaked under various conditions exhibited significant differences in root lengths, shoot lengths, and quantity of roots compared to the controls (Table 22). Treatments with IAA from DR1-2 had a significant influence on the quality of seedling root, with no significant differences identified between standard IAA. The fresh and dry weight of seedlings after treatment with IAA produced by DR1-2 and standard IAA were significantly different from the control. However, all treatments did not affect seed germination. Endophytic actinomycetes utilize root exudates to generate a variety of plant compounds. IAA from DR1-2 resulted in an increase in shoot length, root length, and the number of roots, showing that DR1-2 can produce plant growth regulators such as IAA. Our study results related to screening bacteria for plant growth promoting agents on rice seedling growth as reported by Etesami *et al*. (2015) (Etesami et al., 2015) and IAA-producing bacteria isolated from orchid rhizoplanes of *Dendrobium*

moschatum (Tsavkelova et al., 2007) and *Cymbidium eburneum* (Faria et al., 2013) also have been reported to improve plant seed germination. Our isolates indicated the presence of IAA production as a good option for use as plant growth enhancement in both economic and agricultural systems.

Table 22 Effect of strain DR1-2 on the growth of rice (*Oryza sativa*).

Isolate	Growth parameters of actinomycetes treated rice					
	Root length (cm)	Shoot length (cm)	Number of roots	Seedling fresh weight (g)	Seedling dry weight (g)	% Seed germination
DR1-2	5.45±0.64[a]	5.90±0.83[a]	3.30±0.67[a]	0.81±0.07[a]	0.30±0.05[a]	100
IAA	5.10±0.52[a]	5.25±0.68[ab]	3.80±0.79[a]	0.80±0.08[a]	0.30±0.06[a]	100
Control	4.40±0.66[b]	4.90±0.74[b]	2.40±0.52[b]	0.56±0.08[b]	0.25±0.03[b]	100

Different superscripts indicate significantly different ($P<0.05$) mean ± SD.

4.6.6 Genome sequence analysis

Genome analysis of strain DR1-2 revealed the size of 6,077,423 bp distributed in 23 contigs with G+C content of 74.6 %. The phylogenetic analysis based on whole-genome sequences indicated that strain DR1-2 was phylogenetically closely related to *P. alni* DSM 44104^T and *P. antarctica* DSM 44749^T. The ANIb and ANIm values of the draft genomes between strain DR1-2, *P. alni* DSM 44104^T and *P. antarctica* DSM 44749^T were 95.91 and 97.12 %, and 95.81 and 97.25 %, respectively (Table 23). The digital DNA-DNA hybridization (dDDH) values between strain DR1-2 and its closest strains, *P. alni* DSM 44104^T and *P. antarctica* DSM 44749^T were 72.60 and 74.00 %, respectively (Table 5.3). For genome comparison, ANI and dDDH values are considered well correlated when the values were ≥95 % (ANI) and ≥70% (dDDH), respectively (Fitch, 1971) (Seemann, 2014). Since the dDDH and the ANI values between strain DR1-2 and its closest strains were higher than the species cut off, therefore, strain DR1-2 should be the same species with *P. alni*, *P. antarctica* and *P. carboxydivorans*. Further taxonomic study on *P. antarctica* and *P. carboxydivorans* should be described.

Table 23 Genome statistics, ANIb, ANIm and dDDH values among the draft genomes of strain DR1-2 and closely related strains.

Strains: 1, DR1-2; 2, *P. alni* DSM 44104^{T}; 3. *P. antarctica* DSM 44749^{T}. The genome of type strains was obtained from GenBank.

Genome of	Features				
	Accession no.	Genome size (bp)	G+C content (%)	No. of Contigs	Protein coding genes
1	JAMQOF000000000	6,077,423	74.6	23	5834
2	PHUJ01000000	5,994,807	74.2	3	5777
3	JACCCZ000000000	6,242,493	74.1	2	6015
Genome of	Reference genomes	ANIb%	ANIm%	%dDDH (formula2[a])	Prob. DDH>=70%
1	2	95.91	97.12	72.60%	82.51%
1	3	95.81	97.25	74.00%	84.49%

[a]Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genomes.

4.6.7 Gene function annotation and secondary metabolism gene clusters

The draft genome of DR1-2 has 5,834 protein coding sequences (CDS), 51 transfer RNA (tRNA) genes, and 3 ribosomal RNA (rRNA) genes. The annotation included 2,293 hypothetical proteins and 3,541 proteins with functional assignments. The proteins with functional assignments included 1,246 proteins with Enzyme Commission (EC) numbers (Schomburg et al., 2004), 1,091 with Gene Ontology (GO) assignments (Ashburner et al., 2000), and 983 proteins that were mapped to KEGG pathways (Kanehisa et al., 2016). RAST server annotation of the whole genome describes the subsystem distribution of strain DR1-2. Among the subsystem categories were genes for amino acids and derivatives (390 ORFs), carbohydrate metabolism (344 ORFs), cofactors, vitamin, prosthetic groups, pigment (230), fatty acids, lipids, and isoprenoids (198 ORFs), and protein metabolism (178 ORFs). It also had nucleosides and nucleotides (93), respiration (97), DNA metabolism (97), and stress response genes (51) Figure 19.

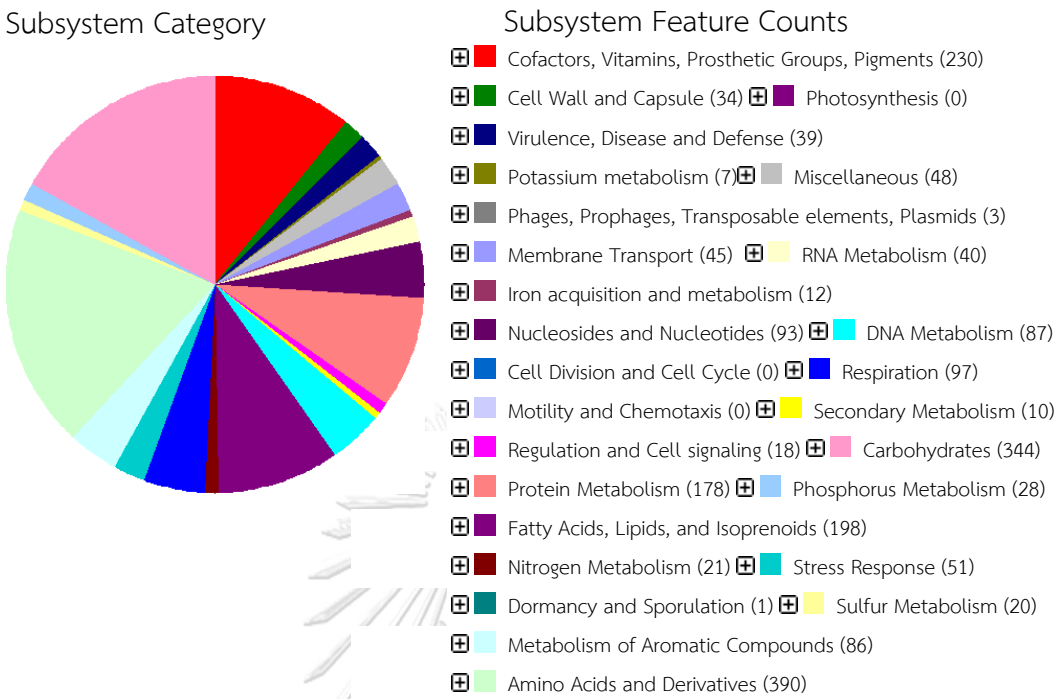


Figure 19 Subsystems distribution statistic of strain DR1-2 based on RAST annotation server.

For phytohormone IAA production, the gene associated to the tryptophan synthase alpha and beta chain (EC 4.2.1.20), which catalyzes the final stage in tryptophan biosynthesis, was discovered. The gene encoding enzymes, aromatic-L-amino-acid decarboxylase (EC 4.1.1.28), anthranilate phosphoribosyl transferase (EC 2.4.2.18), monoamine oxidase (1.4.3.4), and phosphoribosylanthranilate isomerase (EC 5.3.1.24) involved in auxin biosynthetic pathways was also found in strain DR1-2. PATRIC annotation revealed *iaaM* gene in genome DR1-2 that encodes the enzyme tryptophan-2-monooxygenase, which converts tryptophan to the indole-3-acetamide (IAM), intermedia in IAM pathway. In bacteria, the IAM pathway is the best characterized pathway (Sekine et al., 1989) and has been suggested in bacteria by converting exogenous tryptophan to IAA (Perley & Stowe, 1966). The draft genome of DR1-2 was also found mevalonic acid pathway (MVA) and methylerythritol phosphate pathway (MEP), which produced a class of plant growth regulators, brassinosteroids

(BRs) and Gibberellins (GAs), respectively. Results indicated that DR1-2 might be a promising source of plant hormones for agricultural applications.

The draft genome of strain DR1-2 was determined using the antiSMASH server to detect putative biosynthetic gene clusters (BGCs). The 12 gene clusters were observed on DR1-2 genomes related to various BGCs, mainly non-alpha poly-amino acids like e-Polylysin (NAPPA), terpene, and ectoine (Table 24). The secondary metabolite biosynthetic gene clusters (smBGCs) exhibited similar genetic relatedness to the known clusters producing stenothricin (Liu et al., 2014), lankacidin C (Ahsan et al., 2017) and amychelin (Xie et al., 2020) have antimicrobial activity. Strain DR1-2 is predicted to produce anticancer, antitumor, antioxidant agents such as SF2575 (Pickens et al., 2009), CC-1-65 (Cacciari et al., 2000) and isorenieratene (Chen et al., 2019), respectively. In addition, ectoine was predicted in DR1-2, which protects proteins and cellular membranes against damage caused by severe environments like as heat, UV light, strong osmolarity, or dryness (Bilstein et al., 2021) (Richter et al., 2019). Thus, strain DR1-2 might be one of the sources of biological compounds used in pharmaceutical applications. Moreover, the predicted secondary metabolites, streptobactin (Matsuo et al., 2011) and amychelin (Seyedsayamdost et al., 2011), can be used as siderophore, compounds that have a high-affinity iron-chelating ability in agricultural farming. In addition, the genome of strain DR1-2 contained four smBGCs that displayed no similarity to any known smBGCs in antiSMASH (Table 24). These results suggested that strain DR1-2 might be a source of novel secondary metabolites.

Table 24 Biosynthetic gene clusters (BGCs) and secondary metabolites of strain DR1-2 based on the analysis of genome mining with AntiSMASH 5.0.

Region	Type	Most similar known cluster
Region 1	NAPAA	Stenothricin
Region 2	Terpene	SF2575
Region 3	NAPAA	Streptobactin
Region 4	Ectoine	Ectoine
Region 5	NAPAA	CC-1065
Region 6	RiPP-like	-
Region 7	NAPAA, T1PKS, NRPS	-
Region 8	Terpene	Isorenieratene
Region 9	Redox-cofactor	Lankacidin C
Region 10	Ranthipeptide	-
Region 11	RiPP-like	-
Region 12	NRPS, T1PKS, NRPS-like	Amychelin

NAPAA-non-alpha poly-amino acids like e-Polylysin; RiPP-like-Other unspecified ribosomally synthesized; NRPS-Nonribosomal peptide synthetases; NRPS-like-NRPS-like fragment; T1PKS-type 1 polyketide synthetases;

In this study, strain DR1-2 associated with roots of *D. christyanum* orchid was closely related to *Pesudonocardia carboxydivorans*. The strain showed high IAA, ammonia, siderophore production and phosphate solubilizing activity. This strain could promote the number of roots, shoot length, root length and fresh weight of rice seedlings. The draft genome sequence analysis of strain DR1-2 indicated that gene clusters are involved in the plant hormone biosynthesis. This strain will be helpful as phytohormone producing bacteria for seed germination and plant growth improvement.

CHAPTER VI

CONCLUSION

Thai orchids were found to be the interesting source for the actinomycete isolation. In this study, total 62 actinomycetes were isolated from Thai orchids. On the basis of morphology, chemotaxonomy and 16S rRNA gene sequence analysis, they classified in 4 families (Family *Streptomycetaceae*, *Micromonosporaceae, Thermomonosporaceae, Streptosporangiaceae* and *Pseudonocardiaceae*) including 6 genera *Streptomyces* (34 isolates), *Micromonospora* (21 isolate), *Streptosporangium* (2 isolates), *Actinomadura* (1 isolates), *Amycolatopsis* (3 isolates) and *Pseudonocardia* (1 isolates). On the basis of polyphasic approach, strain DR6-1^{T}, new actinomycetes species was proposed name *Amycolatopsis dendrobii*.

The primary antimicrobial activity screening revealed that *Streptomyces* (18 isolates), *Micromonospora* (12 isolates) and *Streptosporangium* (1 isolate) exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231. Only *Streptomyces antibioticus* DR2-2 inhibited all tested pathogens. Based on this study, the production media and strains were the main factor that influenced the antimicrobial activity.

Strains DR5-1, DR7-3, DR8-5, and DR8-8 showed inhibitory activity against against five phytopathogenic fungi including *Fusarium oxysporum*, *Fusarium solani, Alternaria alternata, Curcuria oryzae*, and *C. gloeosporioides.* Strain DR7-3 exhibited a broad-spectrum antifungal activity against five fungi that are causal agents of plant diseases. The ethyl acetate extract from this strain showed a high level of inhibition against *C. oryzae* SA04, compared with a standard chemical fungicide. Moreover, it suppressed mycelial growth and damaged the cell structure of the fungi. Four chemical components with antifungal activity were identified from the extract using the gas chromatography-mass spectrometric (GC-MS) technique. The draft genome sequence analysis of strain DR7-3 indicated that thirteen gene clusters are involved in

the biosynthesis of these antifungal metabolites. This strain appears to be a promising source for developing new antifungal agents against phytopathogenic fungi.

Screening of IAA production show that the ranged of IAA from 0.04±0.36 to 294.10±12.17 µg/mL. DR1-2, DR10-1 and DR9-7 that produced high amounts of IAA were selected for optimization. Maximum IAA values of DR10-1 and DR9-7 were 284.87±8.24 and 132.35±9.39 µg/mL, using 0.4% L-tryptophan, pH 7 with incubation at 30°C for 13 days. DR1-2 showed maximum IAA of 489.73±8.90 µg/ mL, when optimized using 0.5% L-tryptophan, pH 6, with incubation at 30°C for 7 days. This strain exhibited phosphate solubilization (2.20±0.08 solubilization Index, SI), positive siderophore production, and ammonia production (36.99±2.24 µg/ mL). These three isolates promoted root length, shoot length, number of roots and fresh weight of rice seedlings (*Oryza sativa* L. cv. RD49) compared to the control. These strains will be helpful as phytohormone producing bacteria for seed germination and plant growth improvement.

Based on this study, the endophytic actinobacteria associated with the orchid is a promising a new actinomycete species and might be one of the sources of biological compounds used in pharmaceutical and agricultural applications.

APPENDIX A

CULTURE MEDIA

Almost of media were sterilized in autoclave at 121 °C, 15 pounds pressure for 15 min, except skim milk and media for carbon utilization test that were sterilized in autoclave at 110 °C, 15 pounds pressure for 10 min. All media were prepared in 100 mL of distilled water.

1. Gause synthetic No.1 medium

Soluble starch	2.0	g
KNO_3	1.0	g
NaCl	0.05	g
$MgSO_4 \cdot 7H_2O$	0.05	g
K_2HPO_4	0.05	g
$FeSO_4 \cdot 7H_2O$	0.01	g
Gellan gum	1.0	g

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

2. Starch-casein gellan gum medium

Soluble starch	1.0	g
Sodium caseinate	0.03	g
KNO_3	0.2	g
NaCl	0.2	g
K_2HPO_4	0.2	g
$MgSO_4 \cdot 7H_2O$	0.005	g
$CaCO_3$	0.002	g
$FeSO_4 \cdot 7H_2O$	0.001	g
Gellan gum	1.0	g

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

3. Glycerol asparagine gellan gum medium

Glycerol	1.0	g
L-asparagine	0.1	g
K_2HPO_4	0.1	g
NaCl	0.1	g
$MgSO_4 \cdot 7H_2O$	0.1	g
NaCl	0.1	g
Gellan gum	1.0	g

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

4. Gellan gum medium

Gellan gum	1.5	g
$CaCO_3$	0.3	g
$CoCl_2.6H_2O$	0.001	g
$MgSO_4 \cdot 7H_2O$	0.001	g

5. 301 Seed medium

Soluble starch	2.4	g
Glucose	0.1	g
Peptone	0.3	g
Meat extract	0.3	g
Yeast extract	0.5	g
$CaCO_3$	0.4	g

6. Yeast extract-glucose broth

Yeast extract	1.0	g
Glucose	1.0	g

7. Production medium no. 57

Glucose	2.0	g
Peptone	0.5	g
Dry yeast	1.0	g
Meat extract	0.3	g
NaCl	0.3	g
$CaCO_3$	0.3	g

8. Yeast extract-malt extract agar (ISP no.2) plus $CaCO_3$

Yeast extract	0.4	g
Malt extract	0.1	g
Glucose (dextrose)	0.4	g
$CaCO_3$	0.4	g
agar	1.5	g

9. Production medium no. 30

Soluble starch	2.4	g
Glucose	0.1	g
Peptone	0.3	g
Meat extract	0.3	g
Yeast extract	0.5	g
$CaCO_3$	0.4	g

Trace salts solution A

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

10. Yeast extract-malt extract agar (ISP no.2), pH 7.2 ± 0.2

Yeast extract	0.4	g
Malt extract	0.1	g
Glucose (dextrose)	0.4	g
Agar	1.5	g

11. Oatmeal agar (ISP no.3), pH 7.2 ± 0.2

oatmeal	2.0	g
Agar	1.5	g

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

Soluble starch	0.1	g
K_2HPO_4	0.1	g
$MgSO_4 \cdot 7H_2O$	0.1	g
NaCl	0.1	g
$(NH_4)_2SO_4$	0.2	g
$CaCO_3$	0.2	g
Trace salts solution A	0.1	mL
Agar	1.5	g

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

Glycerol	1.0	g
L-Asparagine	0.1	g
K_2HPO_4	0.1	g
Trace salts solution A	0.1	mL
Agar	1.5	g

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

Peptone Iron agar, dehydrated	0.36	g
Yeast extract	0.01	g

Agar	1.5	g

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

Glycerol	1.5	g
L-Tyrosine	0.05	g
L-Asparagine	1.0	g
K_2HPO_4	0.05	g
$MgSO_4 \cdot 7H_2O$	0.05	g
NaCl	0.05	g
$FeSO_4 \cdot 7H_2O$	0.01	g
Trace salts solution A	0.1	mL
Agar	1.5	g

Trace salts solution A

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

16. Nutrient agar (NA), pH 7.2 ± 0.2

Meat extract	1.0	g
Peptone	1.0	g
NaCl	0.1	g
Agar	1.5	g

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

Carbohydrate	1.0	g
$(NH_4)_2SO_4$	0.264	g
$K_2HPO_4 \cdot 3H_2O$	0.565	g
KH_2PO_4 anhydrous	0.238	g
$MgSO_4 \cdot 7H_2O$	0.1	g

Trace salts solution B	0.1	mL
Agar	1.5	g

Trace salts solution B (Pridham and Gottlieb trace salts)

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

18. Gelatin broth, pH 7.2 -7.8

Peptone	1.0	g
Meat extract	0.5	g
NaCl	0.5	g
Gelatin	15	g

19. Peptone KNO_3 broth, pH 7.2 -7.8

Peptone	1.0	g
KNO_3	0.1	g
NaCl	0.5	g

20. 10% Skim milk

Skim milk	10	g

To sterilize at 110 °C for 10 min

21. Mueller-Hinton agar (Difco)

Beef infusion form	3.0	g
Casamino acids, technical	1.75	g
Starch	0.15	g
Agar	1.5	g

22. Potato dextrose agar (Difco)

Potato	20.0	g
Glucose (dextrose)	4.0	g
Agar	1.5	g

23. Pikovskaya (PVK) medium

Glucose	10	g
$Ca_3(PO_4)$	2.5	g
$(NH_4)_2SO_4$	0.5	g
NaCl	0.2	g
$MgSO_4 \cdot 7H_2O$	0.1	g
KCl	0.2	g
Yeast extract	0.5	g
$MnSO_4 \cdot H_2O$	0.002	g
$FeSO_4 \cdot 7H_2O$	0.002	g

APPENDIX B

REAGENTS AND BUFFER

1. Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid100	100	mL

To dissolve by gentle heating in fume hood

N, N-dimethyl-1-naphthylamine solution

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

To dissolve by gentle heating in fume hood.

2. 2 N H_2SO_4

conc. H_2SO_4	2.0	mL
distilled water	34	mL

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

3. 6 N HCl

conc. HCl	50	mL
Distilled water	50	mL

To add conc. HCl (12 N) into the distilled water for cell hydrolysis process in the diaminopimelic acid analysis.

4. Dittmer & Lester reagent

MoO_3	4.011	g
25 N H_2SO_4	100	mL
Molybdenum powder	0.178	g

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

5. Anisaldehyde reagent

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

6. Ninhydrin solution

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

7. Dragendroff's reagent

Solution A

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

Solution B

KI	40	g
Distilled water	100	mL

To mix solution A (10 mL), solution B (10 mL) and acetic acid (10 mL), before use. Reagents for fatty acid analysis

8. Phenol: Chloroform (1: 1, v/v)

Crystalline phenol (melted)	50	mL
Chloroform	50	mL

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

9. 0.2 M Tris-HCl buffer, pH 8.5

Tris	24.22	g
Distilled water	700	mL

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

10. DNA extraction buffer (Grind method)

0.2 M Tris-HCl buffer, pH 8.5	900	mL
NaCl	14.61	g
EDTA2Na	9.31	g
SDS	5	g

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

11. 3 M NaCl

NaCl	17.55	g
Distilled water	100	mL

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

12. RNase A solution

RNase A	20	mg
0.15 M NaCl	10	mL

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

13. 0.1 M Tris-HCl buffer, pH 7.5

Tris	1.2	g
Distilled water	70	mL

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

14. 20 x SSC solution

NaCl	175.3	g
$Na_3C_6H_5O_7$	88.2	g
Distilled water	700	mL

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

15. TE buffer

10 mM Tris HCl, pH 8.0	10	mL
1 m M Na2-EDTA, pH 8.0	10	mL
Distilled water	980	mL

To autoclave at 121 °C, 15 lb/in^2 for 15 minutes.

16. Salkowski reagent

0.5 M Ferric chloride ($FeCl_3$)	1.0	mL
35% Perchloric acid ($HClO_4$)	49	mL

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