IDENTIFICATION AND APPLICATION IN PLANT OF PLANT GROWTH-PROMOTING ENDOPHYTIC BACTERIA

Miss Kanchana Sitlaothaworn

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การระบุเอกลักษณ์และการประยุกต์ในพืชของแบคทีเรียเอนโดไฟท์ที่ส่งเสริมการเจริญของพืช

น.ส.กานต์ชนา สิทธิ์เหล่าถาวร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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By	Miss Kanchana Sitlaothaworn
Field of Study	Biotechnology
Thesis Advisor	Professor ANCHARIDA SAVARAJARA, Ph.D.
Thesis Co Advisor	Professor SOMBOON TANASUPAWAT, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

.....
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.....
Thesis Advisor
(Professor ANCHARIDA SAVARAJARA, Ph.D.)

.....
Thesis Co-Advisor
(Professor SOMBOON TANASUPAWAT, Ph.D.)

.....
Examiner
(Professor SUPACHITRA CHADCHAWAN, Ph.D.)

.....
Examiner
(Associate Professor SEHANAT PRASONGSUK, Ph.D.)

.....
Examiner
(Associate Professor SUCHADA CHANPRATEEP NAPATHORN, Ph.D.)

กานต์ชนา สิทธิ์เหล่าถาวร : การระบุเอกลักษณ์และการประยุกต์ในพืชของแบคทีเรียเอนโดไฟท์ที่ส่งเสริมการเจริญของพืช. (IDENTIFICATION AND APPLICATION IN PLANT OF PLANT GROWTH-PROMOTING ENDOPHYTIC BACTERIA) อ.ที่ปรึกษาหลัก : ศ. ดร.อัญชริดา สวารชร, อ.ที่ปรึกษาร่วม : ศ ดร.สมบูรณ์ ธนาศุภวัฒน์

แบคทีเรียเอนโดไฟท์ 78 ไอโซเลตที่คัดแยกได้จากพืช 15 ชนิดในประเทศไทย รวม 50 ไอโซเลตคัดแยกจากตัวอย่างรากอ้อยจาก 6 จังหวัด และ 28 ไอโซเลตคัดแยกได้จากลำต้นและใบของพืชในจังหวัดกาญจนบุรี ซึ่งจากการระบุเอกลักษณ์โดยอาศัยลักษณะทางฟีโนไทป์และการวิเคราะห์ของ 16S rRNA gene และทดสอบความสามารถในการส่งเสริมการเจริญของข้าว พบว่าแบคทีเรียเอนโดไฟท์ 78 ไอโซเลต ระบุเป็น *Gluconacetobacter* (37 ไอโซเลต) *Pantoea* (14 ไอโซเลต) *Burkholderia* (2 ไอโซเลต) *Pseudomonas* (4 ไอโซเลต) *Priestia* (4 ไอโซเลต) *Enterobacter* (2 ไอโซเลต) *Acinetobacter* (2 ไอโซเลต) *Novosphingobium* (2 ไอโซเลต) *Curtobacterium* (2 ไอโซเลต) *Nguyenibacter, Aureimonas*, *Bacillus*, *Peribacillus*, *Sphingobium*, *Staphylococcus*, *Brevibacillus*, *Aneurinibacillus* และ *Pseudarthrobacter* อย่างละไอโซเลต จากผลของ 16S rRNA gene แสดงให้เห็นว่าสายพันธุ์ Sx8-5^{T}เป็นสมาชิกของสกุล *Novosphingobium* โดยมีความเหมือนของลำดับเบสเมื่อเปรียบเทียบกับ *Novosphingobium barchaimii* LL02^{T} เป็น 99.4% และมีความเหมือนของลำดับเบสเมื่อเปรียบเทียบกับสปีชีส์อื่นๆ ของ *Novosphingobium* น้อยกว่า 99.4% นอกจากนี้ยังมีค่าเฉลี่ยความเหมือนของลำดับเบสนิวคลิโอไทด์ของจีโนม ANIb และ ANIm ระหว่างสายพันธุ์ Sx8-5^{T} และ 7 สายพันธุ์ใกล้เคียงอยู่ระหว่าง 72.33-82.14% และ 83.82-87.38% ตามลำดับ และมีค่าความคล้ายคลึงของดีเอ็นเอ-ดีเอ็นเออยู่ในระหว่าง 21.0-28.6% จากผลการทดลองที่ได้แสดงให้เห็นว่าสายพันธุ์ Sx8-5^{T}เป็นแบคทีเรีย สปีชีส์ใหม่ จึงเสนอชื่อว่า *Novosphingobium kaempferiae* ในการศึกษานี้ได้ทดสอบศักยภาพการส่งเสริมการเจริญพืชของแบคทีเรียเอนโดไฟท์ที่คัดแยกได้ พบว่าสามารถตรึงไนโตรเจนได้ 61 สายพันธุ์ผ่านการทำงานของเอนไซม์ไนโตรจีเนสโดยเปลี่ยนไนโตรเจนในอากาศเป็นแอมโมเนีย ซึ่งถูกยืนยันโดยยีน *nif* ที่พบในจีโนมของ *G. dulcium* PS25 แบคทีเรียเอนโดไฟท์ 59 สายพันธุ์สามารถสลายไตรแคลเซียมฟอสเฟต (SI=1.18-4.4) และ 72 สายพันธุ์สามารถสลายซิงค์ออกไซด์ได้ (SI=1.59-5.6) และ 16 สายพันธุ์สามารถผลิตกรดอินโดลแอซีติก (IAA) (6.13-202.25 มก./มล.) ในอาหาร NF ที่มีทริปโตเฟนเป็นสารตั้งต้นในการสังเคราะห์ IAA โดยสภาวะที่เหมาะสมในการผลิต IAA ได้สูงที่สุด 232.1 มก./มล.จาก *Sphingobium* sp. Sx8-8 คือ pH 7 อุณภูมิ 30 องศาเซลเซียส ในระยะเวลา 48 ชั่วโมง จากการทดสอบ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Kanchana Sitlaothaworn : IDENTIFICATION AND APPLICATION IN PLANT OF PLANT GROWTH-PROMOTING ENDOPHYTIC BACTERIA. Advisor: Prof. ANCHARIDA SAVARAJARA, Ph.D. Co-advisor: Prof. SOMBOON TANASUPAWAT, Ph.D.

Seventy-eight endophytic bacteria isolated from 15 plant samples in Thailand including 50 isolates from sugarcane root samples obtained from 6 Provinces and 28 isolates from stems and leaves of healthy plants in Kanchanaburi Province, were identified and evaluated for plant growth promoting capability using rice as a plant model. They were identified as *Gluconacetobacter* (37 isolates), *Pantoea* (14 isolates), *Burkholderia* (2 isolates), *Pseudomonas* (4 isolates), *Priestia* (4 isolates), *Enterobacter* (2 isolates), *Acinetobacter* (2 isolates), *Novosphingobium* (2 isolates), *Curtobacterium* (2 isolates), and each of *Nguyenibacter, Aureimonas*, *Bacillus*, *Peribacillus*, *Sphingobium*, *Staphylococcus*, *Brevibacillus*, *Aneurinibacillus*, and *Pseudarthrobacter* based on their phenotypic characteristics and 16S rRNA gene analyzes. According to the 16S rRNA gene phylogeny, strain Sx8-5^T was a member of genus *Novosphingobium* and shared the highest sequence similarity to *Novosphingobium barchaimii* LL02^T of 99.4% and other *Novosphingobium* species (<99.4%). The ANIb and ANIm values of whole genome comparison between strain Sx8-5^T and seven closely related type strains were 72.33-82.14%, 83.82-87.38%, respectively, and the digital DNA-DNA hybridization (dDDH) values ranged from 21.0% to 28.6% when compared to the type strains of the member of *Novosphingobium*. Based on the results obtained, demonstrated that strain Sx8-5^T represents a novel species of the genus *Novosphingobium*, the name *Novosphingobium kaempferiae* sp. nov. is proposed. Sixty-one strains were positive in fixing nitrogen through nitrogenase activity by converting of N_2 in the air to ammonia, which was supported by *nif* genes found in the genome of *G. dulcium* PS25. Sixty-nine strains were able to solubilize tricalcium phosphate (SI=1.18-4.4) and 72 strains were able to solubilize zinc oxide (SI=1.59-5.6). Sixteen strains could produce IAA (6.13 to 202.25 µg/mL) in NF medium supplemented with 0.01% L-tryptophan, a main precursor for IAA biosynthesis. The maximum IAA produced by the *Sphingobium* sp. Sx8-8 increased to 232.1 µg/mL at optimized conditions: pH 7, 30°C, 0.5% L-tryptophan and 48 h cultivation. In *in vitro* 2 rice varieties

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2022 Advisor's Signature .....

Co-advisor's Signature .....

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°C	=	degree Celsius
cm	=	centimeter
diam	=	diameter
e.g.	=	for example
et al.	=	et alii (and others)
g	=	gram
L	=	liter
m	=	meter
M	=	molar
min	=	minutes
mL	=	milliliter
mm	=	millimeter
mM	=	millimolar
mg	=	milligram
rpm	=	revolutions per minute
s	=	second
SI	=	solubilization index
sp.	=	species
v/v	=	volume by volume
w/v	=	weight by volume
µg	=	microgram
µL	=	microliter
µm	=	micrometer

CHAPTER 1

INTRODUCTION

1.1 Significance of the study

Endophytic bacteria are microbes that colonize in internal tissue of plants above and underground without negative effect. Relationship between the endophytic bacteria and plants is symbiosis influenced by plant- genotype, growth stage, physiology, type of tissue and environmental conditions. Plant provides habitat such as leaves, root, stem, and root surrounding to bacteria, especially root, due to the rich in nutrient components of root exudate such as amino acids, fatty acids and other organic compounds which attract endophytic bacteria. Most common genera of endophytic bacteria are *Bacillus*, *Rhizobium*, *Sphingomonas*, *Azospirillum*, *Burkholderia*, *Azotobacter*, *Enterobacter*, *Pseudomonas*, *Paenibacillus*, *Streptomyces* and *Microbacterium*. Diverse species of endophytic bacteria were reported from various plants such as rice, corn, maize, sugarcane, cassava, oil palm, cotton, coffee, citrus, Jerusalem artichoke etc. Endophytic bacteria that enhance plant growth are referred to as plant growth-promoting bacteria (PGPB) which involves in several mechanisms such as N_2 fixation, solubilization of insoluble elements and production of phytohormones. As bacteria fix nitrogen in air and convert it to ammonia by nitrogenase enzyme. Therefore, nitrogen fixing bacteria can be used as bio-fertilizer to reduce the use of chemical N-fertilizer. The PGPB isolated from sugarcane belonged to genera *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Herbaspirillum*, *Pseudomonas*, *Rhizobium* and *Gluconacetobacter*. *Acetobacter nitrogenifigens*, *Acetobacter peroxydans*, *Gluconacetobacter azotocaptans*, *Gluconacetobacter johannae*, *Swaminathania salitolerans*, *Asaia bogorensis* and *Asaia siamensis* are acetic acid bacteria (AAB) reported to promote plant growth by fixing nitrogen, solubilizing P and Zn and producing indole-3-acetic acid (IAA), an important

phytohormone used in agriculture to increase plant growth. There are a few studies on plant growth-promoting endophytic bacteria especially the AAB isolated from sugarcane in Thailand. Though, sugarcane is an important economic crop of Thailand that cultivated in at least 47 Provinces.

In this study, endophytic bacteria were isolated from internal tissue and rhizosphere of sugarcane and other plants, identified, then the isolates that fixed nitrogen, solubilized P, solubilized Zn and/or produced IAA were tested for plant growth-promoting ability using rice as a plant model. The identification of endophytic bacteria isolated were based on polyphasic characterization (phenotypic and genotypic characteristics). As species level of bacteria may not be identified by only the phenotypic characteristics. Phenotypic characteristic consists of morphology, physiological characteristics, and biochemical property. The genotypic characteristics were determined by analysis of G+C content, 16S rRNA gene sequence, $(GTG)_5$PCR, DNA-DNA hybridization and whole-genome analysis.

1.2 Research Objectives

1.2.1 To isolate and identify endophytic bacteria from sugarcane and other plants.

1.2.2 To screen for IAA production, nitrogen fixing, P and Zn solubilizing activities and optimize IAA production of the isolates.

1.2.3 To evaluate the potential of selected isolates as bioinoculant for enhancement of rice growth.

1.2.4 To analyze whole genome of selected plant growth-promoting isolates.

CHAPTER 2

LITERATURE REVIEW

Microbial communities that colonize in intercellular plant tissues throughout all or part of their life cycle without causing plant disease were defined as endophyte by De Bary (1866). In 1997, Hallmann *et al*. described that the endophyte live inside plant without negative effect and can be isolated from surface of disinfected plant tissues, Rosenblueth and Martínez-Romero (2006) defined the endophyte as bacteria or fungi that associated with plants, while Hardoim *et al*. (2015) categorized the endophyte based on their colonization niche. Presently, the endophyte have been exhibited in all plant species and participated in complex relationship with their host plants (Strobel and Daisy, 2003; Huang *et al*., 2007).

2.1 Endophytic bacteria and host plants

Endophytic bacteria are found in most healthy plants especially roots because of the rich nutrient components of root exudate such as amino acids, fatty acids and other organic compounds which attract endophytic bacteria that can utilize them. Thus, endosphere of plant roots has the highest frequency of endophytic bacteria (Fig 2.1). Oku *et al*. (2012) showed that amino acid from root exudate of tomato plants attracted *Pseudomonas fluorescens* Pf0-1. Khare *et al*. (2018) reported that flavonoids were one of root exudate obtained from various plant species and had an important role in colonization of bacteria. This agreed well with other reports in that flavonoid as chemotaxis agent for colonization of *Rhizobium* (Dharmatilake and Bauer, 1992; Khandual, 2007; Faure *et al*., 2009). The attachment of endophytic bacterial cells to plants may involve with their structural components such as flagella, fimbriae, pili, and secondary products secreted such as exopolysaccharide (EPS), lipopolysaccharide (LPS) (Sauer and Camper, 2001). *Azospirillum brasilense* used flagella as primary attachment with root surface of wheat

(Croes *et al*., 1993). Janczarek *et al*. (2015) reported that *Rhizobium leguminosarum* produced EPS in early stage of colonization on host plant surface which coincided with *Gluconacetobacter diazotrophicus* that secreted EPS in attachment and colonization of rice root endosphere (Meneses *et al*., 2011). In addition, *Herbaspirillum seropedicae* secreted EPS in attachment and colonization of maize roots (Balsanelli *et al*., 2010). Endophytic bacteria increased their population after penetrating into plant roots (Hallmann, 2001), while pattern and site of colonization were specific for each of endophytic bacterial strain (Zachow *et al*., 2010). However, structure of endophytic bacterial communities is related to plant physiology which is influenced by various factors such as climate in country or location, and environmental variability including fluctuating of temperature, humidity and light which affect nutrients and steady environment (Chesson and Warner, 1981).

Endophytic bacteria that enhance plant growth are referred to as plant growth-promoting bacteria (PGPB). Promotion of plant growth by the PGPB involves with many mechanisms such as fixing nitrogen from air and converting it to ammonia which is utilized by plant (Kirchhorf *et al*., 1997), producing siderophores (under soil Fe-deficiency condition) which form complex with Fe ions and leading to an increase in plant Fe-uptake, producing indole-3-acetic acid (IAA) which involves in cell development, plant root elongation (Normanly *et al*., 1995) and other hormones production, and solubilizing insoluble phosphorus (P), zinc (Zn) and potassium (K) in soil which increases in plant availability of P, Zn and K (Meena *et al*., 2016). Endophytic bacteria predominantly distributed in cultivated plants belonged to genera *Rhizobium*, *Pseudomonas*, *Bacillus*, *Azospirillum*, *Sphingomonas*, *Burkholderia*, *Enterobacter*, *Paenibacillus*, *Nocardia*, *Streptomyces*, *Microbacterium* (Rosenblueth and Martinez-Romero, 2006).

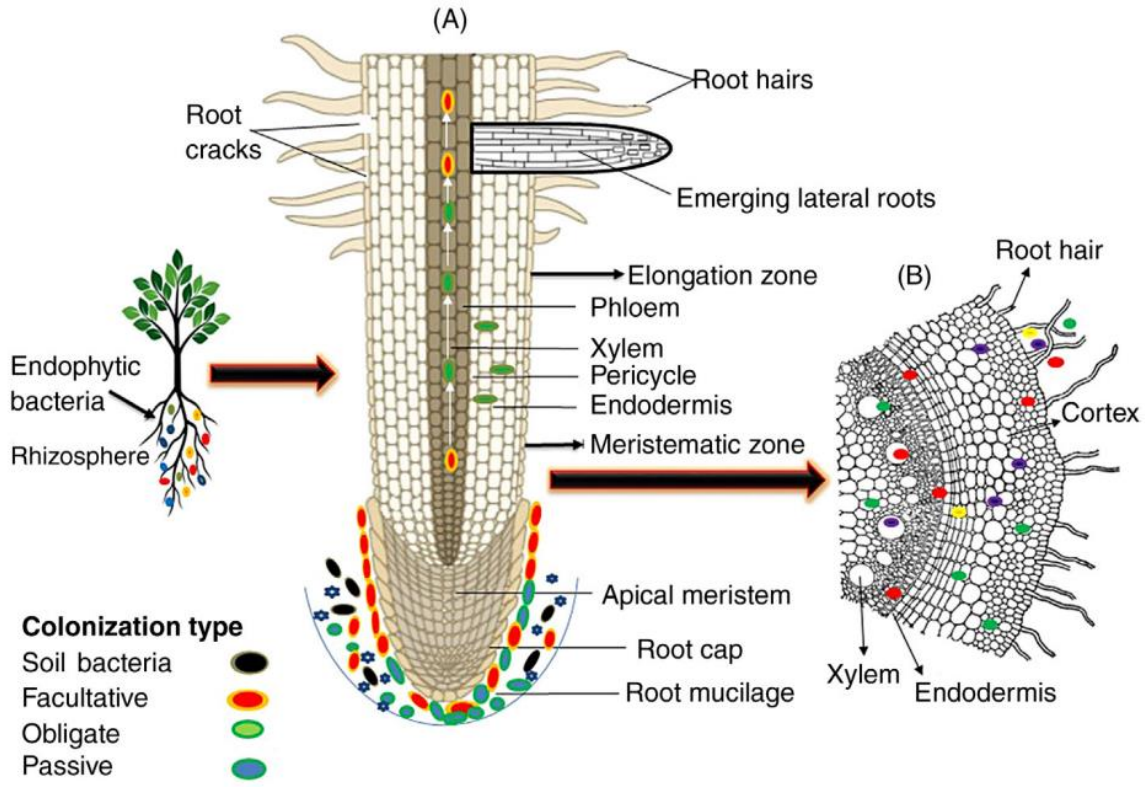

Figure2.1 Schematic representation of endophytic bacterial colonization and distribution in endosphere of plant root. (A) Invasion of bacteria into a plant using several root zones. White arrows show translocation of bacteria into phloem and xylem and colonization types are represented by different colored ovals. (B) Occurrence of endophyte either at the site of entry (indicated in blue) or in intercellular space of cortex and xylem vessels (indicated in green). Red and yellow spheres represent rhizospheric bacteria which are unable to colonize inner plant tissues (Kumar et al., 2020).

2.2 Plant growth-promoting bacteria

Currently, more than 16 phyla or 200 genera of bacteria have been reported as endophytes and isolated from various plant species. Nearly 300,000 plant species that exist on earth are thought to be a host to one or more endophytes (Ryan *et al.*, 2008). Table 2.1 showed example of endophytic bacteria that colonized in plant parts including root, stem, leaves, seed, fruit, tuber, ovule (Hallmann *et al.*, 1997; Benhizia *et al.*, 2004) and diversity of endophytic bacteria communities varied with the difference in host plant

species. Moreover, Yu *et al*. (2016) reported that *Microbacterium* sp. C4 and *Lysinibacillus* sp. C7 isolated from soybean and corn root had an ability to produce IAA over 10 mg/L and showed better performance in promoting soybean and wheat seedling growths in pot experiment. Etminani and Harighi (2018) isolated 61 endophytic bacteria from leaf and stem samples of healthy wild pistachio trees (*Pistacia atlantica* subsp. *kurdica*) in Iran. They belonged to *Pseudomonas*, *Stenotrophomonas*, *Bacillus*, *Pantoea* and *Serratia* genus based on 16S rRNA gene sequence and produced plant growth hormones, auxin and gibberellin in different amounts. Khamwan *et al*. (2018) isolated endophytic bacteria identified as *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Microbacterium* and *Curtobacterium* genera by 16S rRNA sequence analysis from leaves, stem, tuber, and root of *Helianthus tuberosus* L. (Jerusalem artichoke). Six isolates increased height, root and stem weights, and tuber number of 2 varieties of Jerusalem artichoke. Sugarcane, *Saccharum* spp., was reported as habitation of endophytic bacteria as follows; Kruasuwan and Thamchaipenet (2016) isolated 135 endophytic bacteria from roots of sugarcane cultivated in Thailand. Two strains, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21, had high potential to apply for promotion of sugarcane growth. Magnani *et al*. (2010) found that most of endophytic bacteria isolated from internal tissue of sugarcane stem and leaves belonged to genera *Enterobacteriaceae* and *Pseudomonaceae*, respectively. Asis *et al*. (2000) isolated 21 nitrogen-fixing endophytic bacteria from juice of sugarcane cv. NiF-8 in Japan. Four nitrogen-fixing and phytohormone producing-bacteria which isolated from roots and stems were identified as *Enterobacter* sp. and *Klebsiella* sp. based on 16S rRNA gene sequence Mirza *et al*. (2001). Taulé *et al*. (2012) isolated 598 potential endophytic diazotrophs belonged to several genera including *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Acinetobacter*, *Rhanella*, *Enterobacter*, *Pantoea*, *Shinella*, *Agrobacterium* and *Achromobacter* from surface-sterilized sugarcane stems.

2.2.1 Nitrogen fixation

Nitrogen (N) is one of macronutrients for plant growth and development. Plant cannot utilize nitrogen in its gaseous form; thus, the use of nitrogen fertilizers is needed to resolve in nitrogen deficiency. However, application of the N-based fertilizers is subject to loss into soils and environments (Singh *et al*., 2014). Atmospheric nitrogen (N_2)-fixing bacteria that inhabit in both plant tissues (e.g., nodule, root) and soil-root rhizosphere interface, and supply significant N amount for plant growth are called diazotrophs. Several researchers demonstrated that some of N_2 fixing-endophytic bacterial strains such as *Azospirillum* spp., *Bacillus* spp., *Burkholderia* spp., *Enterobacter cloacae*, *Herbaspirillum* spp., *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Pantoea* sp. (Loiret *et al*., 2004; Govindarajan *et al*., 2006; Islam *et al*., 2009) have a role in enhancing of agriculturally important plants; *Brassica napus*, *Leptochloa fusca*, *Oryza sativa*, *Pennisetum glaucum*, *Musa acuminata*, *Saccharum officinarum*, and *Zea mays*. (Anand and Chanway, 2013; Araújo, 2013; Gupta *et al*., 2013; Andrade *et al*., 2014).

Table 2.1 Beneficial endophytic bacteria associated with different plants and their plant growth-promoting properties (Kumar *et al*., 2020).

Endophytic bacteria	Plants	Plant parts	Beneficial features
Bacillus cereus and *B. subtilis*	*Teucrium polium* L.	Leaves	Produced IAA, ammonia, phosphate solubilization
B. subtilis and *Stenotrophomonas* sp.	*Triticum aestivum* L.	Root	IAA production, phosphate solubilization and plant biomass
B. subtilis, Agrobacterium tumefaciens, Pseudomonas putida and *Pseudomonas* sp.	*Cassia tora* L.	Root	Produced IAA, ammonia, siderophore, HCN, and phosphate solubilization
Klebsiella sp. PnB10 and *Enterobacter* sp. PnB11	*Piper nigrum*	Stem	Phosphate solubilization, ACC deaminase* production, and siderophore production
Pseudomonas fluorescens G10 and *Microbacterium* sp. G16	*Brassica napus*	Root	Produced IAA, siderophore and ACC deaminase*
Paenibacillus lentimorbus and *P. macerans*	*Cymbidium eburneum*	Meristem tissue	Phosphate solubilization and potential for plant growth promotion

Table 2.1 Beneficial endophytic bacteria associated with different plants and their plant growth-promoting properties (continued).

Endophytic bacteria	Plants	Plant parts	Beneficial features
Enterobacter sp., *Rahnella* sp., *Rhodanobacter* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., *Xanthomonas* sp. and *Phyllobacterium* sp.	*Lpomoea batatas* (L.) Lam.	Stem	Produced IAA, fix nitrogen and exhibit stress tolerance
B. subtilis and *B. thuringiensis*	*Glycine max* L.	Seed	Increase nodule and soybean weight
Pseudomonas sp. ZoB2	*Zingiber officinale*	Rhizome	Produced IAA, ACC deaminase* and siderophore
Paenibacillus sp. RM	*Tridax procumbens*	Root	Produced secondary metabolites, IAA, siderophore, ACC deaminase*, biosurfactant and solubilized phosphate
B. cereus (ECL1), *B. thuringiensis* (ECL2), *Bacillus sp.* (ECL3), *Bacillus pumilis* (ECL4), *Pseudomonas putida* (ECL5), and *Clavibacter michiganensis* (ECL6)	*Curcuma longa* L.	Rhizomes	Produced IAA, siderophore and solubilized phosphate

Table 2.1 Beneficial endophytic bacteria associated with different plants and their plant growth-promoting properties (continued).

Endophytic bacteria	Plants	Plant parts	Beneficial features
Achromobacter xiloxidans, Alcaligens sp., and *B. pumilus*	*Helianthus annuus* L.	Roots and rhizospheric soil	Phosphate solubilization
Bacillus sp., and *Sphingopyxis* sp.	*Fragaria ananassa*	Meristem tissue	Produced IAA and solubilized phosphate
Peudomonas resinovorans, *Paenibacillus polymaxa* and Acenitobacter calcoaceticus	*Gynura procumbens* (Lour.) Merr.	Leaves	Produced cytokinin compounds
Arthrobacter humicola YC6002	*Zoysia japonica*	Root	Seed germination and stem growth by producing phytotoxic compound
Microbacterium testaceum, Curtobacterium flaccumfaciens, B. subtilis, B. pumilus P. fluorescens, Sphingomonas parapaucimobilis, Serratia sp. and *Pantoea ananatis*	*Panicum virgatum* L.	Leaves	Produced cellulase and solubilized phosphate
B. megatherium, B. pumilus, B. licheniformis, Micrococcus luteus, Paenibacillus sp., Pseudomonas sp. and A. calcoaceticus	*Plectranthus tenuiflorus*	Root, stem and leaves	Exhibited extracellular enzymatic activity

Table 2.1 Beneficial endophytic bacteria associated with different plants and their plant growth-promoting properties (continued).

Endophytic bacteria	Plants	Plant parts	Beneficial features
Serratia nematodiphila, *E. aerogenes*, and *Acinetobacter* sp.	*Solanum nigrum* L.	Root, stem and leaves	Produced ACC deaminase*, IAA, siderophore and solubilized phosphate
Acinetobacter sp., *Agrobacterium* sp., *Brevibacillus* sp., *Bacillus* sp., *Burkholderia sp, Curtobacterium* sp., *Erwinia sp*., *Lactococcus* sp., *Pantoea* sp., and *Pseudomonas* sp.	*Eucalyptus* sp.	Stem	Control of diseases and plant growth promotion, as well as for the production of new metabolites and enzymes
Paenibacillus validus, Lysinibacillus fusiformis, B. licheniformis, P. putida, M. oleivorans and *S. plymutica*	*Citrus sinensis*	Root	Phosphate solubilization, siderophore production, nitrogen fixation, IAA synthesis and antibiotic production
Pantoea ananatis, P. putida, Brevibacillus agri, B. subtilis and B. megaterium	*Oryza sativa*	Leaves, stem and roots	Phosphate solubilization

*1-aminocyclopropane-1-carboxylate (ACC) deaminase

Endophytic bacteria fix nitrogen *via* conversion of atmospheric nitrogen gas (N_2) to ammonia (NH_3) route by using nitrogenase enzyme, a complex enzyme encoded by

nifD and *nifK*, and dinitrogenase reductase subunit encoded by *nifH* (Stacey *et al*., 1992). The *nifH* gene is most widely used as biomarker for confirmation of nitrogenase activity of nitrogen-fixing bacteria. Nitrogen fixation via nitrogenase enzyme complex occurred under available condition of oxygen and metal center, iron, and molybdenum (Fe and Mo) were found in *Azotobacter, Rhizobium* and filamentous cyanobacteria (Kirn and Rees, 1992) as shown in Fig 2.2.

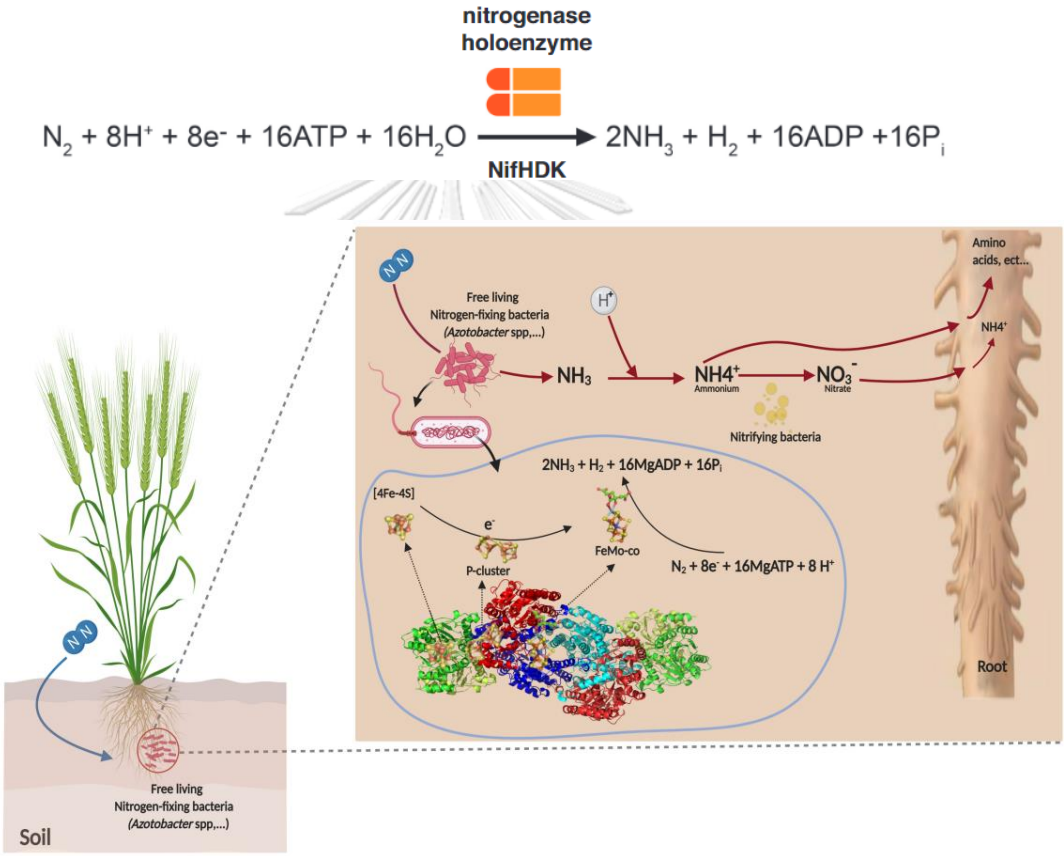

Figure 2.2 General reaction of molecular nitrogen fixation by Azotobacter sp. (Aasfar et al., 2021)

2.2.2 Phosphorus (P) and zinc (Zn) solubilization

Phosphorus (P) and zinc (Zn) are essential nutrients required for cell synthesis, enzyme activity, and protein and vitamin productions of plants. Plants cannot uptake P and Zn existed as insoluble form in soils that bound and precipitated with other minerals

such as calcium, oxide and hydroxide of aluminum and iron, which was a result of using large amount of P and Zn chemical fertilizers. Moreover, the P induced Zn deficiency as P may interfere with translocation of Zn from root to shoot (Cakmak and Marschner, 1987).

Phosphate-solubilizing bacteria

Several bacteria can solubilize inorganic P and mineralize organic P via production of organic acids such as citric, tartaric, succinic, and oxalic acids, production of exopolysaccharide (EPS), phosphatase, phytase and phosphonatase (Zaidi *et al*., 2009) as shown in Fig 2.3. Bacteria having phosphate solubilizing capacity are defined as phosphate-solubilizing bacteria (PSB). The PSB such as *Rhizobium*, *Agrobacterium*, *Pseudomonas*, *Erwinia*, *Bacillus*, *Pantoea* and *Flavobacterium* enhanced P availability to plants and were used as bio-fertilizer to reduce the use of chemical phosphate fertilizers (Matos et al, 2017).

Zhang *et al.* (2019) isolated 2 bacterial strains, *Acinetobacter* sp. RC04 and *Sinorhizobium* sp. RC02, that had positive effect on safflower seed germination from safflower rhizosphere soil. The *Acinetobacter* sp. RC04 showed P solubilization halo ratio (D/d) of 4.08±0.13 on agar medium after 6 days and produced soluble P of 168.5±1.27 mg/L in liquid medium. Co-inoculated of *Acinetobacter* sp. RC04 and *Sinorhizobium* sp. RC02 with safflower seeds increased seedling length, shoot length and root number more than separate inoculation. Matos *et al*. (2017) demonstrated that approximately 67.5% of 40 endophytic bacteria isolated from banana tree roots solubilized tricalcium phosphate in solid medium. Oteino *et al*. (2015) showed that all endophytic bacteria obtained from several sources could solubilize insoluble P through production of gluconic acid (14-169 mM). When selected endophytic bacteria strains (L321, L132, and S10) were inoculated into *P. sativum* L. plant grown in soil under limited soluble P condition, they produced

medium to high level of gluconic acid resulted in enhancement of *P. sativum* L. growth. Yi *et al*. (2008) reported significant production of EPS by highly efficient P-solubilizing bacteria, i.e., *Arthrobacter* sp. (ArHy-505), *Azotobacter* sp. (AzHy-510), *Enterobacter* sp. (EnHy-401), and *Enterobacte*r sp. (EnHy-402).

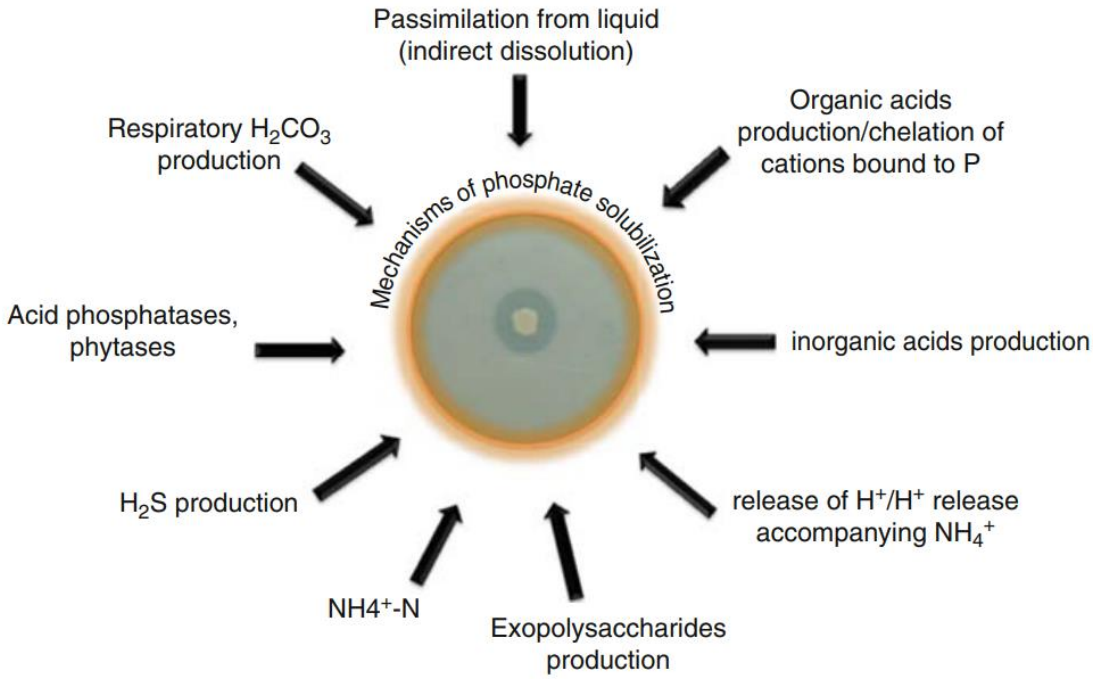

Figure 2. 3 Mechanisms of P solubilization by phosphate-solubilizing bacteria (Zaidi et al., 2009*)*.

Zinc-solubilizing bacteria

There were several reports which demonstrate the potential usage of plant growth-promoting rhizobacteria (GPRs) in enhancing Zn bioavailability in rhizosphere and in plant cells (Subramanian *et al*., 2009). The use of ZSB for improving of plant Zn-deficiency is the most effective approach for sustainable agricultural production system. Zinc-solubilizing bacteria (ZSB) improved Zn bioavailability of plants by several mechanisms as shown in Fig 2.4. Lugtenberg (2015) stated that various signaling mechanisms are involved in Zn solubilization of bacteria such as initiation of nodulation process,

chemoattraction, and release of compounds (organic acids, flavonoids) which their presence give signal for colonization process in crop root rhizospheric zone. After colonizing in crop root zone, microbes start to show their beneficial impacts on the crop. Many studies have confirmed that the ZSB solubilize insoluble form of zinc compounds ($ZnCO_3$, ZnO, and ZnS) in soil (Tariq *et a*l., 2007) and in liquid medium (Saravanan *et al*., 2007). Vaid *et al*. (2014) demonstrated that 3 ZSB strains; BC, AX, and AB; isolated from Zn-deficient rice were effective in significantly increase in rice growth over control and Zn fertilizer treatment.

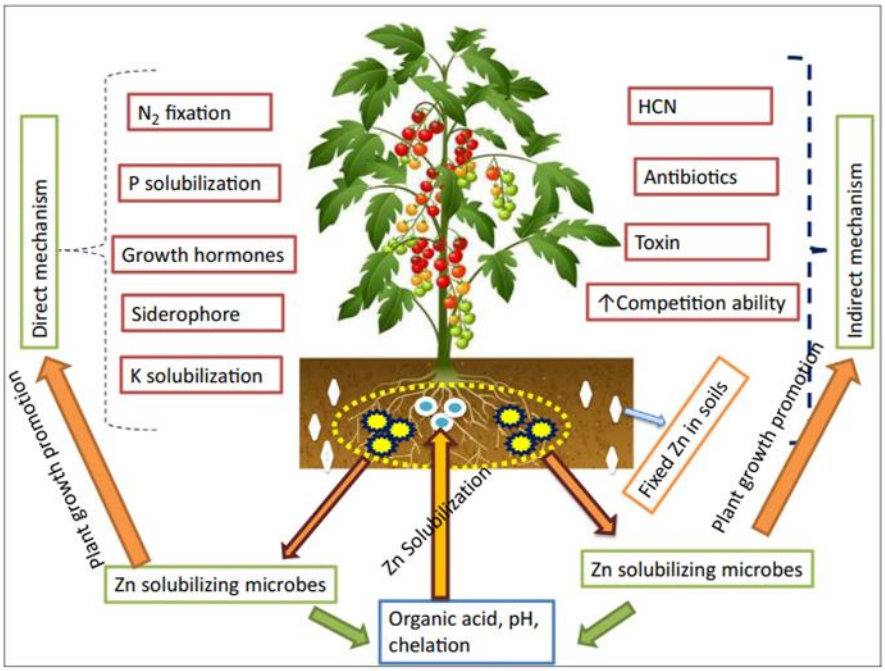

Figure 2.4 Mechanisms of Zn-solubilizing microorganisms (Pradhan et al., 2021).

Saravanan et al. (2004) showed that *Pseudomonas* sp. ZSB-S-2 and *Bacillus* sp. ZSB-O-1 could solubilize insoluble Zn complexes (zinc oxide, zinc sulfide (sphalerite) and zinc carbonate) to many folds in liquid medium. Saravanan *et al*. (2007) revealed that *Gluconacetobacter diazotrophicus* PAI5 had potential in solubilizing of Zn compounds via producing of gluconic acid as analysis by gas chromatography coupled mass

spectrometry (GCMS). Whiting *et al*. (2001) showed that Zn bioavailability in rhizosphere soil of *Thlaspi caerulescens* increased approximately 0.45-folds when inoculated with ZSB strains; *Microbacterium saperdae* BJ1, *Pseudomonas monteilii* BJ5 and *Enterobacter cancerogenes* BJ10. Inoculation of *Azotobacter* and *Azospirillum i*ncreased Zn uptake by 18% into wheat crop Eleiwa *et al*. (2012). This result agreed well with Sadaghiani *et al*. (2008), who reported that recovery of Zn-deficiency symptom and increase of Zn acquisition in wheat and barley occurred when co-cultivated with *Bacillus* M-13 and *P. aeruginosa* 7NSK.

2.2.3 Indole-3-acetic production

The phytohormone auxin is a fundamental compound that modulates plant growth and development (Halliday *et al*. 2009). Naturally occurring members of this hormone group include indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 4-chloro-indole-3-acetic acid (4-Cl-IAA). The IAA is a hetero-aromatic organic acid consisting of indole ring and acetic acid side chain (Fig 2.5). The acetic acid–indole bond at third position of the indole ring in the IAA structure is freely rotating with carboxyl group.

Plant produced IAA which plays an important role in initiation of roots, leaves and flowers, lateral root formation, root elongation and differentiation, particularly in dicots (McSteen, 2010; Phillips *et al*., 2011). IAA deficiency in plant displayed stunted growth compared to normal plants (Tao *et al*., 2008). Not only plants but also microorganisms are able to produce IAA.

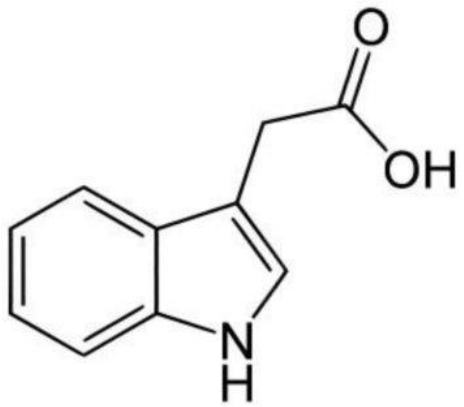

Figure 2.5 Structure of indole-3-acetic acid (IAA) (Han et al., 2018).

2.2.3.1 The IAA biosynthesis pathway of bacteria

Many species of bacteria are capable of synthesizing IAA and are called as IAA-producing bacteria such as *Pseudomonas*, *Rhizobium*, *Azospirillum*, *Enterobacter*, *Azotobacter*, *Klebsiella*, *Alcaligenes*, *Pantoea* and *Streptomyces* (Apine and Jadhav 2011). Kim *et al*. (2011a, 2011b) recommended that most of endophytic bacteria are able to produce IAA which is helpful in improving bacterial surviving in stress conditions such as heat and cold shocks, acidity, UV and salinity (Bianco *et al*., 2006; Donati *et al*., 2013). Bianco *et al*., (2006) showed that IAA-treated cells have increased in production of lipopolysaccharide (LPS), exopolysaccharide (EPS) and biofilm.

L-tryptophan serves as precursor for IAA biosynthesis in several microbes and plants via tryptophan-dependent pathway; however, IAA is also derived from tryptophan-independent pathway. The tryptophan-dependent pathway divided into 3 routes.

1. Indole-3-pyruvic acid (IPA) pathway (Rajagopal, 1971; Pollmann *et al*., 2006)

The IPA pathway is found in plants and bacteria (non-pathogenic, plant-associated and phytopathogenic bacteria) such as *Agrobacterium tumefaciens*, *Pseudomonas syringae* subsp. *savastanoi* and *Erwinia herbicola* pv. *gypsophilae* (Kaper and Veldstra 1958; Manulis *et al*., 1991; Brandl *et al*., 1996; Brandl and Lindow, 1996). Tryptophan is deaminated to IPA by aminotransferase. After that, IPA is converted to indole-3-acetylaldehyde (IAAld) by decarboxylase, which is then, oxidized to IAA by aldehyde dehydrogenase, mutase or oxidase enzyme as shown in Fig 2.6.

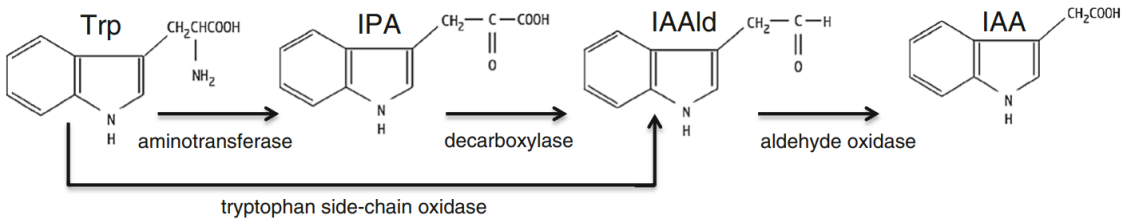

Figure 2.6 Indole-3-pyruvic acid (IPA) pathway. Trp; tryptophan, IPA; indole-3-pyruvic acid, IAAld; indole-3-acetaldehyde, IAA; indole-3-acetic acid (Patten and Glick, 1996).

2. Indole-3-acetamide (IAM) pathway (Pollmann *et al*., 2006)

The IAM pathway is found in phytopathogenic and phytosymbiotic bacteria (Kochar *et al*., 2011). This pathway divided into 2 steps. First, tryptophan is converted to IAM by tryptophan 2-monooxygenase. Second, the IAM is hydrolyzed to IAA by IAM-specific hydrolase/amidase (Fig 2.7).

3. Indole-3-acetonitrile (IAN) pathway

The IAN pathway has been studied in plants. Tryptophan is initially converted to indole-3-acetaldoxime. Then, indoleacetaldoxime dehydratase hydrolyzes the indole-3-acetaldoxime to indole-3-acetonitrile. The indole-3- acetonitrile is consequently converted to IAA by nitrilase enzyme in a single step (Fig. 2.8) or by nitrile hydratase and amidase in two-step process (Fig. 2.7) (Zhao, 2012).

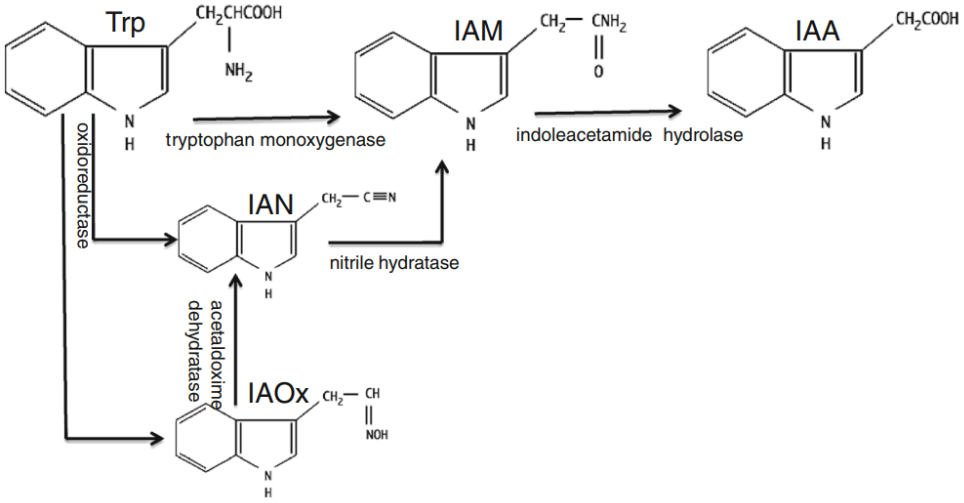

Figure 2.7 Indole-3-acetamide pathway. IAM; indole-3-acetamide, IAN; indole-3-acetonitrile, IAA; indole-3-acetic acid, IAOx; indole-3-acetaldoxime. (Prinsen et al., 1997).

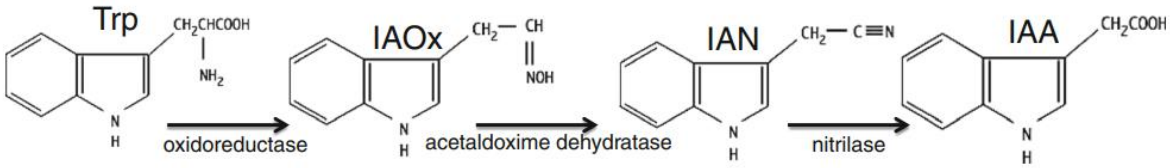

Figure 2.8 Indole-3-acetonitrile. Trp; tryptophan, IAOx; indole-3-acetaldoxime, IAN; indole-3- acetonitrile, IAA; indole3-acetic acid (Patten and Glick, 1996).

2.2.3.2 Application of IAA producing bacteria

The IAA-producing bacteria is used in agriculture to promote plant growth such as elongation of primary roots, cell division and differentiation; therefore, their amount of IAA production is crucial. Bacterial IAA production depends on several parameters; maximum IAA production of *Bacillus subtilis* DR2 isolated from rhizosphere of *Eragrostis cynosuroides* were 137.81µg/mL (96 h incubation), 141.92 µg/mL (35°C), 158.79 µg/mL (pH 7) and 168.09 µg/mL (1.2 g/L L-tryptophan), respectively using mannitol and ammonium sulfate as carbon and nitrogen source Kumari *et al*. (2018). When

mannitol and yeast extract were used as carbon and nitrogen sources, respectively, *Bacillus aryabhattai* MBN3 isolated from root nodules of *Vigna radiate* produced maximum IAA at 500 µg/mL L-tryptophan (Bhutani *et al*., 2018). Immobilized *Arthrobacter agilis* cells grown in optimal conditions (1% mannitol, 30°C, pH 8, 24 h) gave maximum IAA at 520 mg/L. The amount of bacterial IAA production was related to nutrients, L-tryptophan concentration, bacterial species, growth phase (incubation time) and bacterial enzymes which affected by cultural pH and temperature (Ozdal *et al*., 2017).

Microbacterium C4 and *Lysinibacillus* C7 isolated from corn roots which produced IAA over 10 µg/mL increased shoot and root of soybean and wheat seedlings in pot experiment Yu *et al*. (2016). Besides, *Bacillus aryabhattai* MBN3, *Bacillus megaterium* MJHN1 and *Bacillus cereus* MJHN10 which isolated from root nodules of *Vigna radiate* produced high amount of IAA at 92.03 µg/mL, 68.27 µg/mL, and 71.33 µg/mL, respectively. Their IAA production was confirmed by TLC and HPLC analysis. Crude extract of *Bacillus aryabhattai* MBN3 revealed Rf value of 0.78 and retention time peak at 21.54 min the same as those of standard IAA. *In vitro* root growth assay of *Vigna radiata* seedling, *Bacillus megaterium* MJHN1 gave maximum root length, while *Bacillus aryabhattai* MBN3 gave the highest number of lateral roots (Bhutani *et al*., 2018). *Enterobacter cloacae* MG00145 isolated from *Ocimum sanctum* stem which produced IAA at 17.715±0.32 µg/mL promoted growth of four crops including rice, groundnut, black gram and toria by increasing of seed germination, shoot and root lengths (Panigrahi *et al*., 2020). Balliu and Sallaku (2017) revealed that difference in IAA concentration gave different effects on root germination. Root length of grafted cucumber seedling increased when treated with 5-20 ppm IAA but decreased when treated with 50 ppm IAA. Gholamalizadeh *et al*. (2017) showed that *Pantoea ananatis* AEn1 and *Bacillus vietnamensis* MR5 which was IAA-producing bacteria, and *Alcaligenes faecalis* O1R4

which was PSB, were able to increase rice growth and yield, but in different potential in pot experiment. Giassi *et al*. (2016) showed that plant growth-promoting bacteria, *Bacillus* spp. BM16, *Bacillus* spp. CPMO4, *Bacillus* sp. BM17, *Actinobacteria* ACT11, *Bacillus* sp. BM05, were able to promote growth of citrus rootstock. Etesami *et al*. (2014) indicated that IAA-producing strains isolated from canola were consistently more active in colonizing rice seedlings as compared to other isolates. Liu *et al*. (2016) reported that *Bacillus amyloliquefaciens* SQR9 enhanced cucumber growth by increasing of root tryptophan secretion which resulted in sufficient tryptophan to support an increase of IAA production of the *Bacillus amyloliquefaciens* SQR9. Rangjaroen *et al*. (2015) showed that endophytic diazotrophic bacteria isolated from rice roots including *Burkholderia* sp. SS5, *Klebsiella* sp. SS2, *Novosphingobium* sp. TR4 and *Sphingomonas* sp. PS5 significantly increased root germination of commercial rice cultivar Khao Dawk Mali 105. Detraksa (2018) demonstrated that enhancing of plant growth and significantly increasing of shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight and root dry weight of sugarcane seedlings was a result of inoculation with IAA producing *Streptomyces* sp. AS14-2.

2.3 Genes associated with plant growth-promoting endophytic bacteria

Plant growth-promoting bacteria (PGPB) that have positive effect on plants may involve in enhanced availability of nutrients and elements by fixing nitrogen, solubilizing insoluble elements, and stimulating root system development through synthesis of phytohormones. For example, tumor-inducing *Agrobacterium* strains have potential in promoting of non-susceptible plant hosts (Walker, 2013) due to lack of virulence plasmids and are avirulent to susceptible dicot hosts (Raio *et al*., 2004; Hao *et al*., 2011), *Pseudomonas chlororaphis* subsp. *aurantiaca* strain JD37, isolated from potato

rhizosphere soil, was able to produce IAA and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Wang, 2012), and *Klebsiella* sp. are among the major free-living nitrogen fixing bacteria, together with *Azospirillum brasilense*, *Pantoea agglomerans*, *Burkholderia* sp., *Serratia* sp. (Bhattacharjee *et al*., 2008). This plant-beneficial properties suggest the presence of conservative genes potentially encoding plant-beneficial functions, commonly distributed among different genera of bacteria. Bruto *et al*., 2014 reported an existence of plant-beneficial function contributing genes, including 20 *pqqBCDE* genes involved in phosphate solubilization, 5 *ipdC* and 2 *ppdC* genes for auxin synthesis, and *nifHDK* gene clusters for nitrogen fixation in DNA sequences of 25 PGPR strains belonged to genera; *Azospirillum*, *Rhizobium/Agrobacterium*, *Azoarcus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, and *Serratia* (Table 2.2).

Genes responsible for IAA synthesis depend on IAA biosynthetic pathway of bacteria which may be tryptophane-dependent, or tryptophane-independent. In tryptophan-dependent IAA biosynthesis, i) IPA pathway has been postulated from utilization of tryptophan as a precursor, identified IPA pathway genes including *aldA*, and *ipdC* genes code for aldehyde dehydrogenase and indolepyruvate decarboxylase enzyme, respectively. The *aldA* gene was found in genome of *Azospirillum brasilense* Yu62 (Xie *et al*., 2005) and *Gluconacetobacter diazotrophicus* (Go´mez-Manzo *et al*. 2010), ii) IAM pathway, the main genes driving in this pathway are *iaaM/tms-1* encodes tryptophan monooxygenase and *iaaH/tms-2* encodes indoleacetamide hydrolase/amidase enzyme, ii) IAN pathway, genes that involve in this pathway, including *oxd* gene which encodes aldoxime dehydratase enzyme, nitrilase gene and dehydratase gene. Table 2.3 shows example of different genes that encode for IAA biosynthesis pathways in different bacteria.

Genes that involved in P uptake and organic P solubilization were *pst* (Pi-specific transporter), *phoA* (alkaline phosphatase), *glpQ* (glycerophosphoryldiester phosphodiesterase), *phyC* (phytase), and *ushA* (nucleotidase) (Ishige *et al*., 2003; Prágai *et al*., 2004). *Acinetobacter* (Vaid *et al*., 2014), *Pseudomonas* (Di Simine *et al*., 1998; Fasim *et al*., 2002), and *Gluconacetobacter* solubilized insoluble Zn compounds by secreting of gluconic acid (Saravanan *et al*., 2007). The gluconic acid was synthesized extracellularly or direct glucose oxidized via periplasmic glucose dehydrogenase (GDH) which is encoded by *gcd* gene and *pqq* operon encodes products such as "pyrroloquinoline quinone (PQQ)" (Sharma *et al*., 2013; Sashidhar *et al*., 2019).

Table 2.2 Distribution of plant-beneficial function contributing genes according to the primary ecological lifestyle documented for the bacteria studied (Bruto *et al*., 2014).

Gene functions	Genes	PGPR (25*)	Endophytes/symbionts (56)
Phosphate solubilization	*pqqB*	20	36
	pqqC	20	36
	pqqD	20	36
	pqqE	20	36
	pqqF	10	17
	pqqG	7	17
Auxin synthesis	*ipdC*	5	2
	ppdC	2	2
Nitrogen fixation	*nifD*	9	23
	nifH	9	23
	nifK	9	23

* The number of bacteria is indicated in parenthesis.

Table 2.3 Example of IAA biosynthesis pathways that have been identified in different bacteria.

Bacteria	Pathways	Genes identified	Enzyme activities
Azospirillum brasilense Yu62	IPA	*ald A*	Aldehyde dehydrogenase
Azospirillum brasilense Sp245	IPA	*ipdC*	Indolepyruvate decarboxylase
Enterobacter cloacae FERM BP-1529	IPA	*ipdC*	Indolepyruvate decarboxylase
Pseudomonas fluorescens Psd, *Ralstonia solanacearum*	IAM	*iaaM, iaaH*	Tryptophan monooxygenase
Agrobacterium tumefaciens	IAM	*Tms-1, Tms-2*	
Pseudomonas sp. UW4	IAN	*nit* *nthAB*	Indoleacetonitrilase Nitrile hydratase
Bacillus amyloliquefaciens FZB42	IAN	*yhcX*	Ndoleacetonitrilase
Rhodococcus erythropolis JCM 3201	AOX/IAN/ IAM	*oxd* *nha1*	Phenylacetaldoxime dehydratase Nitrile hydratase
Brevibacterium butanicum ATCC 21196	IAOX/IAN/ IAM	*oxd* *nha1*	Phenylacetaldoxime dehydratase Nitrile hydratase

The occurrence of each pathway is based on genes that have been identified in the bacterial genome, enzymatic activities that have been experimentally confirmed or intermediates of the respective pathway that have been identified in the bacterial culture filtrate/supernatant (Duca *et al*., 2014).

2.4 Bacterial identification by polyphasic characteristics

Taxonomic information helps scientists to identify and understand biodiversity and relationship of bacteria isolated from different ecosystems. In late 19th century, bacteria were classified on the basis of morphology by Ferdinand Cohn (1872). At the beginning of 20th century, physiological and biochemical data were used for identification and classification of bacteria, following by chemotaxonomy and DNA-DNA hybridization technique. In 1980, DNA amplification and DNA sequencing techniques were used for classification of bacteria (Table 2.4). In mid-1990, whole genome sequence was used for studying complete genetic information. Therefore, two approaches for identification and classification of bacteria, phenotypic and genotypic characteristics, are combined and this is called polyphasic taxonomy.

Table 2.4 History of classification of bacteria and archaea (Schleifer, 2009).

Time span	Classification mainly based on
Late 19th century	Morphology, growth requirements, pathogenic potential
1900-1960	Morphology, physiology, biochemistry
1960-1980	Chemotaxonomy, Numerical taxonomy, DNA-DNA hybridization
1980-Today	Genotypic analysis, multilocus sequence analysis, average nucleotide identity, whole genome analysis

2.4.1 Phenotypic characteristics

Phenotypic characteristic consists of morphology, physiological characteristic, and biochemical property. Morphology is the first characteristic to be recognized and recorded including cell-, colony-characteristics, and Gram staining. Physiological characteristic and biochemical property describe main physical characteristics of bacteria such as growth conditions, pH, NaCl concentration, components of media,

oxygen requirement, and generate characteristic reaction profiles of substrate utilization. However, limitation of the phenotypic characteristics is lack of resolution at below genus level.

2.4.2 Chemotaxonomic characteristic

Chemotaxonomic characteristic, property of cell-chemical composition, is widely used for identification and classification of prokaryotes. One of the reasons for usefulness of the chemotaxonomic characteristic is accurate and reproducible data to be rapidly obtained.

Lipids are structure that play many important roles in cell. Various kinds of lipids such as fatty acids, mycolic acids, polar lipids, and isoprenoid quinones have been analyzed to provide strong support for bacterial systematics. The use of cellular fatty acid pattern for identification of bacteria is easily and quickly. Fatty acids of bacteria are in the range of C12 to C20; however, fatty acid composition may be affected by many factors such as growth temperature, pH, medium composition, cultivation period and cultural age, etc. Thus, comparison of fatty acid composition within a group of bacteria, all strains have to be cultured in the same conditions.

Isoprenoid quinones have played a crucial role in electron transport of bacteria. Different quinone types depends on different species of bacteria; most strictly aerobic, Gram-negative bacteria produce only ubiquinones, whereas facultatively anaerobic, Gram-negative bacteria additionally contain menaquinones and/or demethylmenaquinones. Aerobic and facultatively anaerobic, Gram-positive bacteria produce only menaquinones. Strictly anaerobic bacteria lack isoprenoid quinones or contain only menaquinones (Schleifer, 2008).

Polar lipids in cell membrane of all bacteria have an enormous variety of structures and are used as strong evidence to support species classification of bacteria (Tindall *et al*., 2010; Rainey, 2011). The most common polar lipids that found in bacteria are phospholipids (phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG)), glycolipids (GLs) and glycophospholipids (GPLs), aminolipids (ALs) and sulfur-containing lipids. To compare polar lipid pattern, bacterial polar lipid is analyzed by two-dimensional thin layer chromatography followed by visualization of spots with several specific reagents.

2.4.3 Genotypic characteristics

Molecular biological technique (molecular tools and sequence databases) is one of strategies to increase number of known microbial species; therefore, bacteria may be grouped using many different methods. The most common analysis such as G+C content, DNA-DNA hybridization, $(GTG)_5$PCR analysis and 16S rRNA gene sequence analysis have been widely used for identification of bacteria. Currently, whole-genome analysis is used to better understand bacterial metabolic capabilities, to compare between related species; thus, selection for appropriate techniques for identification of bacteria is important.

2.4.3.1 $(GTG)_5$ PCR analysis

$(GTG)_5$ PCR is a type of repetitive extragenic palindromic (rep)-PCR that useful to differentiate bacteria by using specific primer which is compatible with conserved repetitive sequences distributed in bacterial genomes. Difference size of DNA amplified products consisting of sequence between repetitive element can be fractionated by electrophoresis which showed as DNA fingerprint pattern. Comparison of the DNA fingerprint pattern is used for determination of genetic relationship between

strains. Therefore, $(GTG)_5$ PCR has become one of the highly powerful molecular tools applicable for identification of bacteria and differentiation of bacterial strains in the same species of broad range of Gram-negative bacteria and a narrow range of Gram-positive bacteria. This method is simple, rapid, inexpensive, and has a high discriminatory power. It is suitable for a high throughput of strains and reliable tool for classifying and typing a wide range of Gram-negative and many Gram-positive bacteria (Versalovic *et al*., 1994; Olive and Bean, 1999).

2.4.3.2 DNA-DNA hybridization

DNA-DNA hybridization (DDH) technique of genomic DNA is recognized as the genotypic gold standard for DNA comparison of prokaryotes. It is stable classification than the phenotypic similarities (Krieg, 1988). This technique has accomplished by comparison similarity of overall DNA base composition of prokaryotes which obtained by measurement of extent and/or stability of hybrid double-stranded DNA resulting from a denatured mixture of DNAs incubated under stringent conditions that allow only renaturation of complementary sequences. The DDH similarity of approximately 70% considered as recommended separation for defining bacterial species (Wayne *et al*., 1987). Nevertheless, this technique has often been criticized as complicated methodology with inherently large degree of error and failure to generate cumulative database (Stackebrandt, 2003). Hence, replacement of the DDH technology (wet lab) with alternative methods that accurate and reproducible is needed.

2.4.3.3 16S rRNA gene sequence analysis

Comparison of small subunit ribosomal RNA (16S) is considered as the gold standard that has been widely used in the identification, determining polygenetic position of bacteria, and studying phylogenetic relationship of bacteria. As it is a

conserved region that exists in all bacteria, function of the 16S RNA gene over time has not changed, and gene sequence is long (1,500 bp) enough for informatic purpose. Moreover, the 16S rRNA gene can be determined rather easily and quickly, provides the highest level of resolution and inexpensive. Two bacterial strains that shared 16S rRNA gene sequence similarity lower than 98.7% considered as belonging to different species, and to a distinct genus if the similarity value was below 95% (Stackebrand and Ebers, 2006) (Figure 2.9). However, there are several arguments that this threshold for new species is not applicable to many of the current bacterial species with validly published names (Fox *et al*., 1992; Beye *et al*., 2018) and resolution at species level is often not enough for some bacterial species. Therefore, species level delineation was better accommodated by comparison of genome sequences and chemotaxonomic characteristics (Konstantinidis and Tiedje, 2005; Kämpfer and Glaeser, 2012).

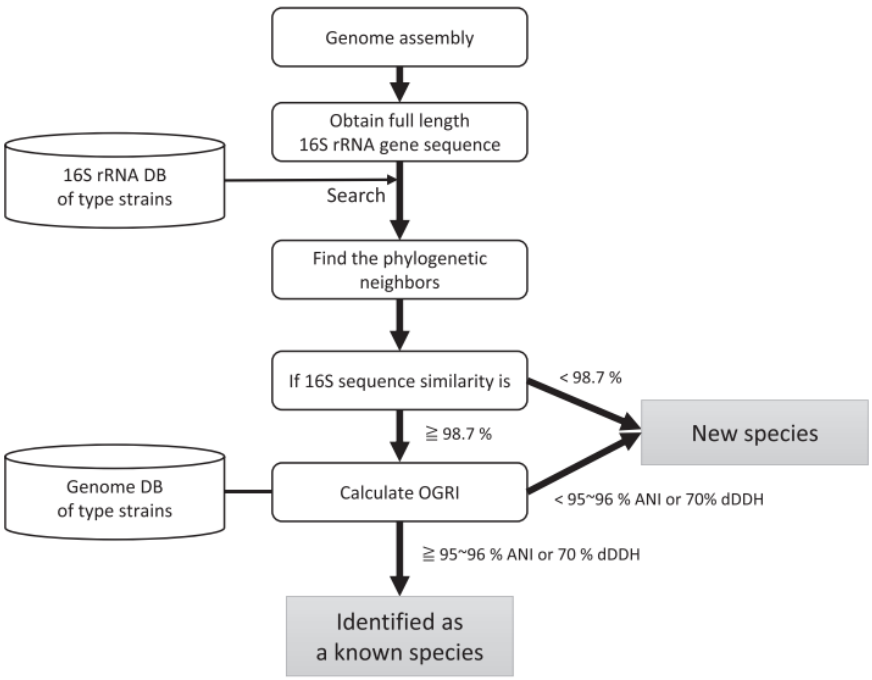

Figure 2.9 Workflow of genome based on classification at the species level. To recognize new genera, phylogenomic tree should be used (Chun et al., 2018).

2.4.3.4 Whole genome analysis

Genome sequence can be used to clearly classify and to infer phylogenetic relationship among prokaryotes. Currently, pairwise comparison of complete genome sequence of all conserved genes between any two genomes which is calculated by bioinformatic method and showed an average nucleotide identity (ANI) value replace DDH (wet lab) for differentiating bacterial species (Kostantinidis and Tiedje, 2005; Goris *et al*., 2007). Due to the ANI has high resolution and accuracy among closely related genomes and is easier to estimate and faster than DNA-DNA hybridization. Goris *et al*., 2007 demonstrated that the ANI correlated well with 16S rRNA sequence identity and DNA-DNA similarity values. It has also been shown that 96% ANI value corresponds to 70% DDH value (Richter and Rosselló-Móra, 2009). Software tools are readily available as web-services and as standard tools for calculation of overall genome relatedness indices (OGRIs) including digital dDDH and ANI values were genome-to-genome distance calculator (GGDC) version 2.1 online service with the recommended formula 2 (Meier-Kolthoff *et al*., 2013) and either BLASTn (ANIb) or MUMMER (ANIm) software on Jspecies (http://imedea.uib-csic.es/jspecies/) online service (Richter *et al*. 2016), respectively as shown in Fig 2.10. Moreover, prediction and annotation of genomes, classification, and analysis of functional genes in genome, and construction of circular genomic map have been done by the Prokka version 1.13 software (Seemann, 2014), rapid annotation of microbial genomes by subsystem technology (RAST) server and SEED Viewer (Aziz *et al*., 2008; Overbeek *et al*., 2014), and CG view sever (Stothard and Wishart, 2005), respectively. Polyphasic taxonomic approach combining phenotypic, chemotaxonomic, and genotypic data is at present most probably the best approach for classifying and identifying of bacteria.

Algorithm	Function	Type	URL/Reference
OrthoANI with usearch	Calculation of ANI	Standalone	https://www.ezbiocloud.net/tools/orthoaniu [9]
OrthoANI with usearch	Calculation of ANI	Web service	https://www.ezbiocloud.net/tools/ani [9]
Genome-to-Genome Distance Calculator	Calculation of dDDH	Web service	http://ggdc.dsmz.de/ggdc.php/ [7]
ANI calculator	Calculation of ANI	Web service	http://enve-omics.ce.gatech.edu/ani/
JSpecies	Calculation of ANI	Standalone	http://imedea.uib-csic.es/jspecies/ [5]
JSpeciesWS	Calculation of ANI	Web service	http://jspecies.ribohost.com/ [30]
CheckM	Checking contamination	Standalone	http://ecogenomics.github.io/CheckM/ [29]
ContEst16S	Checking contamination	Web service	https://www.ezbiocloud.net/tools/contest16s [28]
BBMap	Calculation of sequencing depth of coverage	Standalone	https://sourceforge.net/projects/bbmap/
Amphora2	Phylogenomic treeing	Standalone	http://wolbachia.biology.virginia.edu/WuLab/Software.html [21]
BIGSdb	Phylogenomic treeing	Standalone	https://pubmlst.org/software/database/bigsdb/ [31]
bcgTree	Phylogenomic treeing	Standalone	https://github.com/iimog/bcgTree [32]
Phylophlan	Phylogenomic treeing	Standalone	https://huttenhower.sph.harvard.edu/phylophlan[22]
UBCG	Phylogenomic treeing	Standalone	https://www.ezbiocloud.net/tools/ubcg

Figure 2.10 Web-services and standard software tools for taxonomic purposes (Chun et al., 2018).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents (appendix A)

3.2 Culture Media and Sugar (appendix A)

3.3 Instruments (appendix A)

3.4 Sample collecting and locations

Fifteen samples of plants were collected from different Provinces of Thailand (Table 3.1, Figure 3.1)

Figure 3.1 Map of Thailand.

Table 3.1 Samples and sampling sites.

Plants	Parts	Provinces	Sampling year	Geographical location (lat. N, long. E)
Sugarcane (*Saccharum officinarum* Linn.)	Root	Chumphon	2018	10.4930° N, 99.1800° E
	Root	Si sa ket	2018	15.1186° N, 104.3220° E
	Root	Sa Kaeo	2018	13.8222° N, 102.0660° E
	Root	Phetchaburi	2018	12.9649° N, 99.6426° E
	Root	Prachuap Khiri Khan	2018	11.7938° N, 99.7958° E
	Root	Nong Khai	2018	17.8783° N, 102.7413° E
Swietenia mahagoni (L.) Jacq.	Stem	Kanchanaburi[1]	2017	14.3755° N, 99.1440° E
Bambusa multiplex (Lour.) Raeusch. ex Schult.f.	Stem	Kanchanaburi[1]	2017	14.3755° N, 99.1440° E
Thyrsostachys siamensis Gamble	Stem	Kanchanaburi[1]	2017	14.3755° N, 99.1440° E
Kaempferia marginata Carey	Stem/ Leaf	Kanchanaburi[2]	2017	14.7591° N, 99.1013° E
Crateva religiosa G.Forst.	Stem	Kanchanaburi[2]	2017	14.7591° N, 99.1013° E
Toona ciliata M. Roem.	Stem	Kanchanaburi[2]	2017	14.7591° N, 99.1013° E
Phyllanthus emblica L.	Stem	Kanchanaburi[2]	2017	14.7591° N, 99.1013° E
Afzelia xylocarpa (Kurz) Craib	Stem	Kanchanaburi[2]	2017	14.7591° N, 99.1013° E

[1]Nearby Erawan National Park, [2]Si Sawat District

3.5 Isolation of endophytic bacteria

Plant samples were stored in plastic bags and transported to the laboratory for isolation within 24 h. Seventy-eight endophytic bacteria were isolated from the collected samples by the selective medium. Stems, leaves, and roots were washed with sterile water and aseptically cut into 6 cm long pieces with a sterile knife. Then the cut samples were sterilized by subsequently soaked in a series of solutions as follows: 2% sodium hypochlorite (3 min), sterile distilled water (3 min), 70% ethanol (1 min), and finally washed three times in sterile distilled water. The sterile plant samples were further cut by sterile knife into small pieces, ground, and spread on glucose/ethanol/yeast extract (GEY) agar plate (Yamada *et al*., 1999) containing 0.3%$CaCO_3$, nitrogen-free LGI (Baldani *et al*., 2014) and 1/3 dilute nutrient agar (NA) medium for isolation of acetic acid bacteria (AAB), nitrogen-fixing bacteria and other bacteria, respectively. All plates were incubated at 30°C for 3-7 days (Fig. 3.2). Resultant bacterial colonies were purified using GEY and NA medium, then kept at 4°C for further study.

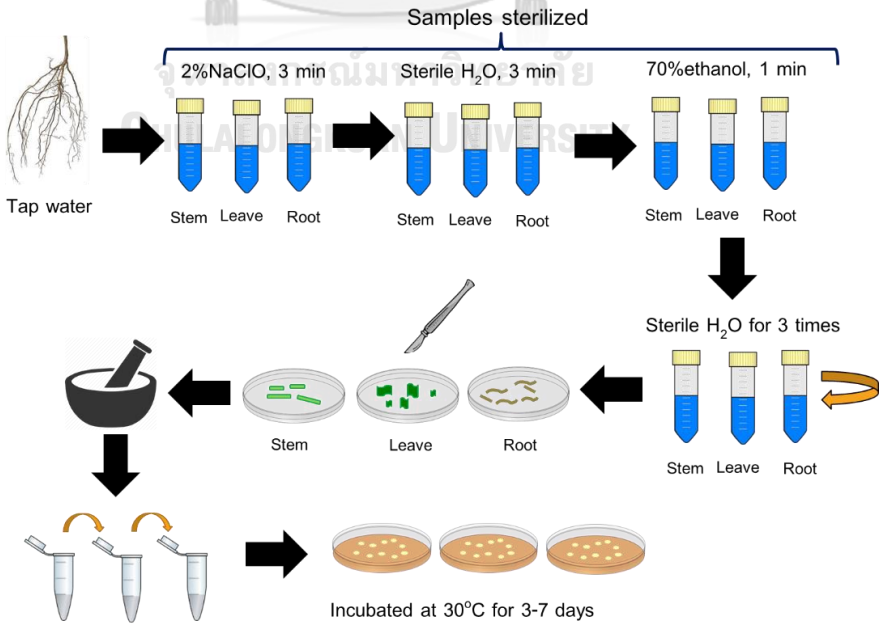

Figure 3.2 Isolation of endophytic bacteria.

3.6 Bacterial identification

3.6.1 Phenotypic characterization

All endophytic bacteria isolated were identified based on morphology (cell and colony morphologies, Gram staining), physiological characteristics including growth at various temperatures (40 and 45°C), pH (5, 6, 8 and 9), and NaCl concentrations (1%, 3% and 5%, w/v); biochemical characteristics, such as catalase and oxidase activities, hydrolysis of starch, lipid, gelatin, casein, arginine and aesculin, formation of acids from different sugars (Tanasupawat *et al*., 1998). Acetate and lactate oxidation were investigated for AAB (Asai *et al*., 1964). Morphological, physiological, and biochemical characteristics data were used for bacterial grouping shown as dendrogram illustration constructed by IBM SPSS statistics software (version 22).

3.6.2 Genotypic characterization

3.6.2.1 $(GTG)_5$-PCR analysis

Repetitive sequence-based polymerase chain reaction (Rep-PCR) using (GTG)5 primer (5´ GTGGTGGTGGTGGTG 3´) targeted against conserved repetitive sequences was performed for strain typing. The PCR reaction was carried out according to Versalovic *et al*. (1994) and Tolieng *et al*. (2018). Reaction mixture consisted of 10 × PCR reaction buffer containing 20 mM $MgCl_2$, 50 ng DNA, 20 pmol (GTG)5 primer, 2.5 mM dNTPs mixture, and 2 U Takara Taq DNA polymerase (Takara Bio Inc, Japan) in total volume 25 µL. Thermocycling program used was initial denaturation at 95°C for 5 min followed by 30 cycles each of denaturation at 94°C for 45 s, annealing at 40°C for 60 s, and primer extension at 65°C for 10 min, then followed by a final extension at 65°C for 20 min using Bio-Rad T100 PCR thermal cycler (Bio-Rad Laboratories, Inc., USA). PCR product (10 µL) was analyzed by electrophoresis using 1% agarose gel (15x25 cm) with

constant voltage of 150 V in 0.5X Tris-boric acid-ethylenediaminetetraacetic acid (TBE) buffer for 2.20 h at 25°C. The gel was stained in 0.5 µg/mL ethidium bromide solution and 100 bp to 10000 bp GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, USA) was used as a molecular size marker. The gel was visualized under a UV transilluminator using Gel document™ XR+ imaging system (Bio-Rad Laboratories, Inc., USA). To investigate for genetic relationship among an endophytic bacterial isolate, the resulting fingerprints were analyzed using software package, GelCompar II version 5.10 (Applied Maths, Belgium). The dendrogram for all isolates was generated by cluster analysis using unweighted pair group method with average linkage (UPGMA) clustering algorithm. A clustering level of 80% was regarded as a significant grouping (Gevers *et al*., 2001; Tolieng *et al*., 2018).

3.6.2.2 Analysis of 16S rRNA gene sequence and phylogenetic tree construction

DNA extraction

Genomic DNA was extracted according to the modified methods of Marmur (1961), Saito & Miura (1963) and Ezaki *et al*. (1983). Loopful of bacterial cells were suspended in 360 µL TE buffer (pH 8.0), small amount lysozyme was added and incubated at 37°C for 30 min. After addition of 40 µL of 10% SDS, the tube was inverted 5-6 times and incubated at 55°C for 10 min, then, 400 µL phenol : chloroform : isoamyl alcohol (25 : 24 : 1) was added, mixed and centrifuged at 13,000 rpm for 15 min. Resultant upper solution was transferred to new microtube, mixed with 1/10 volume of 3 mM sodium acetate followed by 2 times volume of cold ethanol. DNA was pooled by glass rod, dried, dissolved in 100-200 µL of sterile distilled water and stored at -20°C until used.

Amplification and DNA sequence analysis

The 16S rRNA gene was amplified by universal primers “20F” and “1500R” at 10 µL volume for condition optimization and 100 µL for sequencing. PCR reaction composition is shown in Table 3.2 and PCR condition is in Table 3.3. PCR products were analyzed by 1% agarose gel electrophoresis using 1 kb DNA ladder as DNA marker (enzynomics, Korea).

Table 3.2 PCR reaction mixture.

	Stock solutions	Total volume (µL)	
		10	100
Primers*			
20 F	10 pmol / µL	0.5	4
1500R	10 pmol / µL	0.5	4
dNTP	2.0 mM	1.0	10.0
10X *Tag* buffer	10X	1.0	10.0
$MgCl_2$	25 mM	0.8	8.0
Tag DNA polymerase	5 unit / µL	0.05	0.5
dH_2O	-	5.65	59.5
DNA Template	Undiluted 10^{-1}, 10^{-2}	0.5	4.0

*Primers:

Forward: 20F (5’ GAGTTTGATCCTGGCTCAG 3’)

Reward: 1500R (5’ GTTACCTTGTTACGACTT 3’)

Table 3.3 PCR condition.

94°C	3 min	
94°C	1 min	25 cycles
50°C	1 min	
72°C	2 min	
72°C	3 min	
Total time	2.5 h	

DNA sequencing and phylogenetic analysis

PCR products were sequenced by Macrogen®Korea. Universal primer 800R was used for sequencing partial 16S rRNA region and 4 universal primers, 800R, 27F, 518F, and 1492R (appendix B) were used for sequencing 16S rRNA full length. All 16S rRNA gene sequences were blasted with database obtained from NCBI GenBank. The sequences of reference strains were selected from maximum identity score and further aligned by multiple alignment software program, ClustalW. The alignment was manually edited to remove gaps and ambiguous nucleotides before phylogenetic trees were constructed by neighbor-joining (NJ) (Saitou and Nei, 1987), maximum-likelihood (ML) (Felsenstein, 1981), and maximum-parsimony (MP) (Kluge and Farris, 1969) methods using program MEGA11 (Koichiro *et al*., 2021). Evolutionary distances among the strains were computed by Kimura's two-parameter method (Kimura, 1980) for NJ and ML and subtree-pruning-regrafting method for the MP. Confidence values of individual branch in the phylogenetic tree were determined by bootstrap analysis with 1000 replications. Sequence similarity values among the closest strains were determined using EzTaxon server (Yoon *et al*., 2017).

3.6.3 Genome sequencing of selected strains, assembly, and comparisons

Genomic DNA of strain Sx8-8, PS25 and Sx8-5^{T} were extracted by GenepHlow™ Gel/PCR kit (Geneaid, Taiwan). Quality of the extracted DNA was examined using NanoDrop instrument (NanoDrop 2000c Spectrophotometer, Thermo Fisher Scientific Inc., USA) and agarose gel electrophoresis. Genome sequencing was performed using an Illumina MiSeq sequencer at Omics Sciences & Bioinformatics Center, Chulalongkorn University. The assembled genome was achieved using Unicycler (Wick *et al*., 2017),

gene prediction and annotation of the genomes were determined by Prokka software version 1.13 (Seemann, 2014). RAST server (http://rast.nmpdr.org/) for rapid annotation (Aziz *et al*., 2008), SEED Viewer (Overbeek *et al*., 2014) and CG view sever (Stothard and Wishart, 2005) were used for construction of genome circular map and investigation of metabolic features, functional genes, and analysis. A phylogenomic tree construction was achieved using the automated Multi-Locus Species Tree (autoMLST) (https://automlst.ziemertlab.com/) online server (Alanjary *et al*., 2019). Comparative analyses between genome of selected bacterial strains and their closely related type strains were estimated based on average nucleotide identity (ANI), using either BLASTn (ANIb) or MUMMER (ANIm) and digital DNA-DNA hybridization (dDDH). Calculation of ANI, and dDDH were achieved by JspeciesWS web service (Richter *et al*., 2016) and Genome-to-Genome Distance Calculator (GGDC 2.1) by BLAST+ method (Meier-Kolthoff *et al*., 2013), respectively.

3.6.3.1 Phenotypic approach for novel species

Cell morphology of strain Sx8-5^T

Cells of strain Sx8-5^T was grown in NB at 25°C for 20 h were used for investigation of morphology, cell size and presence of flagella by flagella staining (Forbes, 1981) and by transmission electron microscope (TEM) (Hitachi HT-7700). Phenotypic comparison between strain Sx8-5^T and its closely related type strains (Table 3.4) was done under the same conditions. All strains were examined for growth on different media, including tryptic soy agar (TSA), R2A, Luria-Bertani (LB) agar and nutrient agar (NA) at 28°C for 48-96 h. Growth at various pH (5, 6, 8 and 9), temperatures (40°C and 45°C) and NaCl concentrations (1, 3 and 5%, w/v) were performed at 28°C for 48 h in buffered NB, NA, and NB, respectively. Catalase and oxidase tests, hydrolysis of starch, gelatin, urea,

Tween 80 and aesculin, and acid production from various sugars, L-arabinose, maltose, raffinose, and xylose, were performed as described by Tindall *et al*. (2007). The biochemical test was accomplished by API 20NE strips (BIOMÉRIEUX) following the manufacturer's instruction at 28°C.

Table 3.4 Closely related type strains and their accession numbers in GenBank.

No.	Closely related type strains	Accession no.
1.	*N. resinovorum* SA1^T	JFYZ00000000
2.	*N. gossypii* DSM 29615T^T	JGI: 2829944697
3.	*N. barchaimii* LL02^T	JACU00000000
4.	*N. silvae* FGD1^T	WVTD00000000
5.	*N. naphthalenivorans* NBRC 102051^T	BCTX00000000
6.	*N. panipatense* P5^T	MSQB00000000
7.	*N. lindaniclasticum* LE124^T	ATHL00000000
8.	*N. guangzhouense* SA925^T	LYMM00000000
9.	*N. mathurense* SM117^T	FVZE00000000

3.6.3.2 Chemotaxonomic analysis of strain Sx8-5^T

1) Polar lipid

Extraction and analysis of polar lipids

Polar lipid of strain Sx8-5^T grown in NB and incubated at 30°C for 24 h was extracted and analyzed by two-dimensional TLC as described by Minnikin *et al*. (1984). Briefly, 150 mg of freeze-dried cells were put into a test tube with screw cap. Three mL of methanol-0.3% NaCl (100:10) and 3 mL of petroleum ether were added and mixed for 15 min. After removal of upper layer, lower layer was mixed with 1 mL petroleum ether, then resultant upper layer was again removed. The obtained lower layer was heated at 100°C

for 5 min and immediately cooled at 37°C for 5 min then mixed with 2.3 mL of chloroform-methanol-water (90:100:30) for 1 h. Supernatant obtained after centrifugation at 3,000 rpm for 10 min was transferred to new tube and cell precipitate was extracted twice with 2.3 mL chloroform-methanol-water (50:100:40) for 3 min. After centrifugation at 3,000 rpm for 10 min, supernatant was transferred to the previous tube, mixed with 1.3 mL each of chloroform and water and recentrifuged. Lower layer was dried under N_2 gas at temperature less than 37°C.

Analysis of polar lipid by two-dimensional TLC

The polar lipid fraction was dissolved in 60 µL of chloroform-methanol (2:1), and 10 µL was applied to two-dimensional silica gel TLC using chloroform-methanol-water (65:25:4) and chloroform-acetic acid-methanol-water (40:7.5:6:2) as 1st and 2nd solvent systems, respectively.

Detection

Spraying the developed two-dimensional silica gel TLC plate with following reagents:

1) Molybdenum blue reagent (Dittmer & Lester, 1964) for detection of phospholipids (blue spot).

2) Ninhydrin reagent then heat at 110°C for 10 min for detection of phosphatidylethanolamine (PE) and its derivatives (lyso-PE, OH-PE, methyl PE).

3) Anisaldehyde reagent then heat at 110°C for 10 min for detection of glycolipids (green-yellow spots) and their lipids (blue spots).

4) Dragendorff's reagent for detection of choline-containing phospholipids (PC) eg. phosphatidylcholine (orange-red spot).

5) Phosphomolybdic acid (5%) in ethanol then heat at 130°C for 15 min for detection of total lipids.

2) Fatty acid

Cellular fatty acids of the strain Sx8-5^T was analyzed at Thailand Bioresource Research Center, National Center for Genetic Engineering and Biotechnology (BIOTEC) by gas chromatography following protocol of Sherlock Microbial Identification System (MIDI) (Sasser, 1990).

3) Ubiquinone

Ubiquinone of the strain Sx8-5^T was analyzed by HPLC according to method of Tamaoka *et al*. (1983); Komagata and Suzuki (1988). Dried cells of strain Sx8-5^T grown in NB, at 30°C for 24 h (100-300 mg) were mixed with 20 mL of chloroform-methanol (2:1, v/v) in test tube for 12 h, filtrated and the obtained filtrate was evaporated by rotary evaporator at 37°C. After addition of acetone to separate non-polar fraction from lipids, acetone solution was spot on silica gel TLC plate (preparative TLC). The TLC plate was dried, developed in benzene, then visualized under ultraviolet at 254 nm. Quinone spots were scraped from the TLC plate and transferred to tube. Methanol was added to separate the quinones from silica gel. After removal of silica gel by filtration, methanol solution was analyzed by HPLC.

3.7 Screening for potential plant growth-promoting endophytic bacteria

3.7.1 Nitrogen-fixation

Nessler's reagent method (Svehla, 1979) was used for preliminary screening for nitrogen fixing bacteria. All bacterial isolated were individually inoculated into NF broth (9 mL) at $\log_8$ CFU/mL and incubated at 30°C for 48 h. The culture was centrifuged at 3,000

rpm for 10 min and 60 µL of Nessler's reagent was mixed with 30 mL of resultant supernatant. Bacterial isolates showing yellow-orange color were marked as a nitrogen fixer and absorbance at 560 nm of the reaction was used for calculation of ammonia production compared to standard curve of ammonia (expressed as mmol/L) (appendix B).

3.7.2 Phosphorus (P) and zinc (Zn) solubilization

All bacteria isolated were examined for an ability to solubilize P and Zn on Pikovskaya's agar medium (containing 0.5% $Ca_3(PO_4)_2$) and mineral salt agar medium (containing 0.1% ZnO), respectively. The nitrogen fixing-bacteria were grown on NF agar medium, AAB on GEY and other bacteria on NA medium at 30°C for 24-48 h, then the culture-grown agar was cut into 6 mm diameter and placed on Pikovskaya's agar medium and mineral salt agar medium and incubated at 30°C for 7 days and 1 day, respectively. Clear zone around bacterial colony considered as positive result then diameter of the clear zone (solubilization) and colony were measured. Solubilization index (SI) of the bacterial isolates was determined by clear zone diameter/colony diameter (Fig. 3.3).

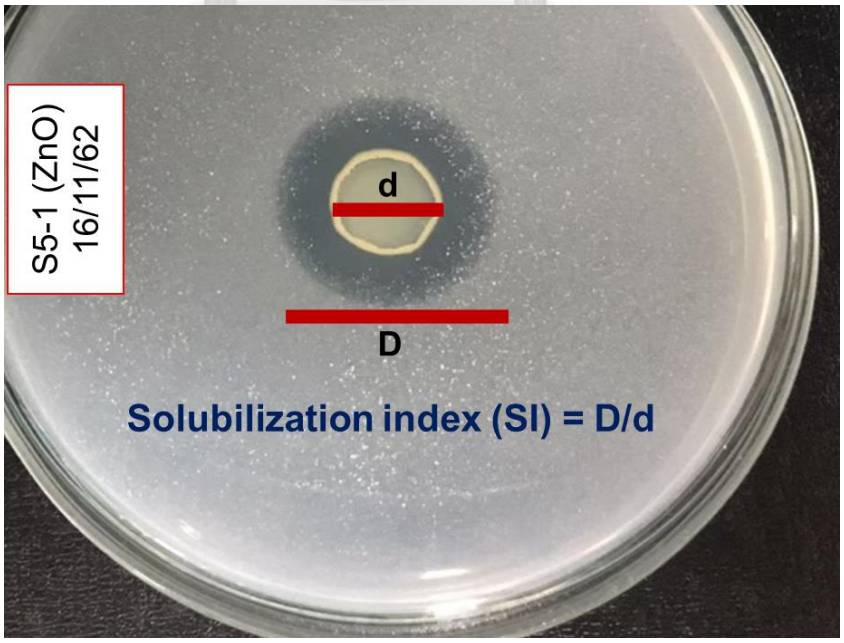

Figure 3.3 Solubilization zone of zinc oxide by isolate S5-1 grown on NA medium.

3.7.3 IAA production

Screening for indole-3-acetic acid (IAA)-producing bacteria was done by colorimetry method using Salkowski's reagent (appendix B). The endophytic bacteria isolated were inoculated into 90 mL of nitrogen free (NF) broth containing 100 µg/mL L-tryptophan at $\log_8$ CFU/mL and incubated at 30°C, 150 rpm for 24-48 h in the dark. After centrifugation at 8,000 rpm for 20 min, 70 µL of resultant supernatant was mixed with 140 µL of Salkowski's reagent and incubated at room temperature for 20 min in the dark. The appearance of pink color indicated IAA production and absorbance at 530 nm of the reaction was compared to standard curve of IAA (appendix B). An uninoculated NF broth containing tryptophan mixed with Salkowski's reagent was used as a control.

3.7.3.1 Confirmation of IAA production of selected strain by high performance liquid chromatography (HPLC)

The IAA production of selected strain was confirmed by modified method developed of Bhutani *et al*. (2018). Briefly, the selected strain was cultured in 100 mL NF broth supplemented with 100 µg/mL L-tryptophane at 30°C, 150 rpm in the dark for 48 h and then centrifuged at 8,000 rpm for 20 min. The supernatant was adjusted to pH 2.5-3 with 1N HCl and extracted twice with an equal volume of ethyl acetate. After extraction, the ethyl acetate was evaporated from the extract in vacuum rotary evaporator at 40°C and the obtained crude extract was dissolved in 2 mL methanol of HPLC grade. Analysis for the presence of IAA in the crude extract was done by reversed-phase HPLC using C18 column equipped with diode-array detector at 280 nm. Solvent system used was deionized water: methanol (55:45) at a flow rate of 1 mL/min (Alvarez *et al*., 2019). Peak was compared to standard IAA.

3.7.3.2 Optimization of IAA production

Production of IAA performed by inoculating 10 mL of selected strain (log_8 CFU/mL) in 90 mL NF broth containing 100 µg/mL L-tryptophan, pH 7 and incubating at 30°C, 150 rpm for 24 h in the dark was optimized by varying pH (4, 5, 6, 7, 8), temperature (25°C, 30°C, 35°C, 40°C), L-tryptophan concentration (0.5, 1, 1.5, 2%, w/v) and incubation time (24, 48, 72, 96, 120 h). One factor at a time method was used. The factor gave maximum IAA production was used in following experiments. Analysis of IAA production was done by Salkowski's reagent method as previously described.

3.8 Effect of selected endophytic bacteria on rice growth

An ability of selected endophytic bacteria on rice growth-promoting was determined *in vitro*. Ten milliliters of selected endophytic bacterial culture (log_8 CFU/mL) were inoculated into 90 mL of NF broth supplemented with 100 µg/mL L-tryptophan and incubated at 30°C, 150 rpm for 48 h in the dark. Two varieties of rice seed, 'RD6' and 'Khao Dawk Mali 105', were dehulled and surface sterilized by soaking in 5% sodium hypochlorite for 15 min, rinsed 5 times with sterile water. (Zhang *et al*., 2019). Then the sterile rice seeds were germinated by placing on sterile filter paper soaked with sterile water in petri dish and incubating at room temperature for 24-48 h in the dark.

3.8.1 'RD6' rice seeds

The germinated 'RD6' rice seeds were inoculated with strain Sx8-5 or S5-1 (IAA producing bacteria) by dipping in culture of the strain Sx8-5 or S5-1 for 3 h (Yu *et al*., 2016). Sterile distilled water and IAA solution at various concentrations (10, 30, and 50 µg/mL) were used as control and positive control, respectively.

3.8.2 'Khao Dawk Mali 105' rice seeds

The germinated 'Khao Dawk Mali 105' rice seeds were inoculated with strain SK2 (IAA-producing bacteria), PS25, or LSG1 (non IAA-producing bacteria) by dipping in culture of the strain SK2, PS25, or LSG1 for 3 h. Sterile distilled water and IAA standard solution (10 µg/mL) were used as control and positive control, respectively.

All inoculated rice seeds were grown on MS (Murashige and Skoog) semi solid medium and incubated at 25°C in the dark. Growth parameters of rice seedlings including total length, root length, shoot length, number of lateral roots, root fresh weight, shoot fresh weight, root dry weight, and shoot dry weight were recorded daily for 15 days.

3.8.3 Data analysis

All inoculated rice seeds were grown on MS (Murashige and Skoog) semi solid medium and incubated at 25°C in the dark. Growth parameters of rice seedlings including total length, root length, shoot length, number of lateral roots, root fresh weight, shoot fresh weight, root dry weight, and shoot dry weight were recorded daily for 15 days.

3.9 Repository

The 16S rRNA gene sequence and draft genome of bacterial strains have been submitted to DDBJ and GenBank. The type strain and Sx8-5^{T}, was deposited at Japan Collection of Microorganisms (JCM) (https://jcm.brc.riken.jp/en/) and Thailand Bioresource Research Center (TBRC).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Plant samples and Isolate number

Seventy-eight endophytic bacteria were isolated from 15 plant samples collected in Thailand. Fifty endophytic bacteria were isolated from 7 sugarcane samples obtained from 6 Provinces obtained from Chumphon (4 isolates), Si Sa Ket (2 isolates), Sa Kaeo (7 isolates), Nong Khai (6 isolates), Phetchaburi (5 isolates) and Prachuap Khiri Khan (26 isolates) (Table 4.1). Twenty-eight endophytic bacteria were isolated from 3 plant samples (*Swietenia mahagoni* (L.) Jacq., *Bambusa multiplex* (Lour.) Raeusch. ex Schult.f. and *Thyrsostachys siamensis* Gamble) collected from nearby Erawan National Park (11 isolates) and 5 plant samples (*Kaempferia marginata* Carey, *Crateva religiosa* G.Forst., *Toona ciliata* M. Roem., *Phyllanthus emblica* L., and *Afzelia xylocarpa* (Kurz) Craib) collected from Si Sawat district (17 isolates) in Kanchanaburi Province (Table 4.1).

Table 4.1 Plant samples for endophytic bacteria isolation.

No.	Sample no.	Provinces	Plant species
1.	CH1	Chumphon	*Saccharum officinarum* Linn. (root)
2.	CH2	Chumphon	*Saccharum officinarum* Linn. (root)
3.	CH3	Chumphon	*Saccharum officinarum* Linn. (root)
4.	CH4	Chumphon	*Saccharum officinarum* Linn. (root)
5.	SK1	Si Sa Ket	*Saccharum officinarum* Linn. (root)
6.	SK2	Si Sa Ket	*Saccharum officinarum* Linn. (root)
7.	PK1	Sa Kaeo	*Saccharum officinarum* Linn. (root)
8.	PK2	Sa Kaeo	*Saccharum officinarum* Linn. (root)
9.	KG1	Sa Kaeo	*Saccharum officinarum* Linn. (root)
10.	KG2	Sa Kaeo	*Saccharum officinarum* Linn. (root)
11.	KG3	Sa Kaeo	*Saccharum officinarum* Linn. (root)
12.	KG4	Sa Kaeo	*Saccharum officinarum* Linn. (root)
13.	KG5	Sa Kaeo	*Saccharum officinarum* Linn. (root)
14.	LSG1	Nong Khai	*Saccharum officinarum* Linn. (root)
15.	LTS2	Nong Khai	*Saccharum officinarum* Linn. (root)
16.	LSS3	Nong Khai	*Saccharum officinarum* Linn. (root)
17.	LSS4	Nong Khai	*Saccharum officinarum* Linn. (root)
18.	LGF5	Nong Khai	*Saccharum officinarum* Linn. (root)
19.	LRF6	Nong Khai	*Saccharum officinarum* Linn. (root)
20.	AM1	Phetchaburi	*Saccharum officinarum* Linn. (root)

Table 4.1 Plant samples for endophytic bacteria isolation (continued).

No.	Sample no.	Provinces	Plant species
21.	AM2	Phetchaburi	*Saccharum officinarum* Linn. (root)
22.	AM3	Phetchaburi	*Saccharum officinarum* Linn. (root)
23.	AM4	Phetchaburi	*Saccharum officinarum* Linn. (root)
24.	AM5	Phetchaburi	*Saccharum officinarum* Linn. (root)
25.	PS1	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
26.	PS2	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
27.	PS3	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
28.	PS4	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
29..	PS5	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
30.	PS6	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
31.	PS7	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
32.	PS8	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
33.	PS9	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
34.	PS10	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
35.	PS11	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
36.	PS12	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
37.	PS13	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
38.	PS14	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
39.	PS15	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
40.	PS16	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)

Table 4.1 Plant samples for endophytic bacteria isolation (continued).

No.	Sample no.	Provinces	Plant species
41.	PS17	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
42.	PS18	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
43.	PS19	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
44.	PS20	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
45.	PS21	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
46.	PS22	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
47.	PS23	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
48.	PS24	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
49.	PS25	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
50.	PS26	Prachuap Khiri Khan	*Saccharum officinarum* Linn. (root)
51.	A1-1	Kanchanaburi[1]	*Thyrsostachys siamensis* Gamble (stem)
52.	A1-2	Kanchanaburi[1]	*Thyrsostachys siamensis* Gamble (stem)
53.	A2-1	Kanchanaburi[1]	*Swietenia mahagoni* (L.) Jacq. (stem)
54.	A2-2	Kanchanaburi[1]	*Swietenia mahagoni* (L.) Jacq. (stem)
55.	A2-3	Kanchanaburi[1]	*Swietenia mahagoni* (L.) Jacq. (stem)
56.	A2-4	Kanchanaburi[1]	*Swietenia mahagoni* (L.) Jacq. (stem)
57.	A2-5	Kanchanaburi[1]	*Swietenia mahagoni* (L.) Jacq. (stem)
58.	A2-6	Kanchanaburi[1]	*Swietenia mahagoni* (L.) Jacq. (stem)
59.	A2-8	Kanchanaburi[1]	*Swietenia mahagoni* (L.) Jacq. (stem)
60.	A2-9	Kanchanaburi[1]	*Swietenia mahagoni* (L.) Jacq. (stem)

[1]Nearby Erawan National Park. [2]Si Sawat District, Kanchanaburi Province, Thailand

Table 4.1 Plant samples for endophytic bacteria isolation (continued).

No.	Sample no.	Provinces	Plant species
61.	A3-1	Kanchanaburi[1]	*Bambusa multiplex* (Lour.) Raeusch. ex Schult.f. (stem)
62.	S4-1	Kanchanaburi[2]	*Afzelia xylocarpa* (Kurz) Craib (stem)
63.	S5-1	Kanchanaburi[2]	*Toona ciliata* M. Roem. (stem)
64.	S5-2	Kanchanaburi[2]	*Toona ciliata* M. Roem. (stem)
65.	S5-3	Kanchanaburi[2]	*Toona ciliata* M. Roem. (stem)
66.	S6-1	Kanchanaburi[2]	*Crateva religiosa* G.Forst. (stem)
67.	S6-2	Kanchanaburi[2]	*Crateva religiosa* G.Forst. (stem)
68.	S7-2	Kanchanaburi[2]	*Phyllanthus emblica* L. (stem)
69.	S7-4	Kanchanaburi[2]	*Phyllanthus emblica* L. (stem)
70.	S7-6	Kanchanaburi[2]	*Phyllanthus emblica* L. (stem)
71.	Sl8-2	Kanchanaburi[2]	*Kaempferia marginata* Carey (leaf)
72.	Sl8-3	Kanchanaburi[2]	*Kaempferia marginata* Carey (leaf)
73.	Sl8-4	Kanchanaburi[2]	*Kaempferia marginata* Carey (leaf)
74.	Sx8-4	Kanchanaburi[2]	*Kaempferia marginata* Carey (stem)
75.	Sx8-5	Kanchanaburi[2]	*Kaempferia marginata* Carey *(stem)*
76.	Sx8-6	Kanchanaburi[2]	*Kaempferia marginata* Carey (stem)
77.	Sx8-7	Kanchanaburi[2]	*Kaempferia marginata* Carey (stem)
78.	Sx8-8	Kanchanaburi[2]	*Kaempferia marginata* Carey (stem)

[1]Nearby Erawan National Park. [2]Si Sawat District, Kanchanaburi Province, Thailand

4.2 Identification of bacteria isolated

The seventy-eight bacterial isolates were grouped and identified based on phenotypic characteristics and 16S rRNA gene sequences.

4.2.1 Identification of the 50 bacteria isolated from roots of sugarcane

The fifty bacterial isolates were rod-shaped, Gram-negative, oxidase-negative, and catalase-positive, grew at pH 5 and 6 and on 1% NaCl medium but did not hydrolyze starch and arginine. Location, isolate no., group, 16S rRNA gene sequence similarity and phenotypic characteristics of the bacteria are shown in Table 4.2 and 4.3.

Group A contained 8 isolates of rod-shaped. All isolates grew at 40 and 45°C which showed the high heat tolerance. All isolates shared high degree of 16S rRNA gene sequence similarity of 99.78-100% to *Pantoea dispersa* LMG 2603^T (Table 4.2); therefore, they were identified as *P. dispersa*.

Group B contained 42 isolates of rod-shaped which divided into 5 subgroups including subgroup B1 (37 isolates), B2 (1 isolate, LTS), B3 (2 isolates), B4 (1 isolate, LRF6) and B5 (1 isolate, SK2).

- Subgroup B1, all isolates oxidized lactate and acetate and showed 98.94-100% 16S rRNA gene sequence similarity to *Gluconacetobacter liquefaciens* IFO12388^T. Hence, they were identified as *G. liquefaciens*.

- Subgroup B2, B4, B5 did not hydrolyze casein, gelatin and aesculin. Isolate LRF6 and SK2 grew on 3 and 5% NaCl media. Isolate LTS2 and LRF6 produced acid from trehalose and arabinose but could not produce acid from lactose, raffinose and ribose. Based on 16S rRNA gene sequence similarity, isolate LTS2, LRF6 and SK2 were closely related to *Nguyenibacter vanlangensis* TN01LGI^T (100%), *Pseudomonas oryzihabitans* NBRC102199^T (100%), and *Aureimonas phyllosphaerae* L9-753^T (97.01%), respectively.

Therefore, isolate LTS2 was identified as *N. vanlangensis* and isolate LRF6 was identified as *P. oryzihabitans*. The isolate SK2 had only 97.01% 16S rRNA gene sequence similarity to *A. phyllosphaerae* which indicated a possibility to be a new species (Stackebrand and Ebers, 2006). Further study of the isolate SK2 is required.

- Subgroup B3 contained two rod-shaped, LGF5 and AM3. These 2 isolates grew on 3% NaCl medium, pH 9 and at 40°C. They hydrolyzed casein and aesculin. The isolate LGF5 and AM3 showed 100 and 99.86 % 16S rRNA gene sequence similarity to *Burkholderia anthina* R-4183^T and *Burkholderia territorii* LMG 28158^T, respectively. Therefore, they were identified as *B. anthina* and *B. territorii*, respectively.

In this study, *G. liquefaciens* was dominant species in all sugarcane root samples except from Si Sa Ket Province (Fig. 4.1 and Table 4.2).

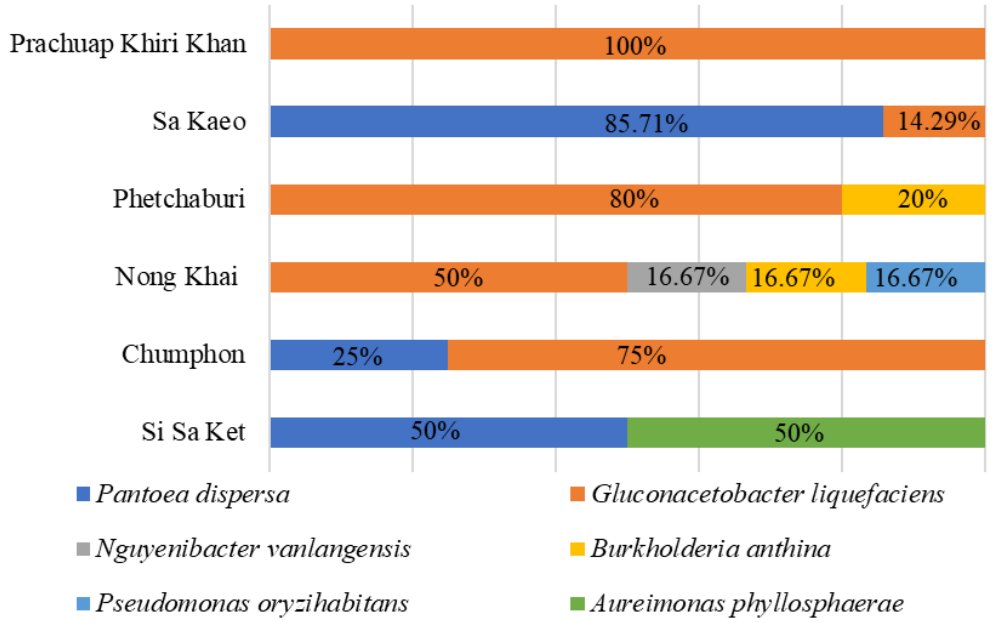

Figure 4.1 Bacterial species (%) isolated from sugarcane roots collected from 6 different provinces. Total number of isolates: Prachuap Khiri Khan (26 isolates), Sa Kaeo (7 isolates), Phetchaburi (5 isolates), Nong Khai (6 isolates), Chumphon (4 isolates), and Si Sa Ket (2 isolates) Provinces.

Table 4.2 Locations, isolate number, group, accession number, 16S rRNA gene sequence similarity (%) and nearest type strains.

Provinces	Isolate no.	Group	Accession no.	% Similarity	Nearest type strains (24/7/2022)
Si Sa Ket	SK1	A	OM742987	99.93	*P. dispersa* LMG 2603^T
Sa Kaeo	KG1	A	OM742989	99.85	*P. dispersa* LMG 2603^T
	KG2	A	OM742990	99.93	*P. dispersa* LMG 2603^T
	KG3	A	OM742991	99.78	*P. dispersa* LMG 2603^T
	KG4	A	OM742992	100	*P. dispersa* LMG 2603^T
	KG5	A	OM742993	100	*P. dispersa* LMG 2603^T
	PK1	A	OM742999	99.93	*P. dispersa* LMG 2603^T
Chumphon	CH2	A	OM743002	100	*P. dispersa* LMG 2603^T
Nong Khai	LSG1	B1	LC618513	100	*G. liquefaciens* IFO 12388^T
	LSS3	B1	LC618514	100	*G. liquefaciens* IFO 12388^T
	LSS4	B1	LC618515	100	*G. liquefaciens* IFO 12388^T
Phetchaburi	AM1	B1	OM742994	100	*G. liquefaciens* IFO 12388^T
	AM2	B1	OM742995	100	*G. liquefaciens* IFO 12388^T
	AM4	B1	OM742997	99.85	*G. liquefaciens* IFO 12388^T
	AM5	B1	OM742998	100	*G. liquefaciens* IFO 12388^T
Sa Kaeo	PK2	B1	OM743000	100	*G. liquefaciens* IFO 12388^T
Chumphon	CH1	B1	OM743001	100	*G. liquefaciens* IFO 12388^T
	CH3	B1	OM743003	100	*G. liquefaciens* IFO 12388^T
	CH4	B1	OM743004	100	*G. liquefaciens* IFO 12388^T
Prachuap Khiri Khan	PS1	B1	LC618520	100	*G. liquefaciens* IFO 12388^T
	PS2	B1	LC618521	100	*G. liquefaciens* IFO 12388^T
	PS3	B1	OM742965	99.85	*G. liquefaciens* IFO 12388^T
	PS4	B1	OM742966	100	*G. liquefaciens* IFO 12388^T
	PS5	B1	OM742967	100	*G. liquefaciens* IFO 12388^T

Table 4.2 Locations, isolate number, group, accession number, 16S rRNA gene sequence similarity (%) and nearest type strains (continued).

Provinces	Isolate no.	Group	Accession no.	% Similarity	Nearest type strains (24/7/2022)
Prachuap Khiri Khan	PS6	B1	OM742968	100	*G. liquefaciens* IFO 12388^T
	PS7	B1	OM742969	100	*G. liquefaciens* IFO 12388^T
	PS8	B1	OM742970	100	*G. liquefaciens* IFO 12388^T
	PS9	B1	OM742971	100	*G. liquefaciens* IFO 12388^T
	PS10	B1	OM742972	100	*G. liquefaciens* IFO 12388^T
	PS11	B1	OM742973	100	*G. liquefaciens* IFO 12388^T
	PS12	B1	OM742974	100	*G. liquefaciens* IFO 12388^T
	PS13	B1	OM742975	100	*G. liquefaciens* IFO 12388^T
	PS14	B1	OM742976	100	*G. liquefaciens* IFO 12388^T
	PS15	B1	OM742977	100	*G. liquefaciens* IFO 12388^T
	PS16	B1	OM742978	100	*G. liquefaciens* IFO 12388^T
	PS17	B1	OM742979	100	*G. liquefaciens* IFO 12388^T
	PS18	B1	OM742980	100	*G. liquefaciens* IFO 12388^T
	PS19	B1	OM742981	100	*G. liquefaciens* IFO 12388^T
	PS20	B1	OM742982	100	*G. liquefaciens* IFO 12388^T
	PS21	B1	OM742983	100	*G. liquefaciens* IFO 12388^T
	PS22	B1	OM742984	100	*G. liquefaciens* IFO 12388^T
	PS23	B1	OM742985	100	*G. liquefaciens* IFO 12388^T
	PS24	B1	LC618522	100	*G. liquefaciens* IFO 12388^T
	PS25	B1	OM742986	99.64	*G. liquefaciens* IFO 12388^T
	PS26	B1	LC618523	100	*G. liquefaciens* IFO 12388^T
Nong Khai	LTS2	B2	LC618516	100	*N. vanlangensis* TN01LGI^T
	LGF5	B3	LC618517	100	*B. anthina* R-4183^T
Phetchaburi	AM3	B3	OM742996	99.86	*B. territorii* LMG 28158^T
Nong Khai	LRF6	B4	LC618518	100	*P. oryzihabitans* NBRC 102199^T
Si Sa Ket	SK2	B5	OM742988	97.01	*A. phyllosphaerae* L9-753^T

Table 4.3 Phenotypic characteristics of bacteria isolated from sugarcane roots.

Characteristics	Group A	Group B				
	(8)	B1 (37)	B2 (1)	B3 (2)	B4 (1)	B5 (1)
Cell form	Rods	Rods	Rod	Rods	Rod	Rod
Growth in						
3% NaCl	+	+ (-7)	-	+	+	+
5% NaCl	+	+ (-7)	-	+(- 1)	+	+
Growth in						
pH 8	+ (-1)	+	+	+	+	+
pH 9	+ (-1)	+ (-4)	+	+	+	-
Citrate utilization	+	+(-9)	-	+	+	-
Hydrolysis of						
Aesculin	+	-	-	+	-	-
Casein	-	-	-	+	-	-
Gelatin	-	-	-	+(- 1)	-	-
Lipid	+(-2)	-	-	+(- 1)	-	+
Acid from						
L-Arabinose	+	- (+7)	+	-	+	-
Lactose	+	-	-	-	-	-
Maltose	+	-	-	-	+	-
Raffinose	+(- 6)	-	-	-	-	-
D-Ribose	+	-	-	-	-	-
D-Sorbitol	+(- 1)	-	-	+(-1)	+	-
Trehalose	+	+	+	+(- 1)	+	-

+, positive reaction; -, negative reaction. Numbers in parentheses indicate number of isolates showing the reaction.

4.2.2 Identification of the 28 bacteria isolated from plants in Kanchanaburi Province

The 11 isolates were Gram-positive and the 17 isolates were Gram-negative (Table 4.4). All isolates were catalase-positive, rod-shaped (except isolate A1-1), grew in 1% NaCl medium, at pH 6 but could not hydrolyze lipid. Eight isolates were spore-forming bacteria and could grow at 40 and/or 45°C. Based on their phenotypic characteristics and 16S rRNA gene sequence similarity, they were divided into 14 groups (Table 4.4 and 4.5).

Group A contained 4 isolates of spore-forming bacteria, A2-2, A2-3, A2-8, and A2-5. They grew in 3% NaCl medium, at 40 and 45°C and hydrolyzed starch. Isolate A2-2, A2-3 and A2-8 hydrolyzed casein, gelatin, and aesculin whereas isolate A2-5 hydrolyzed only arginine. They produced acid from several sugars. Isolate A2-2, A2-3 and A2-8 were closely related to *Priestia aryabhattai* B8W22^{T} (100%) whereas isolate A2-5 was closely related to *Priestia megaterium* NBRC 15308^{T} (100% similarity).

Group B contained 1 isolate, A3-1. This isolate grew in 3% NaCl medium, pH 5, at 40 and 45°C, and hydrolyzed starch, casein, gelatin, and aesculin but could not produce acid from any sugars. Isolate A3-1 was closely related to taxonomic group including *Brevibacillus parabrevis* NRRL NRS 605^{T}, *B. schisleri* ATCC 35690^{T}, *B. brevis* NBRC 15304^{T}, *B. choshinensis* DSM 8552^{T}and *B. reuszeri* DSM 9887^{T} (98.01-99.86% similarity).

Group C contained 1 isolate, SI8-4. This isolate grew in 3 and 5% NaCl media, pH 5 and 8, at 40 and 45°C, and produced acid from only raffinose but could not hydrolyzed starch, casein, gelatin, aesculin and L-arginine. Isolate SI8-4 was closely related to taxonomic group including *Peribacillus butanolivorans* DSM 18926 ^{T}, *P. muralis* DSM 16288^{T} and *P. simplex* NBRC 15720^{T} (98.37-98.64% similarity).

Group D contained 2 isolates, S6-1, and S6-3. They grew in 3 and 5% NaCl media, and pH 5. They hydrolyzed only aesculin and produced acid from various sugars. Isolate S6-1 and S6-3 were closely related to taxonomic group including *Curtobacterium citreum* DSM 20528^{T}, *C. oceanosedimentum* ATCC 31317^{T}, *C. albidum* DSM 20512^{T}, *C. flaccumfaciens* LMG 3645^{T}and *C. luteum* DSM 20542^{T} (99.22-99.85% similarity).

Group E contained 1 isolate, A1-1. This isolate grew in 3 and 5% NaCl media, pH 5, 8 and 9, at 40^{o}C, and produced acid from various sugars, while hydrolyzed only gelatin. Isolate A1-1 was closely related to taxonomic group including *Staphylococcus hominis* subsp. *Hominis* DSM 20328^{T}, *S. hominis* subsp. *novobiosepticus* GTC 1228^{T}, *S. borealis* 51-48^{T}and *S. haemolyticus* MTCC 3383^{T} (99.02-99.86% similarity).

Group F contained 6 isolates, A1-2, S5-1, S5-3, Sx8-4, Sx8-6, and Sx8-7. They could grow in 3 and 5% NaCl media, pH 5, 8 and 9, and at 40 and 45^{o}C which exhibited tolerance to high temperature, hydrolyzed aesculin, and produced acid from various sugars except raffinose and xylose. All isolates were closely related to *Pantoea dispersa* LMG 2603^{T} (100% similarity).

Group G contained 2 isolates, S7-2, and S7-4. They grew in 3 and 5% NaCl media, pH 5 and 8, at 40 and 45^{o}C, hydrolyzed only arginine, and produced acid from various sugars. Isolates S7-2 and S7-4 were closely related to *Acinetobacter baumannii* ATCC 19606^{T} (100% similarity).

Group H contained 2 isolates, S4-1, and S5-2. They grew in 3 and 5% NaCl media, pH 8 and 9, at 40^{o}C, and hydrolyzed only arginine. Isolate S4-1 and S5-2 could produce acid from several sugars but could not produce acid from lactose, raffinose and sorbitol. They were closely related to *Enterobacter hormaechei* subsp. *xiangfangensis* LMG 27195^{T} (100% similarity).

Group I contained 3 isolates, A2-4, A2-6, and A2-9 which could grow in 3 and 5% NaCl media, pH 5, 8, and 9 but could not grow at 40 and 45°C. They hydrolyzed only arginine and produced acid from galactose, sucrose, fructose, and xylose. All isolates were closely related to taxonomic group including *Pseudomonas entomophila* L48^T, *P. asiatica* RYU5^T, *P. taiwanensis* BCRC 17751^T, *P. monteilii* NBRC 103158^T, *P. inefficax* JV551A3^T, *P. mosselii* CIP 105259^{T}and *P. plecoglossicida* NBRC 103162^T (99.29-99.59% similarity).

Group J contained 1 isolate, A2-10. This isolate grew at pH 5, 8, and 9, at 40°C, hydrolyzed arginine, and produced acid from sucrose, fructose, lactose, ribose, and sorbitol. Isolate A2-10 was closely related to taxonomic group including *Aneurinibacillus aneurinilyticus* ATCC 12856^T (99.79% similarity) and *A. migulanus* DSM 2895^T (99.10% similarity).

Group K contained 1 isolate, S7-6. This isolate grew at pH 5, 8, and 9, at 40°C, hydrolyzed arginine, starch, and gelatin, and produced acid from sucrose, fructose, galactose, and xylose. It was closely related to *Bacillus stercoris* JCM 30051^T (100% similarity).

Group L contained 2 isolates, SI8-2, and Sx8-5. They grew at pH 5, and 8, hydrolyzed aesculin, and produced acid from arabinose, galactose, and maltose. Isolate SI8-2 produced acid from lactose, cellobiose and fructose while the isolate Sx8-5 produced acid from xylose. They were closely related to taxonomic group including *Novosphigobium clariflavum* 164^T, *N. barchaimii* LL02^T, N. *naphthalenivorans* NBRC 10205^T, *N. resinovorum* NCIMB 8767^T, AKFJ_s, *N. panipatense* SM16^T, *N. mathurense* SM117^T, *N. gossypii* JM-1396^T, CP030353_s, *N. lindaniclasticum* LE124^T and *N. silvae* FGD1^T (98.40-99.85% similarity).

Group M contained 1 isolate, SI8-5. This isolate grew at pH 5 and 8, hydrolyzed only aesculin and produced acid from arabinose and maltose. It was closely related to taxonomic group including *Pseudarthrobacter phenanthrenivorans* SWC37^{T}, *P. enclensis* NIO-1008^{T}, *P. phenanthrenivorans* Sphe3^{T}, *P. defluvii* 4C1-a^{T}, *P. niigatensis* LC4^{T}, *P. siccitolerans* 4J27^{T}, *P. chlorophenolicus* A6^{T} and *P. equi* IMMIB L-1606^{T} (98.05-99.86% similarity).

Group N contained 1 isolate, Sx8-8. This isolate grew at pH 5 and 8, hydrolyzed only aesculin, and produced acid from arabinose, maltose, galactose, and xylose. Isolate Sx8-8 was closely related to taxonomic group including S*phingobium chungbukens* DJ77^{T}, *S. estronivorans* AXB^{T}, *S. indicum* B90A^{T}, *S. aromaticivastans* UCM-25^{T}, *S. chlorophenolicum* NBRC 16172^{T} and *S. herbicidovorans* NBRC 16415^{T} (97.09-98.59% similarity).

Table 4.4 Plant samples, locations, isolate number, group, accession number, 16SrRNA gene sequence similarity (%), and the nearest type strains.

Plant samples	Parts	Isolate no.	Group	Accession no.	Similarity (%)	Nearest type strains (07/7/2021)	Taxa included in the taxonomic group (https://www.ezbiocloud.net/)
Swietenia mahagoni (L.) Jacq.[1]	Stem	A2-2	A	LC515709	100	*P. aryabhattai* B8W22^T	*P. aryabhattai*, *P. megaterium*, *P. qingshengii*, *P flexa*
Swietenia mahagoni (L.) Jacq.[1]	Stem	A2-3	A	LC515710	100	*P. aryabhattai* B8W22^T	
Swietenia mahagoni (L.) Jacq.[1]	Stem	A2-8	A	LC515714	100	*P. aryabhattai* B8W22^T	
Swietenia mahagoni (L.) Jacq.[1]	Stem	A2-5	A	LC515712	100	*P. megaterium* NBRC 15308^T	
Bambusa multiplex (Lour.) Raeusch. ex Schult.f.[1]	Stem	A3-1	B	LC515716	99.86	*B. parabrevis* NRRL NRS 605^T	*B. parabrevis*, *B. schisleri*, *B. brevis*, *B. choshinensis*, *B. reuszeri*
Kaempferia marginata Carey[2]	Leaf	SI8-4	C	LC618511	98.64	*P. butanolivorans* DSM 18926^T	*P. butanolivorans*, *P. muralis*, *P. simplex*
Crateva religiosa G.Forst.[2]	Stem	S6-1	D	LC515721	99.85	*C. citreum* DSM 20528^T	*C. citreum*, *C. oceanosedimentum*, *C. albidum*, *C. flaccumfaciens*, *C. luteum*
Crateva religiosa G.Forst.[2]	Stem	S6-3	D	ON387760	99.51	*C. oceanosedimentum* ATCC 31317^T	
Thyrsostachys siamensis Gamble[1]	Stem	A1-1	E	LC515706	99.86	*S. hominis* subsp. *hominis* DSM 20328^T	*S. hominis* subsp. *hominis*, *S. hominis* subsp. *novobiosepticus*, *S. borealis*, *S. haemolyticus*
Thyrsostachys siamensis Gamble[1]	Stem	A1-2	F	LC515707	100	*P. dispersa* LMG 2603^T	*P. dispersa*, *P. coffeiphila*, RMVG_s, *P. wallisii*, *P. eucrina*
Toona ciliata M. Roem.[2]	Stem	S5-1	F	LC515718	100	*P. dispersa* LMG 2603^T	
Toona ciliata M. Roem.[2]	Stem	S5-3	F	LC515720	100	*P. dispersa* LMG 2603^T	
Kaempferia marginata Carey[2]	Stem	Sx8-4	F	LC515729	100	*P. dispersa* LMG 2603^T	
Kaempferia marginata Carey[2]	Stem	Sx8-6	F	LC515731	100	*P. dispersa* LMG 2603^T	
Kaempferia marginata Carey[2]	Stem	Sx8-7	F	LC515732	100	*P. dispersa* LMG 2603^T	

[1]Nearby Erawan National Park, [2]Si Sawat District, Kanchanaburi Province, Thailand

Table 4.4 Plant samples, locations, isolate number, group, accession number, 16SrRNA gene sequence similarity (%), and the nearest type strains (continued).

Plant samples	Parts	Isolate no.	Group	Accession no.	Similarity (%)	Nearest type strains (07/7/2021)	Taxa included in the taxonomic group (https://www.ezbiocloud.net/)
Phyllanthus emblica L.[2]	Stem	S7-2	G	LC515724	100	*A. baumannii* ATCC 19606^T	*A. baumannii*, *A. nosocomialis*, *A. seifertii*, MVKX_s,
Phyllanthus emblica L.[2]	Stem	S7-4	G	LC515725	100	*A. baumannii* ATCC 19606^T	OVCN_s, *A. halotolerans*
Afzelia xylocarpa (Kurz) Craib[2]	Stem	S4-1	H	LC515717	100	*E. hormaechei* subsp. *xiangfangensis* LMG 27195^T	*E. hormaechei* subsp. *xiangfangensis*, *E. quasihormaechei*, *E. hormaechei* subsp. *steigerwaltii*,
Toona ciliata M. Roem.[2]	Stem	S5-2	H	LC515719	100	*E. hormaechei* subsp. *xiangfangensis* LMG 27195^T	*E. hormaechei* subsp. *oharae*, *E. hormaechei* subsp. *hoffmannii*
Swietenia mahagoni (L.) Jacq.[1]	Stem	A2-4	I	LC515711	99.58	*P. entomophila* L48^T	*P. entomophila*, *P. asiatica*, *P. taiwanensis*,
Swietenia mahagoni (L.) Jacq.[1]	Stem	A2-6	I	LC515713	99.59	*P. entomophila* L48^T	*P. monteilii*, *P. inefficax*, *P. mosselii*, *P.
Swietenia mahagoni (L.) Jacq.[1]	Stem	A2-9	I	LC515715	99.59	*P. entomophila* L48^T	*plecoglossicida*
Swietenia mahagoni (L.) Jacq.[1]	Stem	A2-10	J	ON387758	99.79	*A. aneurinilyticus* ATCC 12856^T	*A. aneurinilyticus, A. migulanus*
Phyllanthus emblica L.[2]	Stem	S7-6	K	LC618510	100	*B. stercoris* JCM 30051^T	*B. stercoris*, *B. rugosus*, *B. spizizenii*, *B. tequilensis*, *B. halotolerans*, *B. cabrialesii*, *B. inaquosorum*
Kaempferia marginata Carey[2]	Leaf	SI8-2	L	LC618512	99.85	*N. clariflavum* 164^T	*N. clariflavum, N. naphthalenivorans, N. resinovorum,* AKFJ_s, *N. panipatense,*
Kaempferia marginata Carey[2]	Stem	Sx8-5	L	LC662743	99.35	*N. barchaimii* LL02^T	*N. barchaimii, N. mathurense, N. gossypii,* CP030353_s, *N. lindaniclasticum, N. silvae*

[1]Nearby Erawan National Park, [2]Si Sawat District, Kanchanaburi Province, Thailand

Table 4.4 Plant samples, locations, isolate number, group, accession number, 16SrRNA gene sequence similarity (%), and the nearest type strains (continued).

Plant samples	Parts	Isolate no.	Group	Accession no.	Similarity (%)	Nearest type strains (07/7/2021)	Taxa included in the taxonomic group (https://www.ezbiocloud.net/)
Kaempferia marginata Carey[2]	Leaf	SI8-5	M	ON387759	99.86	*P. phenanthrenivorans* SWC37^T	*P. phenanthrenivorans*, *P. enclensis*, *P.defluvii*, *P. niigatensis*, *P. siccitolerans*, *P. chlorophenolicus*, *P. phenanthrenivorans*, *P. equi*
Kaempferia marginata Carey[2]	Stem	Sx8-8	N	OM865399	98.59	*S. chungbukens* DJ77^T	*S. chungbukense*, *S. estronivorans*, *S. indicum*, *S. aromaticivastans*, *S. chlorophenolicum*, *S. herbicidovorans*

[1]Nearby Erawan National Park, [2]Si Sawat District, Kanchanaburi Province, Thailand

Table 4.5 Phenotypic characteristics of bacteria isolated from plant samples in Kanchanaburi Province.

Chracteristics	Group (number of isolates)													
	A (4)	B (1)	C (1)	D (2)	E (1)	F (6)	G (2)	H (2)	I (3)	J (1)	K (1)	L (2)	M (1)	N (1)
Cell form	Rods	Rods	Rods	Rods	Cocci	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Gram stain	+	+	+	+	+	-	-	-	-	+	+	-	-	-
Spore formation	+	+	+	-	-	-	-	-	-	+	+	-	-	-
Growth in:														
3%NaCl	+	+	+	+	+	+	+	+	+	-	-	-	-	-
5%NaCl	+ (-1)	-	+	+	+	+	+	+	+	-	-	-	-	-
pH 5	-	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 8	+	-	+	-	+	+	+	+	+	+	+	+	+	+
pH 9	- (+1)	-	-	-	+	+	-	+	+	+	+	-	-	-
Growth at:														
40°C	+	+	+	-	+	+	+	+	-	+	+	-	-	-
45°C	+	+	+	-	-	+	+	-	-	-	-	-	-	-
Oxidase Test	+	-	+	-	-	-	-	-	+	-	-	+	+	+

+, positive reaction; -, negative reaction. Numbers in parentheses indicate the number of isolates showing the reaction.

Table 4.5 Phenotypic characteristics of bacteria isolated from plant samples in Kanchanaburi Province (continued).

Chracteristics	Group (number of isolates)													
	A (4)	B (1)	C (1)	D (2)	E (1)	F (6)	G (2)	H (2)	I (3)	J (1)	K (1)	L (2)	M (1)	N (1)
Simom citrate	-	+	+	-	-	+	+	+	-	-	+	-	-	-
Hydrolysis of:														
Aesculin	+ (-1)	+	-	+	-	+	-	-	-	-	-	+	+	+
L-Arginine	- (+1)	-	-	-	-	-	+	+	+	+	+	-	-	-
Casein	+ (-1)	+	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	+ (-1)	+	-	-	+	-	-	-	-	-	+	-	-	-
Starch	+	+	-	-	-	-	-	-	-	-	+	-	-	-
Acid from:														
L-Arabinose	+	-	-	-	-	+	+	+	-	-	-	+	+	+
D-Cellobiose	- (+1)	-	-	+	-	+	+	+	-	-	-	+ (-1)	-	-
D-Fructose	+	-	-	-	+	+	-	+	+	+		+ (-1)	-	-
D-Galactose	+	-	-	-	-	+	+	+	+	-	+	+	-	+
Lactose	- (+1)	-	-	+	+	+	-	-	-	+	-	+ (-1)	-	-
Maltose	- (+1)	-	-	+	+	+	+	+	-	-	-	+	+	+

+, positive reaction; -, negative reaction. Numbers in parentheses indicate the number of isolates showing the reaction.

Table 4.5 Phenotypic characteristics of bacteria isolated from plant samples in Kanchanaburi Province (continued).

Chracteristics	Group (number of isolates)													
	A (4)	B (1)	C (1)	D (2)	E (1)	F (6)	G (2)	H (2)	I (3)	J (1)	K (1)	L (2)	M (1)	N (1)
Acid from:														
Raffinose	-	-	+	-	-	-	-	-	-	-	-	-	-	-
D-Ribose	+	-	-	+	-	+	-	+	-	+	-	-	-	-
D-Sorbitol	-	-	-	-	-	+	-	-	-	+	-	-	-	-
Sucrose	+	-	-	+	+	+	-	+	+	+	+	-	-	-
Trehalose	- (+1)	-	-	+	+	+	-	+	-	-	-	-	-	-
D-Xylose	- (+1)	-	-	+	-	-	+	+	+	-	+	+ (-1)	-	+

+, positive reaction; -, negative reaction. Numbers in parentheses indicate the number of isolates showing the reaction.

4.2.3 Strain typing of the 28 endophytic bacteria isolated from plant samples in Kanchanaburi Province

The $(GTG)_5$-PCR for grouping the 28 isolates and dendrogram of the fingerprints is shown in Fig. 4.2. DNA fragment size ranging from 300 to 8,000 bp were generated, yielded 6-25 bands, and displayed 0.5-99.5% similarity. After cluster analysis, 17 groups were defined. Majority of the isolates (21%) included 6 isolates (A1-2, S5-1, S5-3 Sx8-4, Sx8-6, and Sx8-7) were identified as *Pantoea dispersa*. The remainder were distributed in another group. The $(GTG)_5$-PCR fingerprints of the majority was classified into several groups, while those of the remainder did not form cluster based on phenotypic characteristics. This result indicated that rep-PCR is one powerful molecular tool suitable for grouping, identification, and differentiation of bacterial strains of the same species (Gomez-Gil *et al*., 2004). Moreover, the $(GTG)_5$-PCR fingerprint increases our knowledge of bacterial biodiversity in the environment.

Based on partial 16S rRNA gene sequence, the 28 isolates were identified as 14 genera. These diverse bacteria were widely distributed in *Kaempferia marginata* Carey followed by *Swietenia mahagoni* (L.) Jacq.; however, many isolates shared % similarity to related type strains below or close to the cut-off value at 98.7% (Stackebrand, and Ebers, 2006), which might be the novel species and further studies are required.

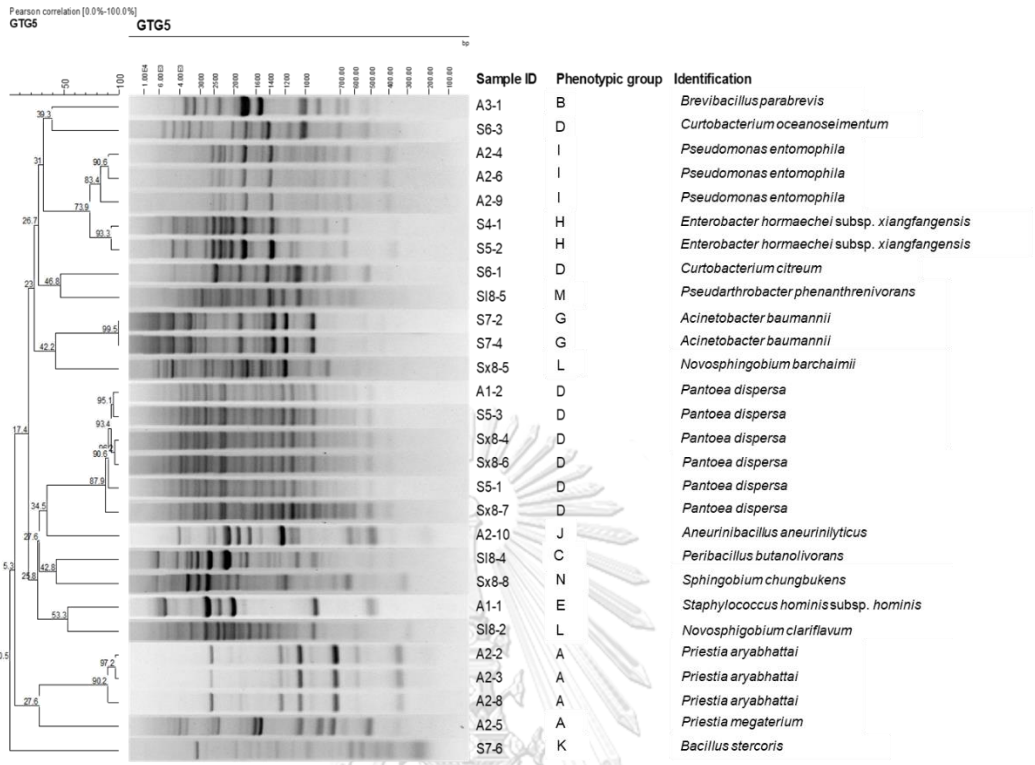

Figure 4.2 $(GTG)_5$-PCR fingerprints of the 28 isolates.

4.3 Genome sequence analysis

The strain Sx8-8, PS25, and Sx8-5 had % similarity of 16S rRNA gene sequence close to cut off level (98.7%) (Stackebrand and Ebers 2006), they might be novel species. Thus, genome analysis was performed for clear classification.

4.3.1 Genomic features of *Sphingobium* sp. Sx8-8

Strain Sx8-8 had genome size of 4.67 Mbp with 83 contigs, N50 size of 150,389 bp, an average G+C content of 64.2 mol%, protein-coding sequences (CDS) of 4,498, tRNA genes of 51, and rRNA genes of 3. Whole genome sequence has been deposited in GenBank under an accession number JAKUJY000000000. Since strain Sx8-8 was IAA-producing bacteria, PSB and ZSB (Fig 4.13, Table 4.13), and it boosted rice growth (Fig.

4.17 and Table 4.15), we concentrated on examining genes that contribute to the potential of plant growth promotion. We found that genome of this strain contained 4 genes related to auxin biosynthesis through enzymes in tryptophan utilization and IAA biosynthesis (Fig 4.3). Based on 16S rRNA gene sequence the strain Sx8-8 (Table 4.4), belonged to genus *Sphingobium* and shared the highest % similarity of 98.59% with *Sphingobium chungbukens* DJ77^{T}. Whole-genome sequence of strain Sx8-8 and its closely related type strains was compared and shown as ANI and dDDH values. The ANIb value obtained between strain Sx8-8 and *Sphingobium indicum* B90A^{T} (83.57%), *Sphingobium chungbukense* DJ77^{T} (83.05%), *Sphingobium chlorophenolicum* NBRC 16172^{T} (82.69%), which were below the cutoff at 95-96% (Chun *et al*., 2018). While the dDDH value between strain Sx8-8 and its related type strains were 27.9-29.6% (Table 4.6), which was also below than 70% threshold (Auch *et al*., 2010). According to the comparison results of genome sequence, the strain Sx8-8 was a new species of the genus *Sphingobium*, thus further characterization should be done.

Table 4.6 ANIb, ANIm and dDDH values among the whole genomes of strain Sx8-8 and its related type strains.

Query genome	Reference genomes	Hit strain	ANIb (%)	ANIm (%)	%dDDH
1	*Sphingobium indicum*	B90A^{T}	83.57	87.03	29.2
1	*Sphingobium chungbukense*	DJ77^{T}	83.05	86.85	29.6
1	*Sphingobium chlorophenolicum*	NBRC 16172^{T}	82.69	86.84	27.9

1 = Strain Sx8-8

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

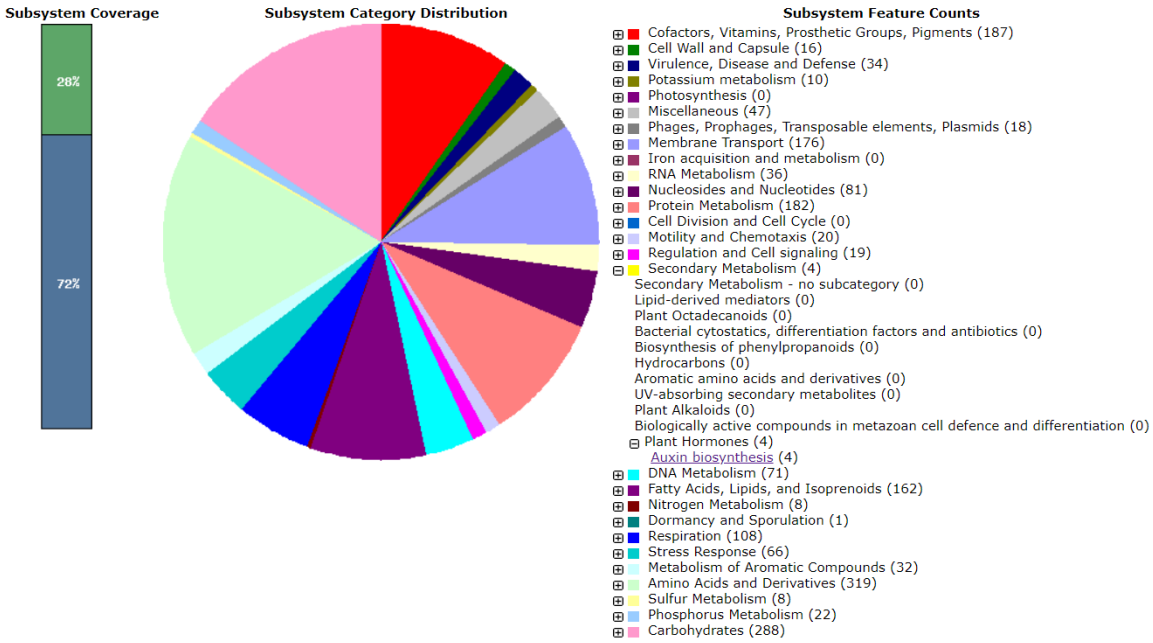

Figure 4.3 An overview of subsystem categories assigned to genes predicted in genome of strain Sx8-8. Whole-genome sequence of the strain Sx8-8 was annotated using the RAST server. The pie chart shows the counts of genes related to each subsystem. The bar graph (on the left) determines the subsystem coverage, the green bar represents the percentage of proteins that could be annotated in the SEED subsystem (28%), and the blue bar represents the proteins that were not annotated in the SEED subsystem (72%).

4.3.2 Genomic features of *Gluconacetobacter* sp. PS25

Genome analysis was done for clear classification of strain PS25. The assembled genome of strain PS25 was 4.4 Mbp in length with 98 contigs and 64.7 mol% G+C content. It has been deposited in GenBank under accession PRJNA808755 (Table 4.7). Genomic sequences of strain PS25 and its related type strains in genus *Gluconacetobacter* were selected for genome comparison and shown as ANI, using either BLASTn (ANIb) or MUMMER (ANIm) software and dDDH value. ANIb value between strain PS25 and *G. dulcium* LMG 1728^{T} was 93.77%, whereas ANIm and dDDH value were 98.74% and 88%, respectively (Table 4.8). According to the ANI criteria (Meier-Kolthoff *et al.*, 2013), this

indicated that the strain PS25 belonged to the same species with *G. dulcium* LMG 1728^{T} and was identified as *G. dulcium*.

The *G. dulcium* PS25 showed positive result for ammonium production in Nessler's reaction test and contained *nif* genes related to nitrogen fixation in genome which indicated potential in nitrogen fixation. Moreover, *G. dulcium* PS25 solubilized P and Zn (Table 4.12-4.13 and Fig 4.10).

Table 4.7 General genomic characteristics of strain PS25 and its related type strains within genus *Gluconacetobacter.*

No.	Type strains	Accession numbers	Size (Mbp)	G+C content (%)	Number of contigs	Number of genes
1	PS25	PRJNA808755	4.44	64.7	98	4417
2	*Gluconacetobacter dulcium* LMG 1728^{T}	NZ_JABEQN000000000	4.42	64.7	76	3,993
3	*Gluconacetobacter liquefaciens* NBRC 12388^{T}	NZ_BJMI00000000.1	4.16	64.4	78	3,728
4	*Gluconacetobacter takamatsuzukensis* LMG 27800^{T}	NZ_JABEQK000000000	3.78	67.0	35	3,377
5	*Gluconacetobacter sacchari* LMG 19747^{T}	NZ_JABEQJ000000000	4.83	66.0	114	4,570
6	*Gluconacetobacter tumulicola* LMG 27725^{T}	NZ_JABEQL000000000	4.3	65.1	90	3,875
7	*Gluconacetobacter asukensis* LMG 27724^{T}	NZ_JABEQE000000000	4.39	65.2	64	3,940
8	*Gluconacetobacter aggeris* LMG 27801^{T}	NZ_JABEQD000000000	4.32	65.2	61	3,908

Table 4.8 ANIb, ANIm and dDDH values among whole genomes of strain PS25 and its related type strains.

Query genome	Reference genomes	Hit strains	ANIb (%)	ANIm (%)	%dDDH
1	*Gluconacetobacter dulcium*	LMG 1728^T	93.77	98.74	88.8
1	*Gluconacetobacter liquefaciens*	NBRC 12388^T	89.13	95.31	62.4
1	*Gluconacetobacter takamatsuzukensis*	LMG 27800^T	82.97	88.53	32.9
1	*Gluconacetobacter sacchari*	LMG 19747^T	80.39	86.85	28.9
1	*Gluconacetobacter tumulicola*	LMG 27725^T	82.63	89.23	35.8
1	*Gluconacetobacter asukensis*	LMG 27724^T	84.12	89.61	36
1	*Gluconacetobacter aggeris*	LMG 27801^T	84.26	89.52	36

1 = Strain PS25

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

4.3.3 Genomic features of *Novosphingobium kaempferiae* sp. nov. Sx8-5^T

4.3.3.1 16S rRNA gene phylogeny

The 16S rRNA gene sequence of strain Sx8-5^T was compared to those of its type strains in genus *Novosphingobium.* The result indicated that the strain Sx8-5^T had the highest 16S rRNA gene sequence similarity to *N. barchaimii* LL02^T (99.4%), while the 16S rRNA gene sequence similarity between the strain Sx8-5^T and other members in the genus *Novosphingobium* ranged from 98.8% to 98.4%, including *N. gossypii* JM-1396^T (98.8%), *N. panipatense* SM16^T (98.6%), *N. lindaniclasticum* LE124^T (98.6%), *N. naphthalenivorans* NBRC 102051^T (98.5%), *N. resinovorum* NCIMB 8767^T (98.4%) and *N. silvae* FGD1^T (98.4%). The phylogenetic analysis based on 16S rRNA gene sequence

revealed that the strain Sx8-5^T belonged to genus *Novosphingobium*. The neighbour-joining tree showed that the strain Sx8-5^T formed a cluster close to *N. barchaimii* LL02^T with a bootstrap value of 61% (Fig 4.4), the phylogenetic tree based on maximum-parsimony and maximum-likelihood also showed the same topology, but the bootstrap values were lower than 50% (appendix C). The phylogenomic analysis based on complete genome sequence of the strain Sx8-5^T were analyzed to explain its relationship with species in genus *Novosphinobium*.

4.3.3.2 Genome Features

The whole genome of strain Sx8-5^T had been submitted to GenBank under accession number CP089301. The genome size of strain Sx8-5^T was 5.7 Mbp, with 1 contig, G+C content of 66 mol %. Phylogenomic tree based on genome data showed that strain Sx8-5^T was closely related to *N. resinovorum* SA1^T, *N. barchaimii* LL02^T, *N. panipatense* P5^T, and *N. lindaniclasticum* LE124^T (Fig. 4.5). According to prediction and annotation obtained from Prokka analysis, total genes of strain Sx8-5^T were 5,083 and RNA genes were 61 (Table 4.9). Based on RAST server, 5,296 protein-coding sequences (CDs) and 55 RNA genes predicted. Genome contained many genes are essential for growth and reproduction such as amino acids and derivatives, carbohydrates, membrane transports, cofactors, vitamins, prosthetic groups, pigments, DNA metabolisms, fatty acids, lipids, isoprenoids, motility, secondary metabolites, ammonia assimilation and phosphorus metabolites (Fig.4.6).

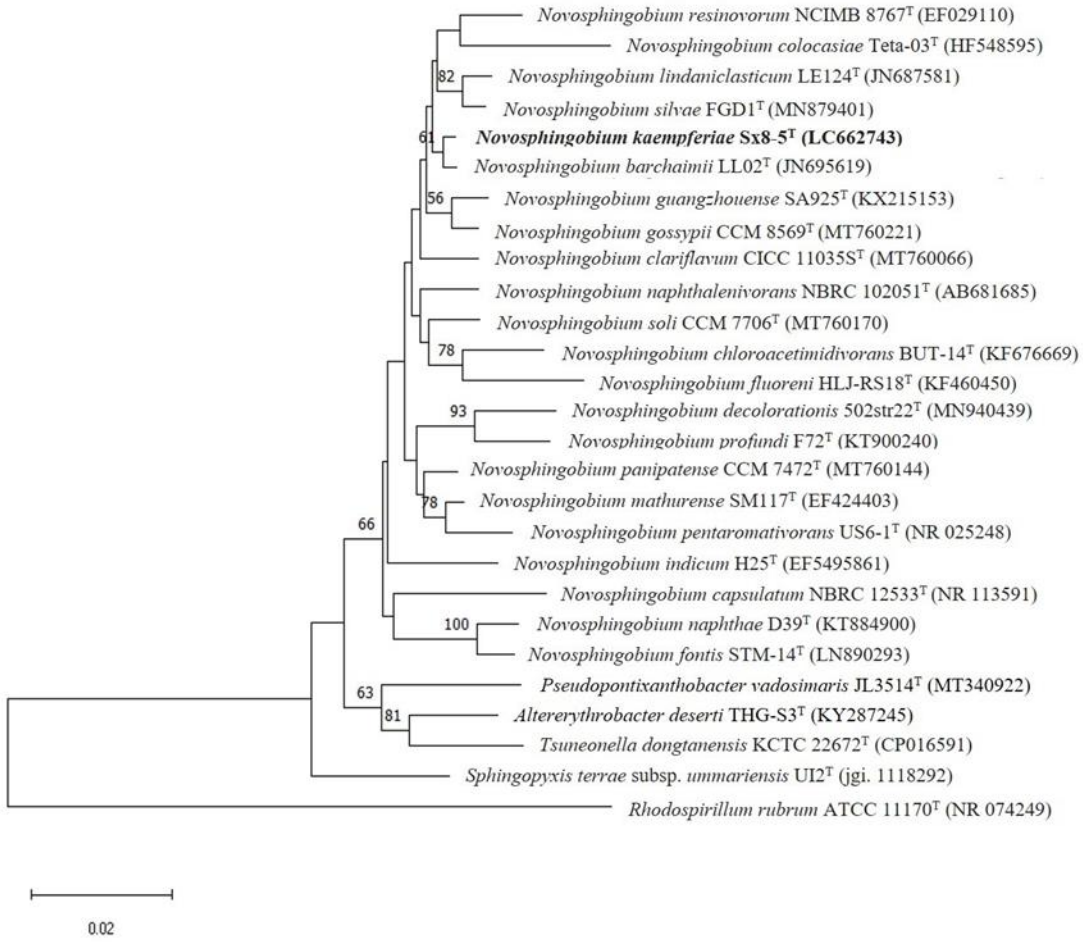

Figure 4.4 The neighbour-joining (NJ) phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain Sx8-5^T and related type strains. *Rhodospirillum rubrum* ATCC 11170^T (NR 074249) was used as an outgroup. Bootstrap values were expressed as a percentage of 1000 replications. Only bootstrap values of more than 50 % are shown. Bar, 0.02 substitutions per nucleotide position.

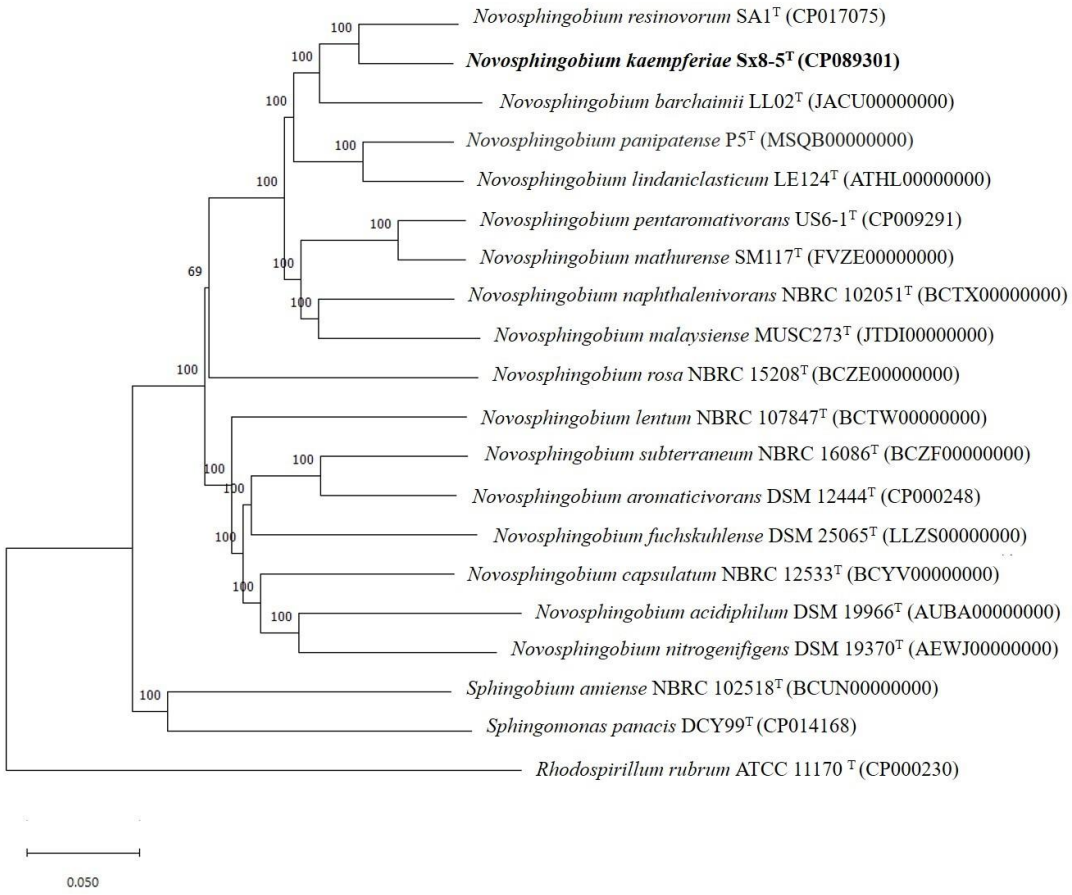

Figure 4.5 Phylogenomic tree constructed by autoMLST based on conserved core genes indicating the phylogenetic position of strain Sx8-5^{T} with the type species of the genus *Novosphingobium* and other related species. *Rhodospirillum rubrum* ATCC 11170^{T} (CP000230) was used as an outgroup. Bootstrap values were expressed as a percentage of 1000 replications. Bar, 0.05 substitutions per site.

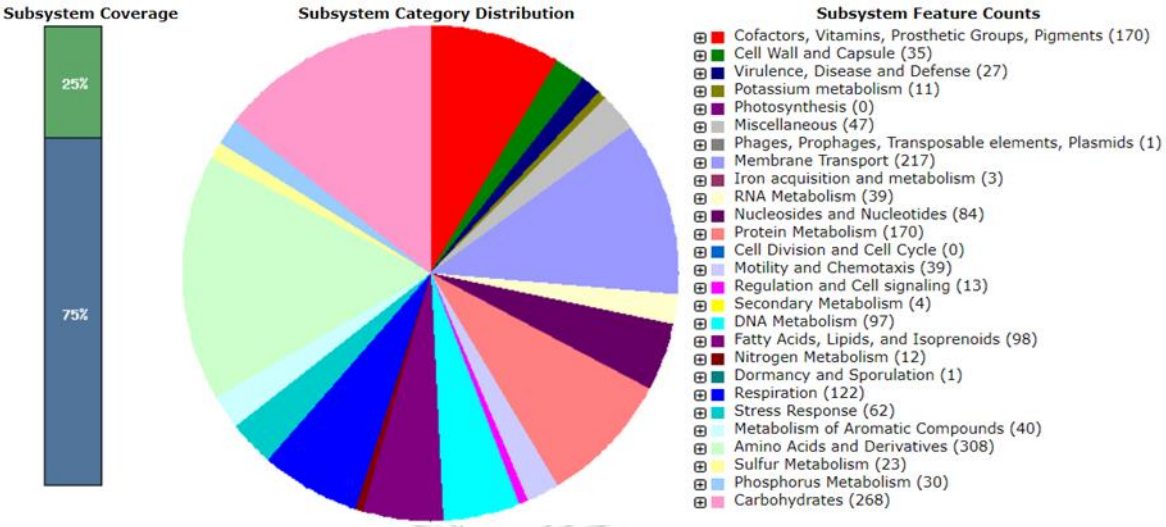

Figure 4.6 An overview of subsystem categories assigned to genes predicted in genome of strain Sx8-5^{T}. The whole-genome sequence of strain Sx8-5^{T} was annotated using the RAST server. The pie chart shows the counts of genes related to each subsystem. The bar graph (on the left) determines the subsystem coverage, the green bar represents the percentage of proteins that could be annotated in the SEED subsystem (25%), and the blue bar represents the proteins that were not annotated in the SEED subsystem (75%).

When compared to genomes of *N. resinovorum* SA1^{T}, *N. gossypii* DSM 29615^{T}, *N. barchaimii* LL02^{T}, *N. silvae* FGD1^{T}, *N. naphthalenivorans* NBRC 102051^{T}, *N. panipatense* P5^{T}, and *N. lindaniclasticum* LE124^{T}, ANIb and ANIm values of strain Sx8-5^{T} were between 72.33-82.14% and 83.82-87.38%, respectively (Table 4.9). Furthermore, calculated dDDH values of strain Sx8-5^{T} compared to genome of seven closely related type strains were 21.0-28.6%. The resulting values obtained was significantly lower than the 96% and 70% identity threshold for species delineation (Goris *et al*., 2007; Richter and Rosselló-Móra, 2009). These results indicated that strain Sx8-5^{T} might be considered as a new species of the genus *Novosphingobium*.

Table 4.9 ANIb and ANIm values (%) and digital (*in silico*) DNA-DNA hybridization (dDDH) values between whole genomes of strain Sx8-5^T and related type strains.

Strains	Accession no.	ANIb (%)	ANIm (%)	% dDDH	Genome sizes (bp)	G+C content (%)	No. Of contigs	Total genes	RNA genes
1	PRJNA781437	-	-	-	5.7	66.0	1	5,083	61
2	JFYZ00000000	82.14	87.38	28.60	6.3	65.1	115	5,881	62
3	JGI: 2829944697	80.87	86.16	26.20	4.78	64.9	31	4,384	63
4	JACU00000000	79.34	85.16	23.60	5.31	64.0	26	5,082	77
5	WVTD00000000	78.58	85.22	23.2	4.58	65.1	65	4,433	58
6	BCTX00000000	76.50	85.21	22.4	5.24	63.8	234	5,103	52
7	MSQB00000000	73.29	84.59	23.00	5.74	64.7	123	5,265	61
8	ATHL00000000	72.33	83.82	21.00	4.86	64.6	156	4,620	66

*Number of total gene and RNA gene were determined by Prokka

Strains: 1, Sx8-5^T; 2, *N. resinovorum* SA1^T, 3, *N. gossypii* DSM 29615^T,4, *N. barchaimii* LL02^T, 5, *N. silvae* FGD1^T, 6, *N. naphthalenivorans* NBRC 102051^T, 7, *N. panipatense* P5^T, 8, *N. lindaniclasticum* LE124^T.

4.3.3.3 Phenotypic characteristics

Cells of strain Sx8-5^T were rod-shaped approximately 0.6-0.7 x 1.3-1.5 µm and motile by peritrichous flagella (Fig. 4.7). Phenotypic and physiological characteristics of strain Sx8-5^T and related strains are shown in Table 4.10. Strain Sx8-5^T grew well on TSA, R2A, LB and NA under aerobic condition at 30^{o}C after 48 h cultivation. Colonies were yellow, circular, and smooth. It grew at pH 6 to 9, at 25-37^{o}C on NA and in 1% NaCl. Strain Sx8-5^T was catalase and oxidase positive like related type strains except *Novosphingobium barchaimii* DSM 25411^T, which gave negative in oxidase test. Strain Sx8-5^T and related type strains could hydrolyze aesculin which used as biochemical key

in identification of this genus but could not hydrolyze urea. Hydrolysis of tween 80 was observed at only strain Sx8-5^T. Based on the results from ANI 20 NE for 24 h, strain Sx8-5^T and related type strains assimilated D-maltose but did not assimilate capric acid, negative for hydrolysis of L-arginine, urea and gelatin, and acid production from D-glucose. Strain Sx8-5^T hydrolysed 4-nitrophenyl-β-D-galactopyranoside (PNPG) and assimilated D-glucose, L-arabinose, D-mannose, D-maltose, potassium gluconate, malic acid and trisodium citrate. According to these characteristic data, the strain Sx8-5^T was different from its seven closely related type strains.

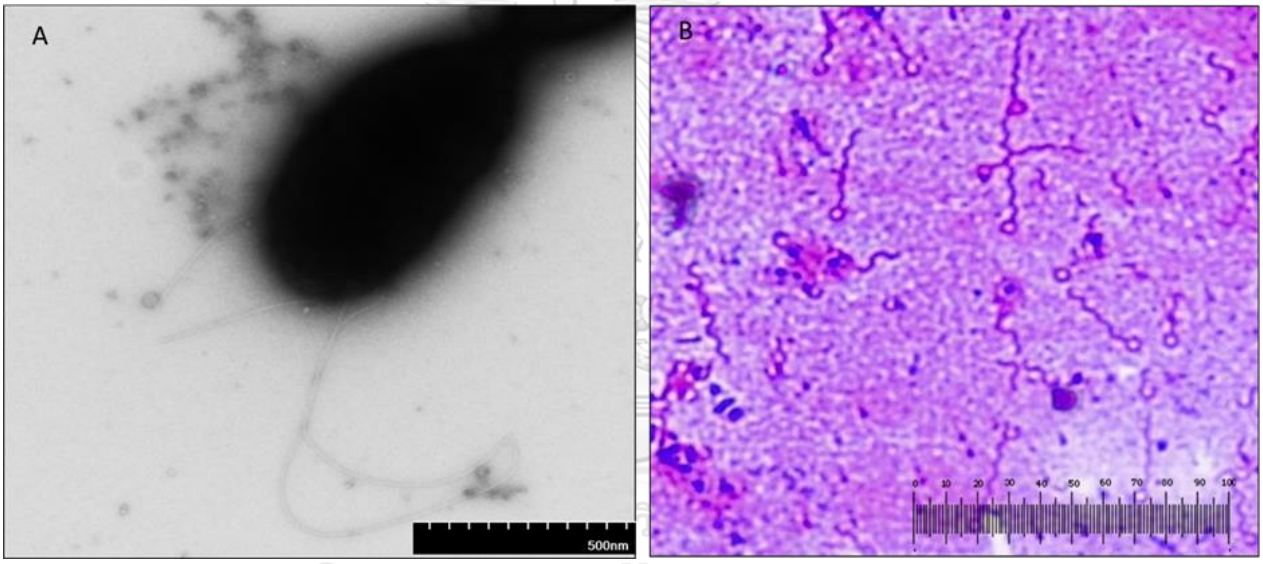

Figure 4.7 Transmission electron micrograph showing flagellum of strain Sx8-5^T grown in NB, 25^{o}C for 20 h (A) and flagella staining (B).

Table 4.10 Differential characteristics of strain Sx8-5^T and its related type strains.

Characteristics	1	2	3	4	5	6	7	8
Colony pigment	Yellow	Yellow	Yellow	Yellow	Mustard	Pale yellow	Yellow	Yellow
Temperature range (°C)	25-37	25-37	25-36	25-28	25-35	25-37	25-37	25-37
Growth in								
3% NaCl	-	-	+	+	-	-	+	-
5% NaCl	-	-	+	-	-	-	-	-
Growth at								
pH 5	-	-	-	-	-	-	w	+
pH 8	+	+	+	+	-	w	+	+
pH 9	+	+	+	+	-	w	+	+
Oxidase test	+	+	-	+	+	+	+	+
Hydrolysis of								
Urea	+	-	-	-	-	-	w	+
Tween 80	+	-	-	-	w	-	w	-
Aesculin	+	+	+	+	+	+	+	+
API 20NE (24 h)								
Nitrate reduction	-	-	-	-	-	+	w	-

+, positive; w, weakly positive; -, negative. All characteristics are determined in this study. All strains are positive for D-maltose assimilation and negative for acid production from D-glucose, hydrolysis of L-arginine and urea, and capric acid assimilation.

Strains: 1, Sx8-5^T; 2, *N. resinovorum* LMG 08367^T, 3, *N. gossypii* LMG 28605^T,4, *N. barchaimii* DSM 25411^T, 5, *N. silvae* KACC 21283^T, 6, *N. naphthalenivorans* JCM 13951^T, 7, *N. panipatense* KACC 14599^T, 8, *N. lindaniclasticum* CCM 7976^T

Table 4.10 Differential characteristics of strain Sx8-5^T and its related type strains (continued).

Characteristics	1	2	3	4	5	6	7	8
Hydrolysis of								
Aesculin	+	+	+	+	+	+	+	+
4-Nitrophenyl-β-D-galactopyranoside (PNPG)	+	w	+	w	+	-	w	+
Assimilation of								
Adipic acid	-	-	-	w	-	-	-	-
L-Arabinose	+	+	+	-	+	+	+	+
N-Acetyl-glucosamine	-	w	-	w	-	-	-	-
D-Glucose	w	+	w	-	w	-	-	+
Malic acid	+	+	+	-	+	-	-	+
D-Mannitol	-	w	-	-	-	-	-	-
D-Mannose	+	-	+	-	-	-	-	-
Phenylacetic acid	-	-	-	+	-	-	-	-
Potassium gluconate	+	-	w	+	-	-	-	+
Trisodium citrate	+	+	+	-	w	-	-	-

+, positive; w, weakly positive; -, negative. All characteristics are determined in this study. All strains are positive for D-maltose assimilation and negative for acid production from D-glucose, hydrolysis of L-arginine and urea, and capric acid assimilation.

Strains: 1, Sx8-5^T; 2, *N. resinovorum* LMG 08367^T, 3, *N. gossypii* LMG 28605^T,4, *N. barchaimii* DSM 25411^T, 5, *N. silvae* KACC 21283^T, 6, *N. naphthalenivorans* JCM 13951^T, 7, *N. panipatense* KACC 14599^T, 8, *N. lindaniclasticum* CCM 7976^T

4.3.3.4 Chemotaxonomic characteristics

Major fatty acids of strain Sx8-5^T were summed feature 8 ($C_{18:1}\omega$7c and/or $C_{18:1}\omega$6c), feature 3 ($C_{16:1}\omega$7c and/or $C_{16:1}\omega$6c), and $C_{16:0}$, which were similar to those of closely related type strains of genus *Novosphingobium*, however, the presence of saturated fatty acid $C_{17:0}$ iso distinguished strain Sx8-5^T from the closely related type strains (Table 4.11). The predominant respiratory quinone was ubiquinone 10 (Q-10).

Polar lipid profile of strain Sx8-5^T consisted of diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, two unidentified phospholipids and twelve unidentified polar lipids as shown in Fig. 4.8. According to all data obtained from polyphasic analysis, including genomic comparison, phenotypic and chemotaxonomic investigation, strain Sx8-5^T represented a novel species of the genus *Novosphingobium*. The name of *Novosphingobium kaempferiae* sp. nov is proposed for strain Sx8-5^T. The type strain is Sx8-5^T (=JCM 35076^T, =TBRC 15600 ^{T}). *Novosphingobium kaempferiae* (kaemp. fe' ri. ae. N.L. gen. n. *kaempferiae* of *Kaempferia* referring to an isolation of the type strain from stem tissue of *Kaempferia marginata* Carey).

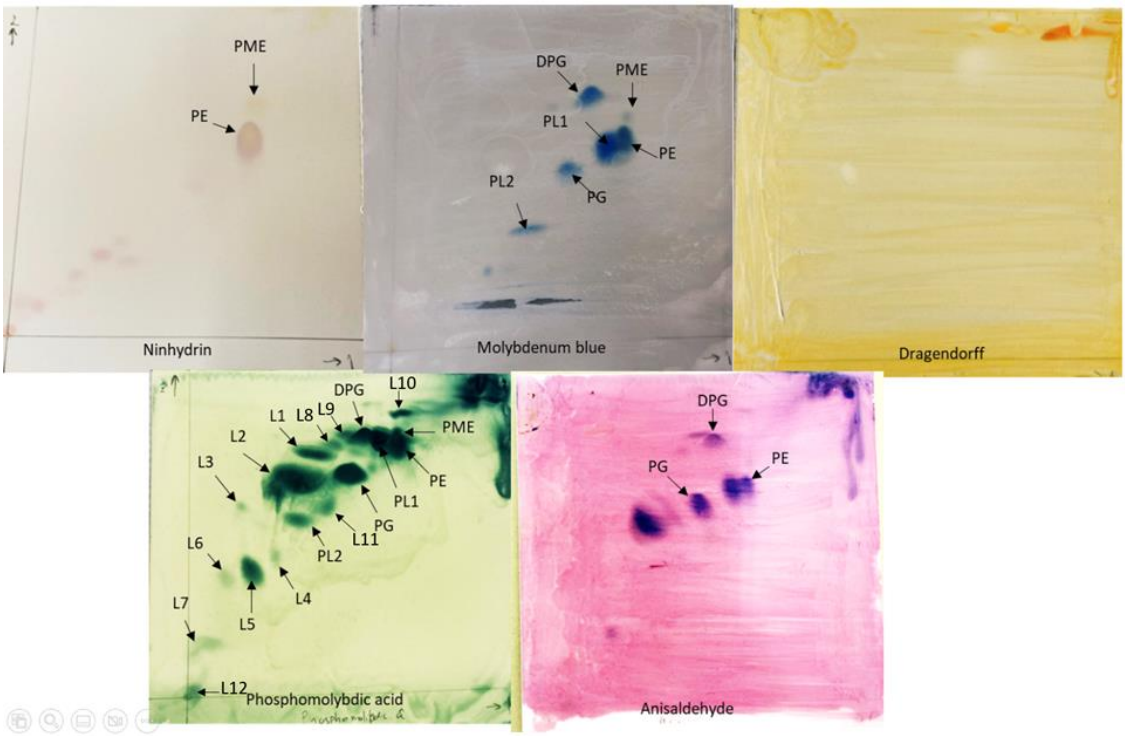

Figure 4.8 Polar lipid chromatograms of strain Sx8-5^{T} based on two dimensional thin-layer chromatograms visualized by spraying with ninhydrin, molybdenum blue, Dragendorff, phosphomolybdic acid and anisaldehyde.

Abbreviations: PE, Phosphatidylethanolamine; DPG, Diphosphatidylglycerol; PME, phosphatidylmonomethylethanolamine; PG, Phosphatidylglycerol; PL, unidentified phospholipids; L, unidentified polar lipids.

Table 4.11 Cellular fatty acid composition (%) of strain Sx8-5^T and its related type strains.

Fatty acids	1	2	3	4	5	6	7	8
Saturated fatty acids								
$C_{16:0}$	**13.6**	8.1	9.7	6.3	**10.4**	3.1	4.2	9.3
$C_{18:0}$	0.5	3.4	4.1	25.3	6.1	2.6	4.2	4.1
$C_{19:0}$ cyc ω8c	1.5	1.6	1.2	1.2	0.7	0.5	0.8	1.2
Unsaturated fatty acids								
$C_{15:1}$ ω6c	0.6	1.6	0.6	7.3	2.4	0.5	-	1.6
$C_{16:1}$ ω5c	1.2	1.6	1.3	1.2	1.8	0.7	3.6	1.3
$C_{17:1}$ ω6c	2.8	0.2	1.0	-	1.3	35.5	2.1	-
$C_{18:1}$ ω5c	-	1.5	1.7	-	2.0	1.6	1.8	1.3
$C_{18:1}$ ω7c 11-methyl	3.4	7.0	9.2	-	7.0	2.8	4.9	13.6
Hydroxy								
$C_{14:0}$ 2OH	-	10.2	7.4	-	6.9	2.2	8.4	7.5
$C_{15:0}$ 2OH	1.2	-	-	-	0.5	6.6	0.6	-
$C_{16:0}$ 2OH	-	-	-	1.8	-	-	1.2	-
$C_{16:1}$ 2OH	0.6	-	0.7	3.2	-	0.5	0.7	
$C_{17:0}$ iso 3OH	-	-	-	-	1.0	0.4	-	0.8
$C_{18:0}$ 3OH	-	-	-	1.6	0.8	-	-	-
*Summed feature 3[a]	**12.9**	**11.8**	9.9	**13.2**	**14.6**	3.1	**15.5**	9.8
*Summed feature 8[b]	**53.8**	**49.6**	**44.9**	**35.2**	**41.7**	**25.2**	**49.5**	**41.8**

Strains: 1, Sx8-5^T; 2, *N. resinovorum* LMG 08367^T, 3, *N. gossypii* LMG 28605^T,4, *N. barchaimii* DSM 25411^T, 5, *N. silvae* KACC 21283^T, 6, *N. naphthalenivorans* JCM 13951^T, 7, *N. panipatense* KACC 14599^T, 8, *N. lindaniclasticum* CCM 7976^T. All strains were cultured on R2A plate at 28°C for 48 h. Major components (≥10%) are highlighted with bold type. -, The amount of fatty acid less than 1.0% in all strains or not detected was omitted. All data are from this study. * Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total. [a]$C_{16:1}$ω7c and/or $C_{16:1}$ω6c; [b]$C_{18:1}$ω7c and/or $C_{18:}$1ω6c.

4.4 Determination of plant growth-promoting activity

4.4.1 Nitrogen fixation

The seventy-eight isolates were evaluated for an ability to fix nitrogen using Nessler's reagent method. Sixty-one isolates were positive in fixing nitrogen (Table 4.12), including all isolates that isolated from roots of sugarcane (50 isolates) and 11 isolates from other plant samples. Twelve isolates that isolated from roots of sugarcane produced ammonia in a range of 4.41-11.4 mmol/L (Fig. 4.9). These results indicated that bacterial isolates that colonized in sugarcane roots exhibited majority of nitrogen fixation through nitrogenase activity by converting N_2 in the air to ammonia, which was supported by *nif* genes found in genome of isolate PS25 (Fig. 4.10). Several researchers suggested that stem, root, leaves and rhizosphere soil of sugarcane were sources of nitrogen fixing-bacteria, especially *G. diazotrophicus* (James and Olivares, 1997; Boddey *et al*., 2003; Pedraza 2008). Our work revealed that nitrogen fixing-bacteria, *G. liquefaciens* and *G. dulcium,* were other species of *Gluconacetobacter* that were dominantly found in sugarcane roots. Nitrogen is a crucial limiting factor for plant growth because it is an essential component of enzymes and nucleic acids that create DNA and chlorophyll in plants. Plants cannot utilize nitrogen in atmosphere for growth and metabolism; thus, the nitrogen must be reduced to ammonia by nitrogen-fixing bacteria.

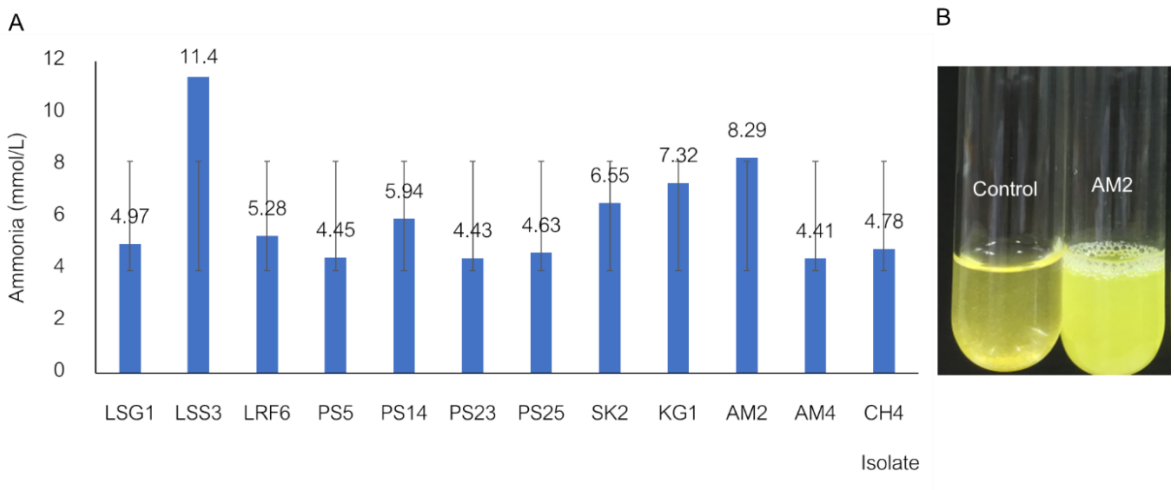

Figure 4.9 Ammonium production of endophytic bacteria isolated (A). Nitrogen fixation test using Nessler's reagent (B). The data are average of 3 independent experiments ± standard deviation (SD).

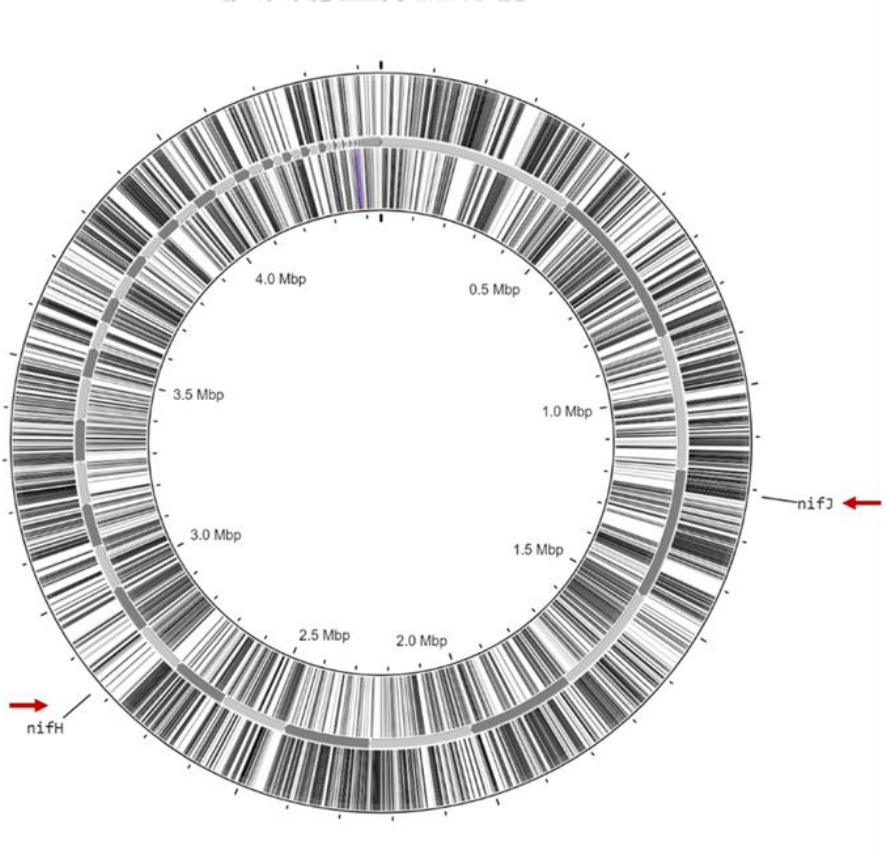

Figure 4.10 Circular genomic map of isolate PS25 with *nif* genes (red arrow).

Table 4.12 Nitrogen fixation of bacteria isolated.

Isolate no.	Plant samples	N_2 fixation		Isolate no.	Plant samples	N_2 fixation
CH1	Sugarcane	+		AM3	Sugarcane	+
CH2	Sugarcane	+		AM4	Sugarcane	+
CH3	Sugarcane	+		AM5	Sugarcane	+
CH4	Sugarcane	+		PS1	Sugarcane	+
SK1	Sugarcane	+		PS2	Sugarcane	+
SK2	Sugarcane	+		PS3	Sugarcane	+
PK1	Sugarcane	+		PS4	Sugarcane	+
PK2	Sugarcane	+		PS5	Sugarcane	+
KG1	Sugarcane	+		PS6	Sugarcane	+
KG2	Sugarcane	+		PS7	Sugarcane	+
KG3	Sugarcane	+		PS8	Sugarcane	+
KG4	Sugarcane	+		PS9	Sugarcane	+
KG5	Sugarcane	+		PS10	Sugarcane	+
LSG1	Sugarcane	+		PS11	Sugarcane	+
LTS2	Sugarcane	+		PS12	Sugarcane	+
LSS3	Sugarcane	+		PS13	Sugarcane	+
LSS4	Sugarcane	+		PS14	Sugarcane	+
LGF5	Sugarcane	+		PS15	Sugarcane	+
LRF6	Sugarcane	+		PS16	Sugarcane	+
AM1	Sugarcane	+		PS17	Sugarcane	+
AM2	Sugarcane	+		PS18	Sugarcane	+

+, positive reaction; -, negative reaction. Ammonia production was determined from absorbance at 560 nm compared to control (NF broth).

Table. 4.12 Nitrogen fixation of bacteria isolated (continued).

Isolate no.	Plant samples	N_2 fixation	Isolate no.	Plant samples	N_2 fixation
PS19	Sugarcane	+	A2-6	*Swietenia mahagoni* (L.) Jacq.	-
PS20	Sugarcane	+	A2-8	*Swietenia mahagoni* (L.) Jacq.	+
PS21	Sugarcane	+	A2-9	*Swietenia mahagoni* (L.) Jacq.	-
PS22	Sugarcane	+	A2-10	*Swietenia mahagoni* (L.) Jacq.	+
PS23	Sugarcane	+	A3-1	*Bambusa multiplex* (Lour.) Raeusch. ex Schult.f.	-
PS24	Sugarcane	+	S4-1	*Afzelia xylocarpa* (Kurz) Craib	-
PS25	Sugarcane	+	S5-1	*Toona ciliate* M. Roem.	-
PS26	Sugarcane	+	S5-2	*Toona ciliate* M. Roem.	-
A1-1	*Thyrsostachys siamensis* Gamble	-	S5-3	*Toona ciliate* M. Roem.	-
A1-2	*Thyrsostachys siamensis* Gamble	-	S6-1	*Crateva religiosa* G.Forst.	+
A2-2	*Swietenia mahagoni* (L.) Jacq.	+	S6-3	*Crateva religiosa* G.Forst.	+
A2-3	*Swietenia mahagoni* (L.) Jacq.	+	S7-2	*Phyllanthus emblica* L.	-
A2-4	*Swietenia mahagoni* (L.) Jacq.	-	S7-4	*Phyllanthus emblica* L.	-
A2-5	*Swietenia mahagoni* (L.) Jacq.	-	S7-6	*Phyllanthus emblica* L.	-

+, positive reaction; -, negative reaction. Ammonia production was determined from absorbance at 560 nm compared to control (NF broth).

Table. 4.12 Nitrogen fixation of bacteria isolated (continued).

Isolate no.	Sample plant	N_2 fixation	Isolate no.	Sample plant	N_2 fixation
SI8-2	*Kaempferia marginata* Carey	-	Sx8-5	*Kaempferia marginata* Carey	+
SI8-4	*Kaempferia marginata* Carey	+	Sx8-6	*Kaempferia marginata* Carey	-
SI8-5	*Kaempferia marginata* Carey	+	Sx8-7	*Kaempferia marginata* Carey	+
Sx8-4	*Kaempferia marginata* Carey	+	Sx8-8	*Kaempferia marginata* Carey	-

+, positive reaction; -, negative reaction. Ammonia production was determined from absorbance at 560 nm compared to control (NF broth).

4.4.2 Qualitative analysis of phosphate and zinc solubilization

Phosphorus (P) is an essential element for plants as they are required for cell synthesis, cell activity, protein, and vitamin productions. Zinc (Zn) is an essential micronutrient required in small amount for living organisms. In soil, P and Zn form insoluble complex with aluminum and iron minerals (Singh, 2001) which limits plant availability. Zn deficiency in soil leads to retarded flower and fruit development, and decrease in synthesis of phytohormones and carbohydrates (Welch and Graham, 2004). Applied chemical fertilizers such as Zn-sulfate, Zn-ammonia complex, Zn-nitrate, Zn-oxide, Zn-oxysulfate, Zn-carbonate, and Zn-chloride are remained found in soil surface and unavailable to plants (Khanghahi *et al*., 2018). Several researches have reported that phosphate-solubilizing bacteria (PSB) and zinc-solubilizing bacteria (ZSB) had a potential to increase P and Zn availability for plants through various mechanisms such as production of phosphatase enzymes and chelating agents, and secretion of organic acids

to convert the insoluble phosphate complex into soluble monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{-2}) ions (Richardson *et al*., 2009; Bashan *et al*., 2013; Khan *et al*., 2014; Ramanuj and Shelat, 2018). Organic acids such as oxalic, citric, butyric, malonic, lactic, succinic, malic, gluconic, acetic, fumaric, etc. (Seshachala and Tallapragada, 2012) were reported to chelate cations bound to phosphate or zinc and their hydroxyl and carboxyl groups (Kpomblekou and Tabatabai, 1994).

In this study, the seventy-eight isolates were examined for P solubilization ability using solid medium containing 0.5% $Ca_3(PO_4)_2$. As shown in Table 4.13, 69 isolates were able to solubilize tricalcium phosphate and were considered as PSB composed of 46 isolates (67%) obtained from sugarcane and 23 isolates (33%) from other plants (Fig 4.11 A). Four isolates, PS3, PS6, PS15 and KG1, from roots of sugarcane were classified as high phosphate solubilizer as their SI values were in a range of 3.89-4.4. While isolate Sx8-5 (*N. barchaimii*) from other plant sample gave the highest SI at 2.5 (Table. 4.13) which agreed well with Chen *et al*. (2021) and Song *et al*. (2022) who reported that *Novosphingobium* showed zone of tricalcium phosphate solubilization. However, the use of culture media and phosphate compound as P source affected the PSB-screening (Oliveira *et al*., 2009).

Zn solubilization capacity of the seventy-eight isolates was assessed using zinc oxide (ZnO) in mineral salt agar medium. Most isolates (92%) exhibited extraordinary ability to solubilize zinc oxide consisted of 49 isolates (68%) and 23 isolates (32%) obtained from sugarcane and other plants, respectively (Fig 4.11 B). High zinc solubilization activity was observed in isolate SI8-5 at SI value of 6, followed by isolate LTS2, KG1 and CH1 at SI value of 5.38, 5.6 and 5.09, respectively (Table 4.13). The results indicated that most of the isolates increased zinc availability to plants. These isolates

might solubilize Zn through production of acids, siderophores, or proton (Alexander, 1997; Saravanan *et al*., 2007).

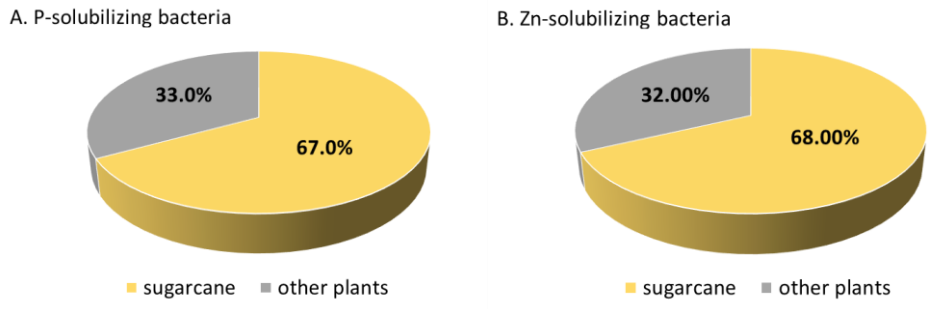

Figure 4.11 Percentage of P and Zn solubilizing bacteria isolated from sugarcane and other plants. A, phosphate-solubilizing bacteria B, zinc-solubilizing bacteria.

Clear zone around bacterial colonies on agar medium revealed that the bacteria produced acid (Fig. 4.12). Dominant bacteria isolated in this study belonged to genera *Gluconacetobacter*, *Pantoea*, *Bacillus*, and *Pseudomonas* which occur in significant number in ecological environments. These bacteria could solubilize both P and Zn. *Pantoea*, *Rahnella*, *Enterobacter*, *Acinetobacter*, *Bacillus* and *Gluconacetobacter* reportedly possessed solubilizing capability of both P and Zn. (Kumari *et al*., 2018; Khamwan *et al*., 2018; Ramanuj and Shelat, 2018).

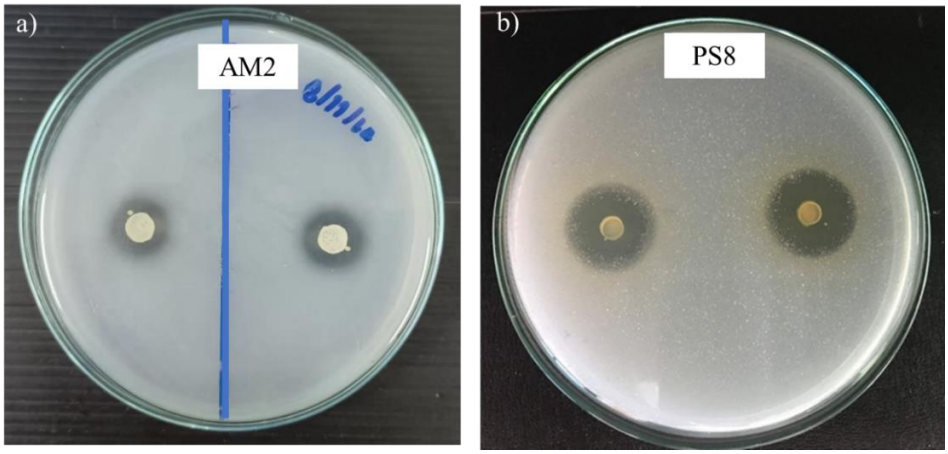

Figure 4.12 Clear zone around bacterial colonies, isolate AM2 and PS8, on agar medium containing (a) tricalcium phosphate, (b) zinc oxide.

Table 4.13 Phosphate and zinc solubilization of bacteria isolated.

Isolate no.	Plant samples	P solubilization Index (SI)	Zn solubilization Index (SI)
CH1	Sugarcane	2.14	5.09
CH2	Sugarcane	1.83	2.42
CH3	Sugarcane	2.28	5.40
CH4	Sugarcane	2.13	4.15
SK1	Sugarcane	1.25	2.79
SK2	Sugarcane	-	-
PK1	Sugarcane	-	3.08
PK2	Sugarcane	2.31	4.83
KG1	Sugarcane	3.89	5.6
KG2	Sugarcane	1.38	2.44
KG3	Sugarcane	-	3.29
KG4	Sugarcane	1.97	1.59
KG5	Sugarcane	-	3.51
LSG1	Sugarcane	2.67	3.50
LTS2	Sugarcane	2.62	5.38
LSS3	Sugarcane	1.71	4.54
LSS4	Sugarcane	2.28	4.60
LGF5	Sugarcane	1.67	3.00
LRF6	Sugarcane	1.54	2.92
AM1	Sugarcane	1.18	3.09
AM2	Sugarcane	1.43	3.08
AM3	Sugarcane	2.14	3.67
AM4	Sugarcane	3.25	4.10
AM5	Sugarcane	1.54	3.42
PS1	Sugarcane	1.46	2.67
PS2	Sugarcane	1.57	2.92
PS3	Sugarcane	4.00	3.25

-, No clear zone around colony

Table 4.13 Phosphate and zinc solubilization of bacteria isolated (continued).

Isolate no.	Plant samples	P solubilization Index (SI)	Zn solubilization Index (SI)
PS4	Sugarcane	3.90	3.40
PS5	Sugarcane	3.73	3.40
PS6	Sugarcane	4.40	3.36
PS7	Sugarcane	3.64	3.40
PS8	Sugarcane	1.73	3.80
PS9	Sugarcane	3.57	4.20
PS10	Sugarcane	1.55	3.90
PS11	Sugarcane	3.23	2.71
PS12	Sugarcane	2.69	3.60
PS13	Sugarcane	3.54	3.41
PS14	Sugarcane	1.15	3.50
PS15	Sugarcane	4.20	3.80
PS16	Sugarcane	1.83	3.80
PS17	Sugarcane	2.31	3.75
PS18	Sugarcane	2.57	3.42
PS19	Sugarcane	1.70	3.90
PS20	Sugarcane	1.33	3.58
PS21	Sugarcane	2.83	3.70
PS22	Sugarcane	2.53	3.42
PS23	Sugarcane	2.18	3.45
PS24	Sugarcane	3.00	3.75
PS25	Sugarcane	2.64	3.42
PS26	Sugarcane	2.60	3.42
A1-1	*Thyrsostachys siamensis* Gamble	1.10	2.6
A1-2	*Thyrsostachys siamensis* Gamble	1.18	2.4
A2-2	*Swietenia mahagoni* (L.) Jacq.	1.36	1.7
A2-3	*Swietenia mahagoni* (L.) Jacq.	1.26	1.5

-, No clear zone around colony

Table 4.13 Phosphate and zinc solubilization of bacteria isolated (continued).

Isolate no.	Plant samples	P solubilization Index (SI)	Zn solubilization Index (SI)
A2-4	*Swietenia mahagoni* (L.) Jacq.	2.09	4.6
A2-5	*Swietenia mahagoni* (L.) Jacq.	1.84	2.3
A2-6	*Swietenia mahagoni* (L.) Jacq.	1.25	3.9
A2-8	*Swietenia mahagoni* (L.) Jacq.	-	1.4
A2-9	*Swietenia mahagoni* (L.) Jacq.	1.36	3.4
A2-10	*Swietenia mahagoni* (L.) Jacq.	1.34	1.5
A3-1	*Bambusa multiplex* (Lour.) Raeusch. ex Schult.f.	2	2.8
S4-1	*Afzelia xylocarpa* (Kurz) Craib	1.3	3.5
S5-1	*Toona ciliata* M. Roem.	-	2.2
S5-2	*Toona ciliata* M. Roem.	1.87	1.8
S5-3	*Toona ciliata* M. Roem.	2.12	2.4
S6-1	*Crateva religiosa* G.Forst.	-	-
S6-3	*Crateva religiosa* G.Forst.	-	-
S7-2	*Phyllanthus emblica* L.	1.12	2.1
S7-4	*Phyllanthus emblica* L.	1.27	3.3
S7-6	*Phyllanthus emblica* L.	1.18	-
SI8-2	*Kaempferia marginata* Carey	1.14	-
SI8-4	*Kaempferia marginata* Carey	1.17	3.5
SI8-5	*Kaempferia marginata* Carey	1.52	6
Sx8-4	*Kaempferia marginata* Carey	1.14	2.2
Sx8-5	*Kaempferia marginata* Carey	2.5	-
Sx8-6	*Kaempferia marginata* Carey	1.11	2.3
Sx8-7	*Kaempferia marginata* Carey	-	2.4
Sx8-8	*Kaempferia marginata* Carey	1.42	1.9

-, No clear zone around colony

4.4.3 Screening for IAA-producting bacteria

All endophytic bacteria (78 isolates) were screened for IAA producting isolates. It was found that 16 isolates (20%) could produce IAA in NF medium supplemented with 0.01% L-tryptophan, a main precursor for IAA biosynthesis in try-dependent pathway of bacteria (Spaepen *et al*., 2007). From the sixteen IAA-producing isolates, 9 isolates from sugarcanes consisting of SK1, SK2, KG1, KG2, KG3, KG4, KG5, AM3, and PK1 produced IAA ranging from 67.25 to 202.25 µg/mL (Fig 4.13 A), and 7 isolates from other plants consisting of A1-2, S5-1, S5-2, S6-3, SI8-5, Sx8-6, and Sx8-8 produced IAA ranging from 6.13 to 67.29 µg/mL (Fig. 4.13 B). Maximum IAA-producing isolate was KG5 (202.25 µg/mL) followed by KG4 (152.79 µg/mL), SK1 (141.18 µg/mL), KG3 (109.27 µg/mL) and PK1 (107.79 µg/mL), respectively (Fig. 4.13 A), which were isolated from sugarcanes and most of them was identified as *P. dispersa*. These results coincided with *Pantoea* sp. KRZ5 isolated from rhizosphere of sugarcane (RB 867515) which produced IAA at 69.36 µg/mL (Rodrigues *et al*. 2016) and *Pantoea* sp. LMA 28 isolated from Brazilian semi-arid region which produced IAA at 113.57 µg/mL. Moreover, *Pantoea* have been described as plant growth promoter (Kavamura *et al*., 2013). Other species, including *A. phyllosphaerae* (isolate SK2) and *B. territorii* (isolate AM3) could produce IAA at 78.86 and 67.25 µg/mL, respectively (Fig.4.13 A In this experiment, *G. liquefaciens* which found as dominant species in different of sugarcane samples could not produce IAA. For bacteria isolated from other plants in Kanchanaburi province, it was found that *Sphingobium* sp. Sx8-8 produced the highest IAA (67.29 µg/mL) (Fig. 4.13 B). Similar results were reported by Rodrigues *et al*. (2018), in that *S. yanoikuyae* BU32 gave the highest IAA compared to *Burkholderia*, *Sphingobium, Rhizobium* and *Enterobacter* genera isolated from rhizosphere soil of sugarcane. Several factors such as bacterial species, IAA biosynthetic pathway, precursor concentration, media composition,

temperature, and growth stage etc. affected IAA biosynthesis of bacteria (Jasim *et al*., 2014).

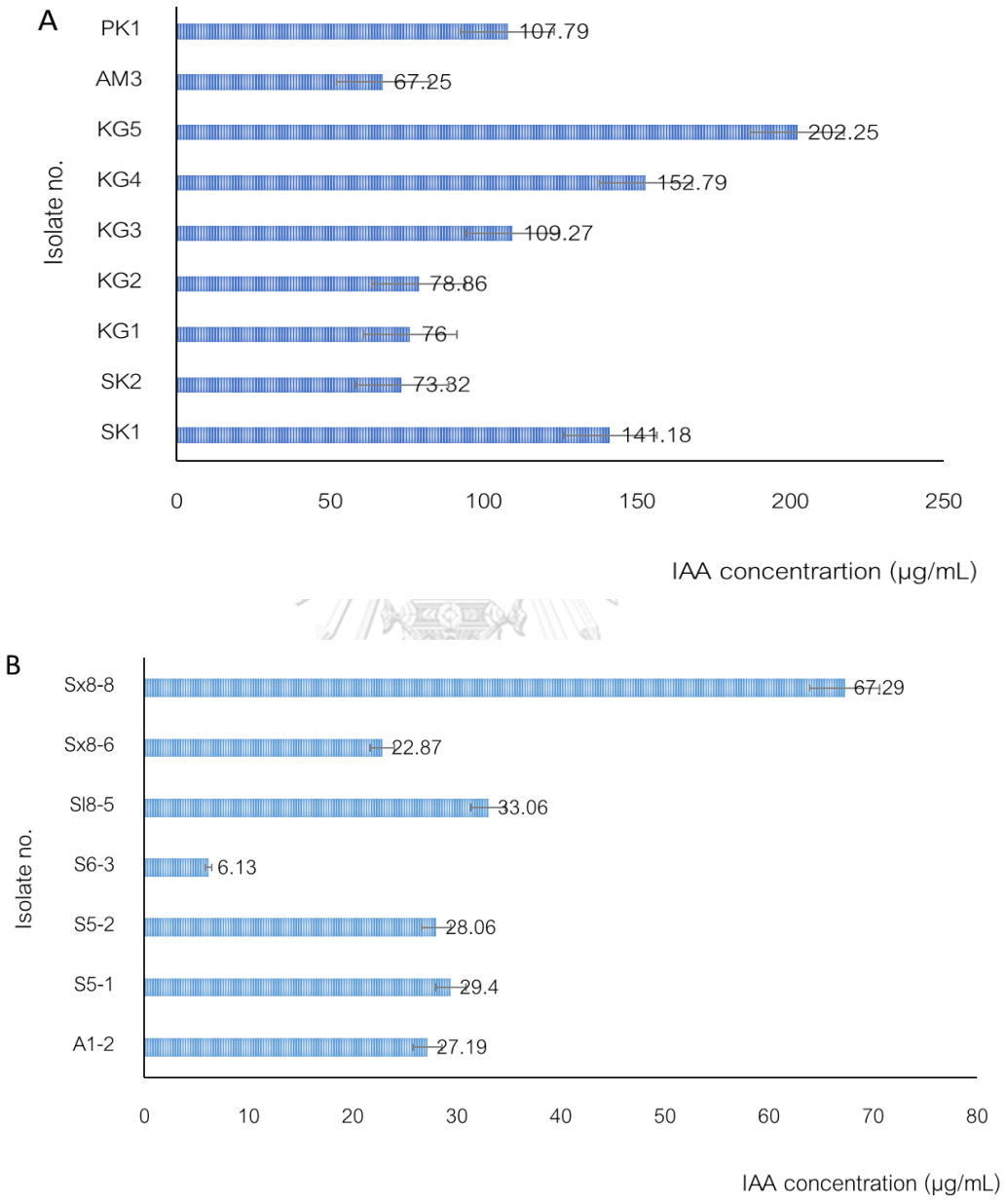

Figure 4.13 Bacterial Indole-3-acetic acid (IAA) production in NF broth containing 0.01% L-tryptophan. A, bacteria isolated from sugarcanes; B, bacteria isolated from other plants. The data represent the average of independent experiments (n=3), each with replicates ± standard deviation (SD).

4.4.3.1 Confirmation of IAA production of the selected strain by high performance liquid chromatography (HPLC)

The strain SK2 (*Aureimonas* sp.) and Sx8-8 (*Sphingobium* sp.) were selected to confirm for the IAA production by HPLC analysis. Crude extract of the strain SK2 showed peak at 2.74 min retention time, while peak of standard IAA was at 2.69 min retention time (Fig 4.8 A), and the strain Sx8-8 displayed peak at 2.54 min retention time corresponded to peak of standard IAA at 2.58 min. (Fig. 4.8 B). This result confirmed that the strain SK2 and Sx8-8 produced IAA in the presence of L-tryptophan which agreed well with other previous reports (Harikrishnan *et al*., 2014; Bhutani *et al*., 2018; Baliyan *et al*., 2021). Khakipour *et al*. (2008) suggested that HPLC is a more assuring method in recognition and analysis of IAA than the mass spectrophotometry.

4.4.3.2 Optimization of IAA production

The *Sphingobium* sp. Sx8-8 and *Pantoea* sp. S5-1 were able to produce IAA at pH 6 and pH 7, whereas at high acidic (pH 5) and strong alkaline (pH 8) pHs were considered as unsuitable for IAA production. Maximum IAA of strain Sx8-8 (76.19 µg/mL) and strain S5-1 (21.9 µg/mL) were produced at pH 7 (Fig. 4.15 A). Kumari *et al*. (2018) has reported that pH 7 was optimal for IAA production of *B. subtilis* DR2 (158.79 µg/mL). Apine and Jadhav (2011) reported that *P. agglomerans* strain PVM showed maximum IAA production (1.441 g/L) at pH 7, while maximum IAA production of *Pseudomonas putida* UB1 was at pH 7.5 (Bharucha and Patel 2013). However, pH 6 has also been reported as optimal for *Acetobacter diazotrophicus* L1 isolated from sugarcane (Patil *et al*., 2011). The *Sphingobium* sp. Sx8-8 and *Pantoea* sp. S5-1 could produce IAA at 25, 30, 35, and 40^{o}C; nevertheless, production of IAA was affected by high temperature as shown in Fig. 4.15 B, *Sphingobium* sp. Sx8-8 and *Pantoea* sp. S5-1 produced the highest IAA at 30^{o}C and 25^{o}C, respectively. The maximum IAA production of *Sphingobium* sp. Sx8-8 was

35.41% increased when concentration of L-tryptophan in NF broth was increased from 0.01% to 0.5%. The decrease in IAA production was observed at high concentration of tryptophan (Fig. 4.15 C), whereas maximum IAA production of *Pantoea* sp. S5-1 was achieved at 1.5% L-tryptophan. L-tryptophan at 2% had significant negative effect on IAA production of both strains. Different bacterial species had different IAA synthetic pathway, such as indole-3-pyruvic acid (IPA) pathway was found in non-pathogenic plant-associated bacteria, while indole-3acetamide (IAM) pathway was found in phytopathogenic bacteria (Duca *et al*., 2014; Shaik *et al*., 2016). Our results indicated that IAA production affected by tryptophan utilization was different in different species. Production of IAA by *Sphingobium* sp. Sx8-8 was highest (232.1 µg/mL) in NF broth supplemented with 0.5% L-tryptophan (pH 7) at 30°C after 48 h of incubation, then slightly decreased as shown in Fig. 4.15 D. At 48 h, *Sphingobium* entered stationary phase of growth (Flood *et al*., 2018), and IAA is secondary metabolite. This might be a reason why the *Sphingobium* sp. Sx8-8 produced maximum IAA at 48 h. Rodrigues and Forzani (2016) have demonstrated that *S. yanoikuyae* BU32 grown in TSB supplemented with 5 mM L-tryptophan for 72, 96, and 120 h gave IAA more than those grown for 24 h.

Based on the results obtained; pH, temperature, L-tryptophan concentration, and incubation time; that are affected by species, growth phase, and enzyme activity had influenced on the IAA production. In addition, the differences in carbon and nitrogen sources could affect both growth and IAA production of microorganisms because C/N ratio has a crucial role in increasing of cell yield and IAA production (Mohite, 2013; Nutaratat *et al*., 2017; Kumari *et al*., 2018).

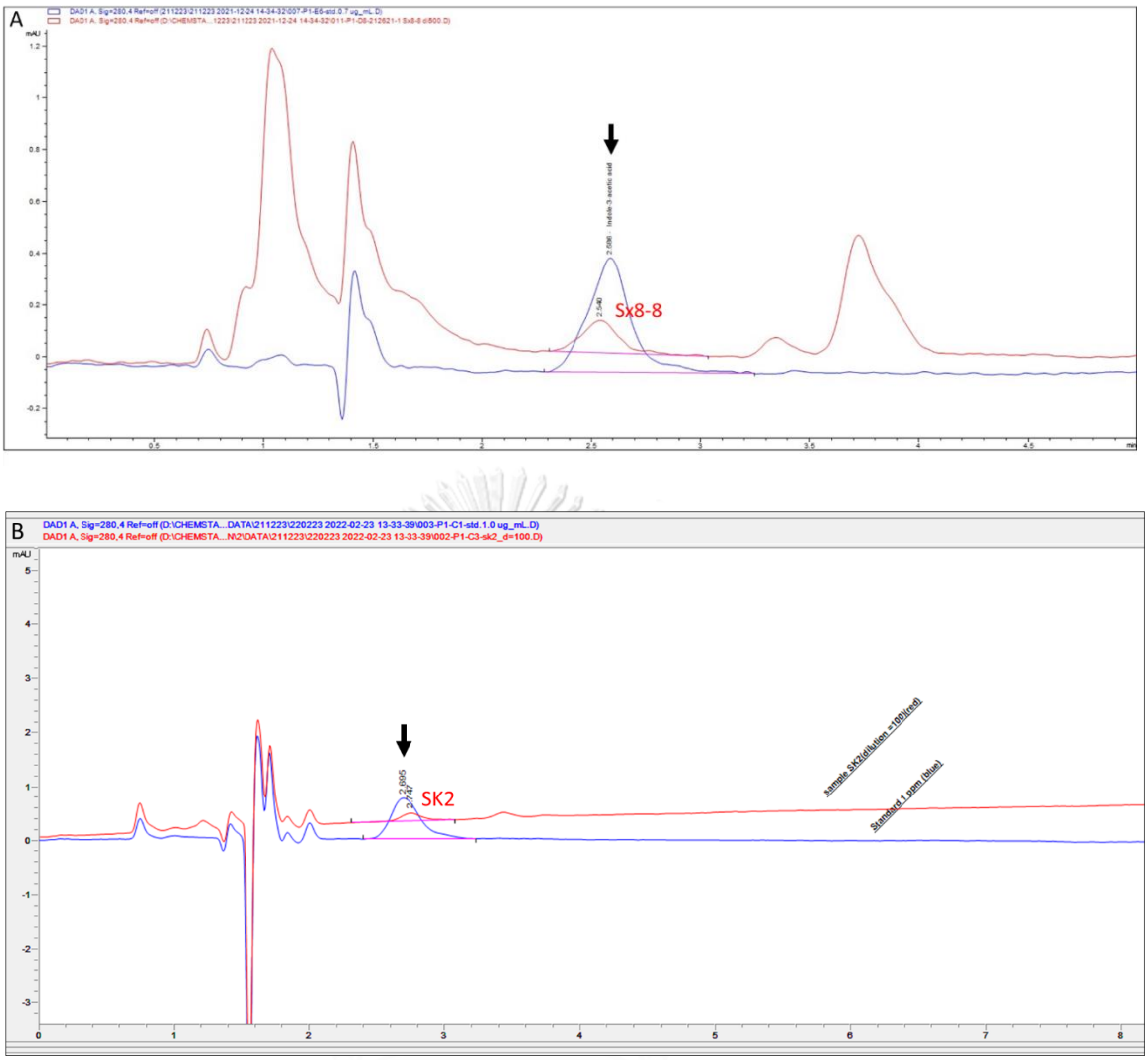

Figure 4.14 HPLC analysis of IAA in crude extract of selected strains. A, overlay peak IAA of strain Sx8-8 and standard IAA; B, overlay peak IAA of strain SK2 and standard IAA.

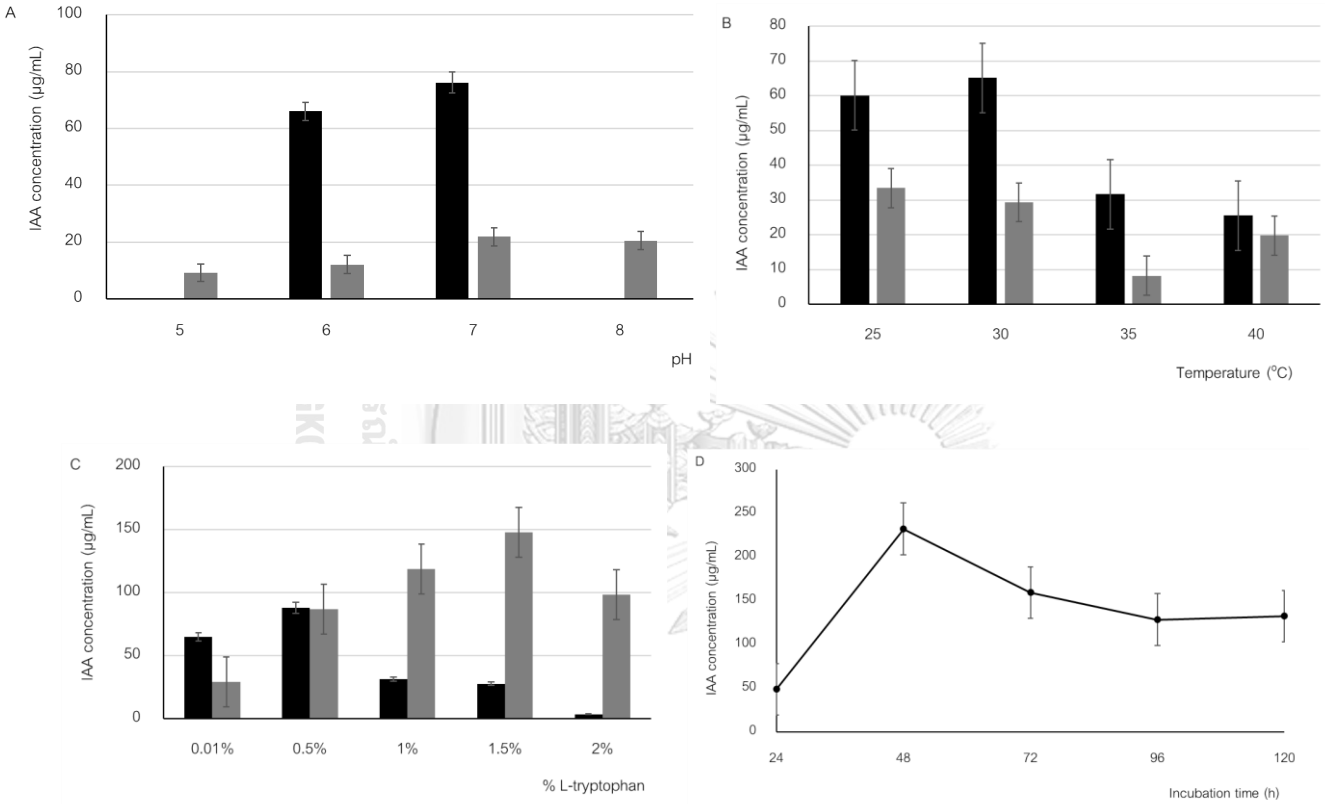

Figure 4.15 Optimization of IAA production (µg/mL) in NF medium supplemented with 0.01% L-tryptophan at 30°C for 24 h based on variation of pH (A), temperature (B), L-tryptophan concentration (C), incubation time (D), ■ ; Strain Sx8-8 and ■ ; Strain S5-1. The data represent the average of independent experiments, each with replicates ± standard deviation (SD).

4.4.3.3 Plant growth promotion activity of strain Sx8-5^T and its closely related type strains

Several species of genus *Novosphingobium* inhabited with plants and promoted plant growth have been reported (Krishnan *et al*., 2017; Rangjaroen *et al*., 2017; Chen *et al*., 2021). In this study, an ability to solubilize P and Zn of strain Sx8-5^T and its closely related type strains were examined. The strain Sx8-5^T solubilized tricalcium phosphate (SI=2.5) but not solubilize Zn (Table 4.14). Genome analysis based on CG server revealed genes involved in phosphate uptake such as *pst* (Pi-specific transporter), and phosphate starvation genes such as *phoA* (alkaline phosphatase), *glpQ* (glycerophosphoryldiester phosphodiesterase) which stimulated organic phosphate solubilization (Ishige et al., 2003; Zeng et al., 2017) as shown in Fig. 4.16.

The strain Sx8-5^T did not produce IAA, whereas IAA production by *N. gossypii* LMG 28605^T (84.39 µg/mL), *N. silvae* KACC 21283^T (56.43 µg/mL) and *N. naphthalenivorans* JCM 13951^T (17.36 µg/mL); similarly, results have been previously reported (Krishnan et al., 2017; Rangjaroen et al.,2017; Chen et al., 2021; Song et a., 2022) (Table 4.14). Therefore, this novel strain Sx8-5^T will be useful as bioinoculant that enhances P-availability for plant growth and development.

Table 4.14 IAA production, P and Zn solubilizations of strain Sx8-5^{T} and its related type strains.

Strains	IAA production (μg/mL)	Solubilization index (SI)	
		P	Zn
N. kaempferiae Sx8-5^{T}	-	2.5	-
N. resinovorum LMG 08367^{T}	-	-	-
N. gossypii LMG 28605^{T}	84.39	-	-
N. barchaimii DSM 25411^{T}	-	-	-
N. silvae KACC 21283^{T}	56.43	-	1.48
N. naphthalenivorans JCM 13951^{T}	17.36	-	-
N. panipatense KACC 14599^{T}	-	-	-
N. lindaniclasticum CCM 7976^{T}	-	-	-

-, negative

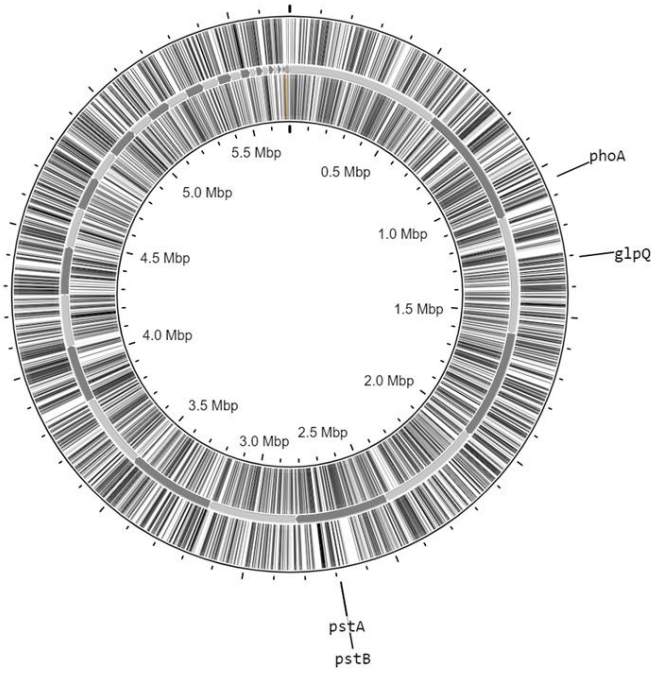

Figure 4.16 Circular genomic map of strain Sx8-5^{T} with genes involved in phosphate uptake and organic phosphate solubilization, *pst*; Pi-specific transporter, *phoA*; alkaline phosphatase, *glpQ*; glycerophosphoryldiester phosphodiesterase.

4.5 Rice growth-promoting ability of selected strains

4.5.1 Effects of IAA-producing strains on 'RD6' rice growth

Two selected strains, *Sphingobium* sp. Sx8-8 and *Pantoea* sp. S5-1, were tested for an ability to promote rice growth. After 15 days of cultivation, a significant increase in root length and number of lateral roots were observed in germinated rice seeds treated with the two IAA-producing isolates, *Sphingobium* sp. Sx8-8 and *Pantoea* sp. S5-1, and standard IAA. The *Sphingobium* sp. Sx8-8 increased root length 80.22% higher than control and 22.9% higher than 50 µg/mL standard IAA. The *Pantoea* sp. S5-1 increased root length lower than *Sphingobium* sp. Sx8-8 and standard IAA but 20.9% higher than control (Table. 4.15). Ten µg/mL standard IAA gave the highest root length. Increased of standard IAA concentration to 30 and 50 µg/mL resulted in decrease of root length (Fig. 4.17 A) which coincided with Kukavica *et al.* (2007). High IAA concentration has an inhibitory effect on root elongation due to synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase for ethylene formation is induced. The ethylene formation and transport of precursor in plants are inhibited by low concentration of IAA (Hansen and Grossmann, 2000; Wei *et al*., 2000). Besides, *Sphingobium* sp. Sx8-8 gave the highest shoot length (18.7 cm) (Fig. 4.17 B) and a high number of lateral roots similar to standard IAA. Increase of all parameters including; root and shoot lengths, number of lateral roots, root and shoot fresh weights, and root and shoot dry weights of germinated rice seeds treated with *Pantoea* sp. S5-1 were higher than control. The results indicated that *Sphingobium* sp. Sx8-8 and *Pantoea* sp. S5-1, an IAA-producing bacteria, had positive effect on rice growth. Plant growth-promoting ability of bacteria were reported in several studies. Kalaiselvi and Priya (2020) reported that *S. yanoikuyae* MH394206 and mixed consortia enhanced plant height and root volume of rice CO51 in moisture deficiency condition. Makar *et al*. (2021) reported that 12 bacterial strains, isolated from wheat

grains, belonged to genera *Staphylococcus*, *Pantoea*, *Sphingobium*, *Bacillus*, *Kosakonia,* and *Micrococcus* could synthesize indole-related compounds (IRCs) which is generally considered as IAA precursor. *Pantoea* has been found in many species of plants such as sugarcane, olive knots, Cactaceae family and has potential in promoting plant growth (Dastager *et al*., 2009; Rodrigues and Forzani, 2016; Luziatelli *et al*., 2020). In this study, *Sphingobium* sp. Sx8-8 and *Pantoea* sp. S5-1, isolated from stem of *Kaempferia marginata* Carey and *Toona ciliata* M. Roem., respectively, revealed capability to enhance rice growth. This result supported other previous studies that endophytic bacteria that were isolated from one kind of plant was able to promote growth in another kind of plant. Giassi *et al*. (2016) reported that IAA producing *Bacillus* spp. (BM05 BM16, MB17) isolated from strawberry leaves and *Bacillus* CPM04 isolated from coffee leaves were able to promote growth of citrus rootstocks, while IAA-producing endophytic bacteria, *Microbacterium* C4 and *Lysinibacillus* C7 isolated from corn roots, showed a potential to promote growth of both soybean and wheat seedlings (Yu *et al*., 2016). Moreover, endophytic bacteria isolated from plant stem was found to be able to enhance rice root gemination (Yu *et al*., 2016). Khamwan *et al*. (2018) reported that bacteria isolated from stem and leaves of Jerusalem artichoke had growth-promoting activity in Jerusalem artichoke tuber which implied that the endophytic bacteria might spread-out over-all parts of the plant and function in different systems. An endophytic bacteria colonized in plant roots gives beneficial effects to plant in different way depends on bacterial property, plant species, and interaction between bacteria and plants (Liu *et al*., 2017).

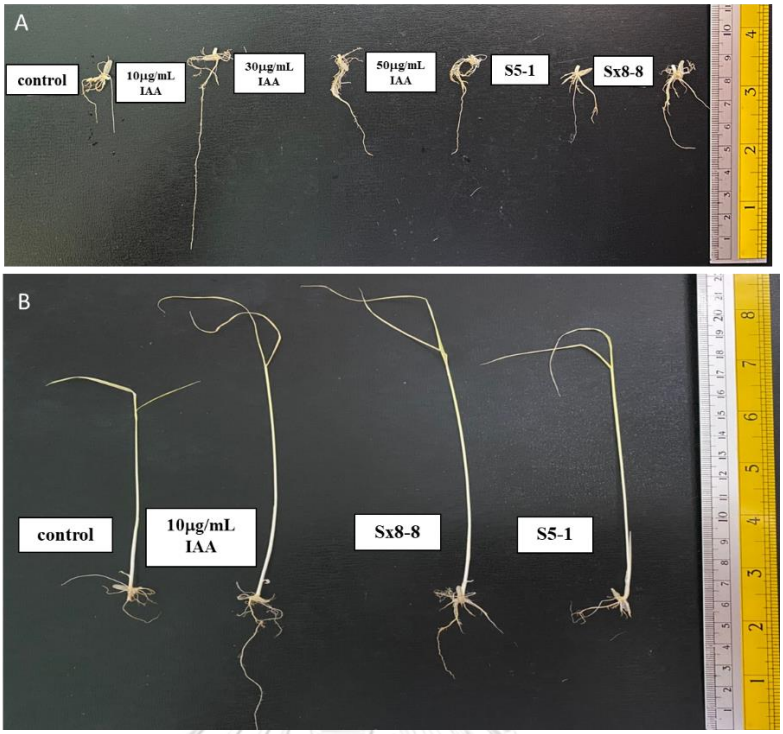

Figure 4.17 Fifteen days-old 'RD6' rice seedlings treated with strain Sx8-8 and S5-1, an IAA-producing bacteria, and standard IAA. A) roots B) rice seedlings.

Table 4.15 Fifteen days-old 'RD'6 rice seedlings treated with strain Sx8-8 and S5-1.

Strain no. / IAA	Root length (cm)	Shoot length (cm)	Number of lateral roots	Root fresh weight (g)	Shoot fresh weight (g)	Root dry weight (g)	Shoot dry weight (g)
Sphingobium sp. Sx8-8	4.83^{c}	18.7^{d}	48^{c}	0.049^{ab}	0.051^{a}	0.0031^{a}	0.0048^{a}
Pantoea sp. S5-1	3.24^{ab}	13.75^{c}	36^{b}	0.058^{b}	0.057^{a}	0.0057^{b}	0.0071^{b}
Standard IAA							
10 µg/mL	9.64^{d}	17.99^{d}	48^{c}	0.063^{b}	0.13^{b}	0.0033^{a}	0.0063^{b}
30 µg/mL	4.53^{c}	17.89^{d}	50^{c}	0.057^{b}	0.057^{a}	0.0038^{a}	0.007^{b}
50 µg/mL	3.93^{bc}	10.52^{b}	30^{b}	0.048^{ab}	0.057^{a}	0.0039^{a}	0.0068^{b}
Control	2.68^{a}	9.47^{a}	19^{a}	0.038^{a}	0.058^{a}	0.0031^{a}	0.0066^{b}

The same alphabets in each column mean no significant differences ($p \leq 0.05$) according to the Duncan's multiple range test.

4.5.2 Effect of endophytic bacteria isolated from sugarcane roots on 'Khao Dawk Mali 105' rice growth

Three selected strains, *Aureimonas* sp. SK2 (IAA-producing strain), *Gluconacetobacter* sp. PS25 and *Gluconacetobacter* sp LSG1 (non IAA-producing strains), were investigated for an ability to promote rice growth. After cultivation of treated germinated rice seed for 15 days, all three strains significantly increased total length, shoot length and number of lateral roots of rice seedlings more than control, whereas increase of root length was observed in rice seedlings treated with strain SK2 and PS25. However, the highest total length, root length and shoot length were observed in rice seedlings treated with 10 µg/mL of standard IAA. Strain SK2, an IAA-producing bacteria, increased total length (19.86 cm), root length (3.49 cm) and fresh shoot weight (0.043 g) of rice seedling more than strain PS25 and LSG1, which did not produce IAA. No difference of total length and root length of rice seedlings treated with strain PS25 and LSG1, while rice seedlings treated with strain LSG1 had the highest number of lateral roots (Table 4.16, Fig. 4.18). The results indicated that endophytic bacteria both IAA-producing bacteria and non IAA-producing bacteria had potential to promote rice growth, and they were beneficial in increasing of crop yield as biofertilizer or bioinoculant. Furthermore, *Aureimonas* sp. SK2, *Gluconacetobacter* sp. PS25 and *Gluconacetobacter* sp. LSG1 were isolated from sugarcane roots but provided positive effect on rice (another plant) which was similar to *Spingobium* sp. Sx8-8 and *Pantoea* sp. S5-1 and several previous reports of Deivanai *et al*. (2014), Etesami *et al*. (2014) and Khan *et al*. (2020). However, the germination of rice in this experiment was performed under the dark condition that may cause the abnormal growth. Further studies on the gemination of rice seeds with cultivation under natural light to produce adequate nutrient *via* photosynthesis and the

studies of gemination of rice seeds with coated husk are required for these endophytic bacteria.

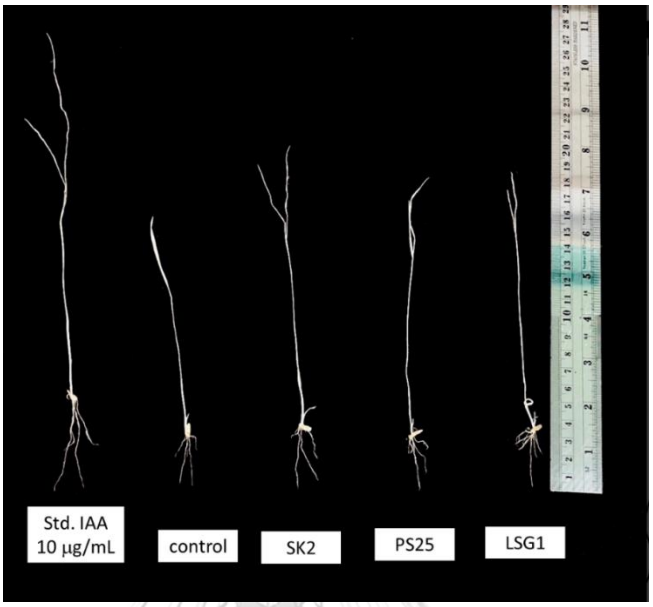

Figure 4.18 Fifteen days-old 'Khao Dawk Mali 105' rice seedlings treated with selected strains.

Table 4.16 Fifteen days-old 'Khao Dawk Mali 105' rice seedlings treated with strain SK2, PS25 and LSG1.

Strain no. / IAA	Length (cm)			Number of lateral roots	Fresh weight (g)		Dry weight (g)	
	Total	Root	Shoot		Root	Shoot	Root	Shoot
10µg/mL std. IAA	29.81[a]	6.16[a]	11.96[a]	9[a]	0.037[a]	0.060[a]	0.0051[a]	0.0059[a]
Control	15.51[b]	3.14[b]	7.25[b]	7[b]	0.011[b]	0.022[b]	0.001[b]	0.0021[b]
SK2	19.86[c]	3.49[c]	11.04[c]	8[a]	0.031[c]	0.043[c]	0.0028[c]	0.0038[c]
PS25	18.22[d]	3.17[b]	10.14[d]	9[a]	0.033[c]	0.030[d]	0.0026[c]	0.0038[c]
LSG1	18.05[d]	3.09[b]	11.21[c]	15[c]	0.036[a]	0.040[c]	0.0041[d]	0.0050[d]

The same alphabets in each column mean no significant differences ($p \leq 0.05$) according to the Duncan's multiple range test.

CHAPTER 5
CONCLUSION

Seventy-eight endophytic bacteria were isolated from 7 sugarcane samples in 6 Provinces and 8 plant samples in Kanchanaburi Province of Thailand. Fifty isolates were obtained from sugarcane roots and 28 isolates from stems and leaves of other plants. Among the fifty isolates; 92%, 98%, 100% and 18% were able to solubilize P, Zn, fix nitrogen and produce IAA. Based on 16S rRNA gene sequence, they belonged to genera *Gluconacetobacter, Pantoea, Nguyenibacter, Burkholderia, Pseudomonas* and *Aureimonas w*hile *G. liquefaciens* was dominant species. Genome analysis indicated that strain PS25 was *G. dulcium.* This is the first report that *G. liquefaciens and G. dulcium* were isolated from sugarcane roots and able to fix nitrogen. Three strains; SK2, PS25 and LSG1; exhibited 'Khao Dawk Mali 105' rice seedlings growth promotion. Based on 16S rRNA gene sequence, the 28 isolates from other plant samples belonged to 14 genera and 17 species. The highest capability to fix nitrogen, P and Zn solubilization were isolate A2-2, Sx8-5, and Sl8-5, respectively. *Sphingobium* sp. Sx8-8 produced the highest IAA and solubilized P and Zn at optimized condition, medium supplemented with 0.5% L-tryptophan, pH 7.0, 30°C and 48 h incubation, the IAA produced was 232.1 µg/mL. Its draft genome was 4.67 Mbp with 83 contigs, 150,389 bp of N50 size, and 64.2 mol% G+C content. The *Sphingobium* sp. Sx8-8 showed 'RD6' rice seedlings growth promotion indicated potential to use as biofertilizer or bioinoculant. Further studies on an effect of the *Sphingobium* sp. Sx8-8 on plant growth promotion under pot and field conditions including mechanisms involved are required. Polyphasic characterization revealed that the strain Sx8-5^{T}, were Gram-negative, aerobic, non-spore forming, rods (0.6-0.7x1.3-1.5 µm). Colonies were yellow, circular, smooth and motiled by peritrichous flagella. Grew at 25-37°C (optimum at 30°C), pH 6-9 (optimum at 7) and in 1% NaCl (w/v) medium.

Catalase and oxidase were positive. Hydrolyzed tween 80 and aesculin but not urea, starch, and gelatin, and produced acid from L-arabinose, D-maltose, raffinose and D-xylose. API 20 NE test for 24 h showed positive for hydrolysis of aesculin, 4-nitrophenyl-β-D-galactopyranoside, assimilation of D-Glucose (weakly), L-arabinose, D-mannose, D-maltose, potassium gluconate, malic acid, trisodium citrate; whereas negative for nitrate reduction, L-tryptophan, acid production from D-glucose, hydrolysis of L-arginine, urea, gelatin, and no assimilation of D-mannitol, *N*-acetyl-glucosamine, capric acid, adipic acid and phenylacetic acid. The major fatty acids were summed feature 8 ($C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$), feature 3 ($C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$), and $C_{16:0}$. Polar lipid profile contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmonomethylethanolamine, two unidentified phospholipids and twelve unidentified polar lipids. The predominant respiratory quinone was Q-10. These results indicated that the strain Sx8-5^T represented a novel species of genus *Novosphingobium*. The name *Novosphingobium kaempferiae* sp. nov was proposed for the strain Sx8-5^T.

From the results obtained in this study, endophytic bacterial isolates from sugarcane roots have potential in nitrogen fixation which could help the host plant to obtain N directly from atmosphere and fulfill its nutritional requirements. Moreover, the findings of IAA-producing bacteria will provide their potential for application on rice growth. Extensive research on the investigations of plant improvement in pot by the influence of endophytic bacteria under natural condition will give new insights and a better understating of endophytes and host relationships in agricultural application.

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Appendix

APPENDIX A

1. Chemicals and Reagents

- Acetic acid glacial, Qchemical Co.Ltd., New Zealand
- Ammonium sulfate ($(NH_4)_2SO_4$), Sigma-Aldrich Co., Inc., Singapore
- Bromocresol purple, Elago Enterprises Pty Ltd, Australia
- Bromothymol blue, ECP LABCHEM-ECP Ltd., New Zealand
- Calcium carbonate ($CaCO_3$), Loba Chemie Pvt Ltd., India
- Calcium phosphate ($(Ca_3PO_4)_2$), Kao Industrial Co., Ltd., Thailand
- Calcium sulfate ($CaSO_4$), Univar Solutions Inc., US
- Chloroform, RCI Labscan Ltd., Thailand
- Crystal violet, Elago Enterprises Pty Ltd, Australia
- Dipotassium phosphate (K_2HPO_4), Univar Solutions Inc., US
- Ethyl acetate, Univar Solutions Inc., US
- Ethyl alcohol, Merck KGaA, Germany
- Ferric chloride ($FeCl_3$), Qchemical Co.Ltd., New Zealand
- Gelatin, ECP LABCHEM-ECP Ltd., New Zealand
- Glycerol, Qchemical Co.Ltd., New Zealand
- Hydrogen peroxide, Qchemical Co.Ltd., New Zealand
- Indole acetic acid, Merck KGaA, Germany
- Iodine, Univar Solutions Inc., US
- L-Tryptophan, HiMedia Laboratories, India
- Magnesium sulfate ($MgSO_4$), Univar Solutions Inc., US
- Mercuric chloride ($HgCl_2$), Sigma-Aldrich Co., Inc., Singapore
- Methanol, Merck KGaA, Germany
- Nessler's reagent, HiMedia Laboratories, India

- N, N-Diethyl-ρ-phenylenediamine, Sigma-Aldrich Co., Inc., Singapore
- Potassium hydroxide (KOH), Sigma-Aldrich Co., Inc., Singapore
- Potassium chloride (KCl), Merck KGaA, Germany
- Potassium dihydrogen phosphate (KH_2PO_4), Univar Solutions Inc., US
- Potassium iodine (KI), Merck KGaA, Germany
- Sanfranin O, Elago Enterprises Pty Ltd, Australia
- Sodium chloride (NaCl), Univar Solutions Inc., US
- Sodium hypochlorite (NaClO), Kao Industrial Co., Ltd., Thailand
- Sodium molybdate (Na_2MoO_4), Univar Solutions Inc., US
- Sulfuric acid (H_2SO_4), Qchemical Co.Ltd., New Zealand
- Tri-calcium phosphate, Sdfcl Sd Fine Chem Ltd., India
- Tween 80, Sigma-Aldrich Co., Inc., Singapore
- Zinc oxide (ZnO), Merck KGaA, Germany

2. Culture Media and Sugar

- Agar powder, HiMedia Laboratories, India
- Beef extract, HiMedia Laboratories, India
- Luria-Bertani (LB), HiMedia Laboratories, India
- Nutrient ager (NA) and Nutrient broth (NB), HiMedia Laboratories, India
- Murashige Skoog basal medium with vitamins, Phyto Techonology Laboratories, US
- R2A medium, Merck KGaA, Germany
- Simmons citrate agar, HiMedia Laboratories, India
- Skim milk agar, HiMedia Laboratories, India
- Tributyrin agar, HiMedia Laboratories, India
- Tryptic soy agar (TSA), Merck KGaA, Germany

- D (+)-Cellobiose, Fluka, Fisher Scientific, UK
- D (-)-Fructose, Univar Solutions Inc., US
- D (+)-Galactose, Univar Solutions Inc., US
- D-glucose, Univar Solutions Inc., US
- D (-)-Ribose, TCI Europe N.V., Belgium
- D-Sorbitol, UNILAB PTY. Ltd., Australia
- D (+)-Trehalose dihydrate, Fluka, Fisher Scientific, UK
- D (+)-Xylose, Merck KGaA, Germany
- Lactose-Monohydrate, Merck KGaA, Germany
- L-arabinose, TCI Europe N.V., Belgium
- L-arginine, Fluka, Fisher Scientific, UK
- Peptone (bacteriological), HiMedia Laboratories, India
- Raffinose, Difco BBL, USA
- Soluble starch, Univar Solutions Inc., US
- Sucrose, Elago Enterprises Pty Ltd, Australia
- Yeast extract Powder, HiMedia Laboratories, India

3. Media composition

1) Glucose-ethanol-calcium carbonate agar (GECA)

Glucose	25.00	g
Peptone	5.00	g
Yeast extract	3.00	g
$CaCO_3$	3.00	g
Agar	15.00	g
Absolute ethanol	5.00	mL
Distilled water	1.00	litre

2) Luria Bertani Broth (LB)

Tryptone	10.00	g
Yeast extract	5.00	g
NaCl	10.00	g
Final pH (at 25°C) 7.5±0.2		

3) Nutrient agar (NA)

Peptone	5.00	g
Beef extract	3.00	g
Agar	15.00	g
Distilled water	1.00	litre
Adjust to pH 7±0.1		

4) Nitrogen free (NF)

K_2HPO_4	0.80	g
KH_2PO_4	0.20	g
$MgSO_4$	0.20	g
$CaSO_4$	0.13	g
$FeCl_3$	0.00145	g
Na_2MoO_4	0.000253	g
sucrose	20.00	g
Agar	15.00	g
Distilled water	1.00	litre

Adjust pH to 5.5 with acetic acid

5) Mineral salt agar

Glucose	10.00	g
$(NH_4)_2SO_4$	1.00	g
KCl	0.20	g
K_2HPO_4	0.10	g
$MgSO_4$	0.20	g
Agar	15.00	g
Distilled water	1.00	litre

Adjust pH to 7±0.1 and 0.1% Zinc Oxide (ZnO) was added to the medium. Medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

6) Pikovskaya's agar

Glucose	10.00	g
$Ca_3(PO_4)_2$	5.00	g
$(NH_4)_2SO_4$	0.50	g
KCl	0.20	g
$MgSO_4$	0.10	g
Yeast extract	0.50	g
Agar	15.00	g
Distilled water	1.00	litre

Adjust pH to 7±0.1

7) R2A

Acicase#	0.50	g
Yeast extract	0.50	g
Proteose peptone	0.50	g
Glucose (Dextrose)	0.50	g
Starch soluble	0.50	g
K_2HPO_4	0.30	g
$MgSO_4$	0.024	g
Sodium pyruvate	0.30	g
Agar	15.00	g
Distilled water	1.00	litre

Final pH (at 25°C) 7.2±0.2, # Equivalent to casein acid hydrolysis

8) Tryptone Soya Broth (TSB)

Pancreatic digest of casein	17.00	g
Papaic digest of soyabean meal	3.00	g
NaCl	5.00	g
Dextrose	2.50	g
K_2HPO_4	2.50	g
Distilled water	1.00	litre

Final pH (at 25°C) 7.3±0.2

9) Simmom citrate medium

Simmom citrate agar	24.28	g
Distilled water	1.00	litre

10) Starch agar

Tryptic Soy Broth	30.00	g
Starch	10.00	g
Agar	15.00	g
Distilled water	1.00	litre

11) Skim milk agar

Skim milk agar	100.00	g
Distilled water	1.00	litre

12) Gelatin agar

Beef extract	3.00	g
Peptone	5.00	g
Gelatin	7.00	g
Agar	15.00	g
Distilled water	1.00	litre

13) Lipolytic medium

Peptone	10.00	g
Yeast extract	5.00	g
$CaCl_2 \bullet {}_2H_2O$	0.10	g
Agar	15.00	g
Distilled water	1.00	litre
Tween 80	0.10	g

14) Aesculin agar

Beef extract	3.00	g
Yeast extract	5.00	g
Esculin	1.00	g
Glucose	2.50	g
Ferric citrate	0.50	g
$MnSO_4 \bullet {}_4H_2O$	0.10	g
Tween 80	1.00	g
Distilled water	1.00	litre

4. Instruments

- Autoclave (model autoclave SX-700, Tomy Kogyo Co., Ltd, Japan)

-4-Digit precision weighting balance (model MS3002 TS/00, Mettler Toledo, Switzerland)

- Electrophoresis chamber set (model Mupid®ex U, Advance, Japan)

- High speed refrigerated centrifuge (model 5804R, Eppendorf, Germany)

- Low temperature incubator (model IF30, Memmert GmbH and Co. KG., Germany)

- Low temperature incubator shaker (model MaxQ 481R HP, Thermo Scentific, USA)

- Laminar flow (model 25 Manometer, Boss Tech, USA)

- Microscope (model Leica DM750, Leica, Singapore)

- Microwave (Toshiba, Japan)

- Microplate reader (model spectra Max M2^{e}, Molecular Devices, LLC, USA)

- Nanodrop (model NanoDrop 2000C, Thermo Scientific, USA)

- pH meter (Metter Toledo, Switzerland)

- Microcentrifuge (model HERAEUS Pico 17, Thermo Scientific, USA)

- UV Vis Spectrophotometer (model GENESYS^{TM}20, Spectonic, Thermo Scientific, USA)

- Vortex mixer (model G-560E, Scientific Industries, Inc., Bohemia USA)

- Water bath (model WTB50, Memmert GmbH and Co. KG., Germany)

- Rotary evaporator (model Rotavapor R-20, BUCHI, Switzerland)

- Laboratory feezer -80°C (model FORMA 900 series, Thermo Scientific, USA)

- Hot air oven (model HERA THERM, Thermo Scientific, USA)

- Gel documentation system (Bio-Rad Laboratories Gel Doc TM XR, USA)

APPENDIX B

CHEMICAL AGENTS

1) Universal primers

800R	5'-TACCAGGGTATCTAATCC - 3'; 802-785
27F	5'-AGAGTTTGATCCTGGCTCAG-3'; 8-27
518F	5'-CCAGCAGCCGCGGTAAT-3'; 542-518
1492R	5'-GGTTACCTTGTTACGACTT-3'; 1492-1507

2) TE buffer

Tris. Cl pH 8.0, 10 mM	1 M (stock)	10	mL
EDTA 2Na, 1mM	250 mM (stock)	4.0	mL
Distilled water		986.0	mL
Total volume		1000	mL

3) 100X TAE buffer (autoclave)

Tris base	242.0	mL
Glacial acetic acid	57.5	mL
0.5 M EDTA pH 8.0	100.0	mL
Distilled water	110.5	mL
Total volume	500.0	mL

4) Phenol/chloroform/isoamyl alcohol (25:24:1)

Phenol (65°C)	240.0	mL
Chloroform	230.4	mL
Isoamyl alcohol	9.6	mL
0.1 M Tris-Cl, pH 8.0	20.0	mL
Stored in light-tight bottle and place art 4°C		

5) Agarose gel (2.5%)

Agarose powder	0.8	g
1X TAE buffer	100.0	mL

6) Rapid flagella staining (Forbes, 1981)

Mixture A

Basic fuchsin	0.4	g
Acid fuchsin	0.2	g
Tannic acid	0.2	g
Aluminum ammonium sulfate	0.5	g

Solvent B

95% Ethanol	2.0	mL
Glycerol	0.5	mL
Tris buffer (0.05 M pH7.6 adjust with HCl)	7.5	mL

Mixture of A and B for 3-5 min and centrifuge at 2,500 rpm for 2min. Supernatant is transferred into another tube and left at room temperature for 30 min before use.

7) Salkowski solution

Solution A: Dissolve 0.45 g of $FeCl_3$ (MW=162.2) in DI water 100 mL

Solution B: Mixture of 11 mL of H_2SO_4 and 79 mL of DI water

Solution A is mixed well with solution B

8) Indole 3 acetic acid (IAA) standard solution at 100 µg/mL

To dissolve the 0.025 g of IAA completely, add 2-5mL of 95% ethanol and mixed well. Bring final volume to 50 mL with DI water and mixed well (stock IAA at 500 µg/mL). Stock IAA solution diluted as 100 µg/mL concentration. Different concentrations of IAA were prepared (0, 10, 20, 30, 40, 50, 60, 70, 80, 80, 100) as shown in Table B.1

Table B.1 Different concentration of standard IAA solution

Conc. (µg/mL)	100	90	80	70	60	50	40	30	20	10	0
IAA 100 µg/mL	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.0
DI. (mL)	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0

8.1) Absorbance at 530 nm and standard curve of IAA for calculation IAA production of bacterial strains isolated from root of sugarcane

Table B.2 Absorbance of standard IAA solution at 530 nm for calculation IAA production of bacterial strains isolated from root of sugarcane

Conc.(µg/mL) / OD (530nm)	0	10	20	30	40	50	60	70	80	90	100
Repeat 1	0.042	0.069	0.11	0.139	0.165	0.191	0.21	0.24	0.265	0.305	0.335
Repeat 2	0.041	0.073	0.11	0.137	0.166	0.193	0.21	0.238	0.267	0.307	0.334
Repeat 3	0.043	0.073	0.11	0.136	0.164	0.189	0.21	0.239	0.265	0.307	0.336

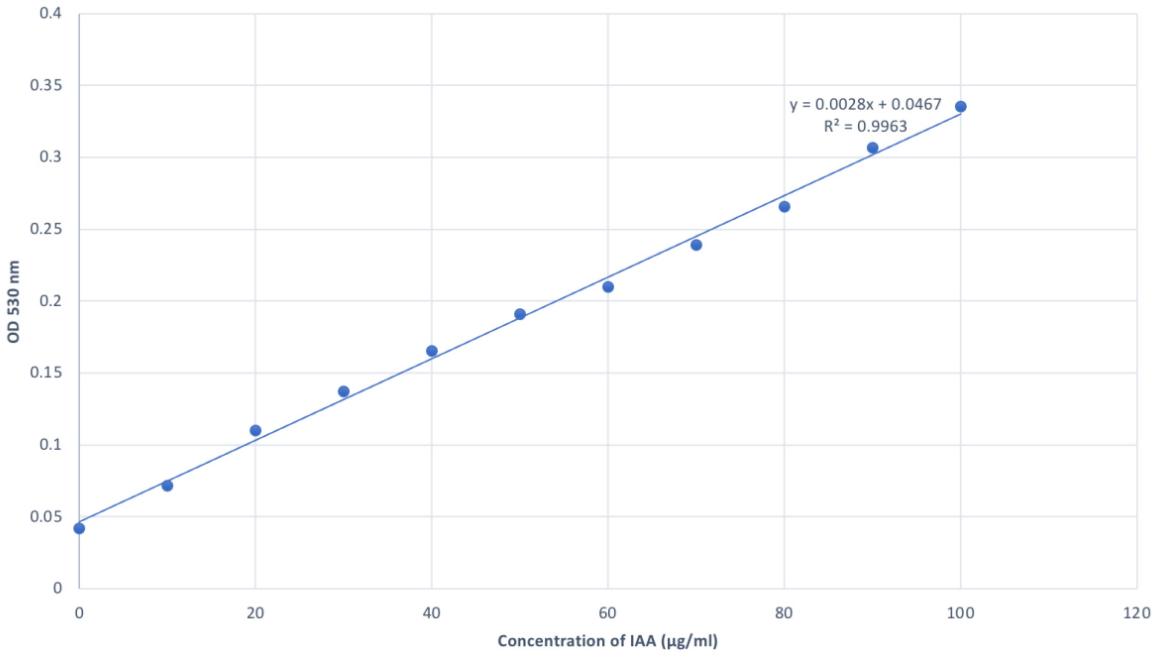

Figure B.1 Standard curve of IAA (µg/mL) for calculation IAA production of bacterial strains isolated from root of sugarcane

8.2) Standard curve of IAA for calculation IAA production of bacterial strains isolated from plants in Kanchanaburi Province

Table B.3 Absorbance of standard IAA solution at 530 nm for calculation IAA production of bacterial strains isolated from plants in Kanchanaburi Province

Conc. (µg/mL) / OD (530nm)	0	10	20	30	40	50	60	70	80	90	100
Repeat 1	0.042	0.095	0.158	0.219	0.275	0.324	0.379	0.427	0.469	0.519	0.550
Repeat 2	0.042	0.095	0.187	0.21	0.275	0.326	0.381	0.431	0.473	0.517	0.560
Repeat 3	0.042	0.095	0.158	0.214	0.275	0.324	0.377	0.422	0.473	0.518	0.559

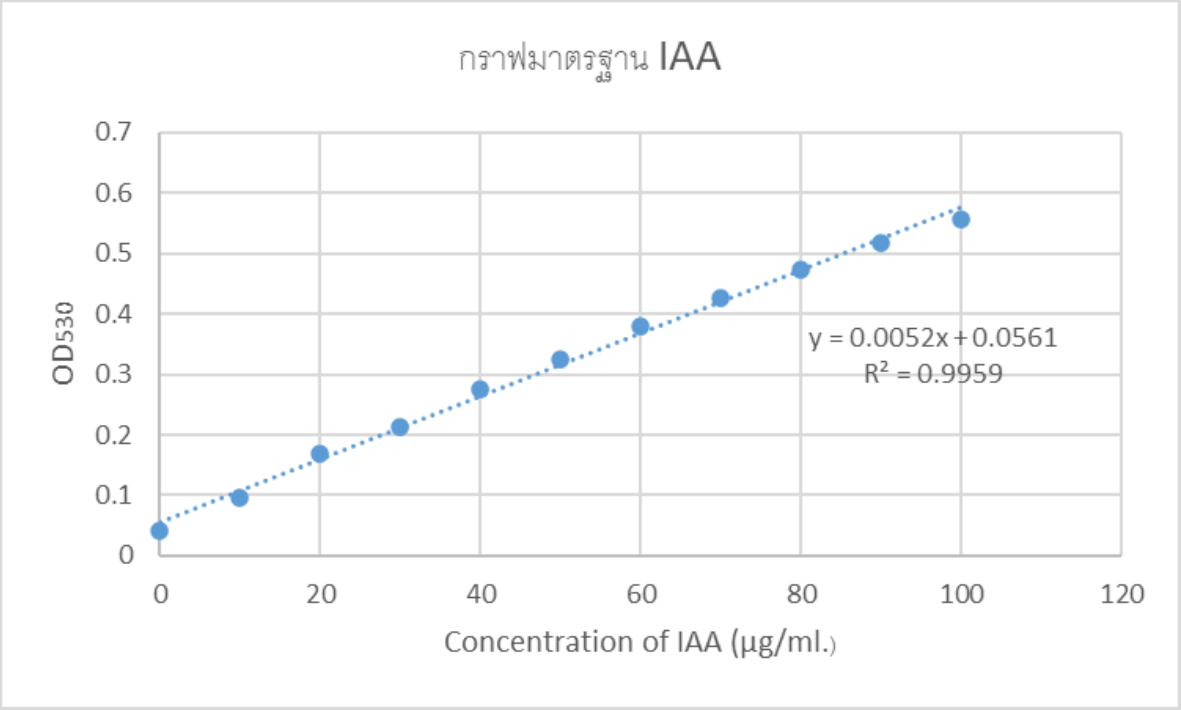

Figure B.2 Standard curve of IAA (µg/mL) for calculation IAA production of bacterial strains isolated from plants in Kanchanaburi Province

9) Ammonia standard curve

Table B.4 Absorbance of ammonium sulfate ($(NH4)_2SO_4$) solution at 560 nm

Conc. (mmol/L)	1.072	2.501	4.912	10.717
Repeat 1	0.081	0.3356	2.0737	3.1612
Repeat 2	0.0656	0.3332	2.0604	3.2039
Repeat 3	0.0688	0.3298	2.0594	3.2374
Repeat 4	0.0662	0.3217	2.0384	3.2486
Repeat 5	0.078	0.3397	2.0512	3.2061

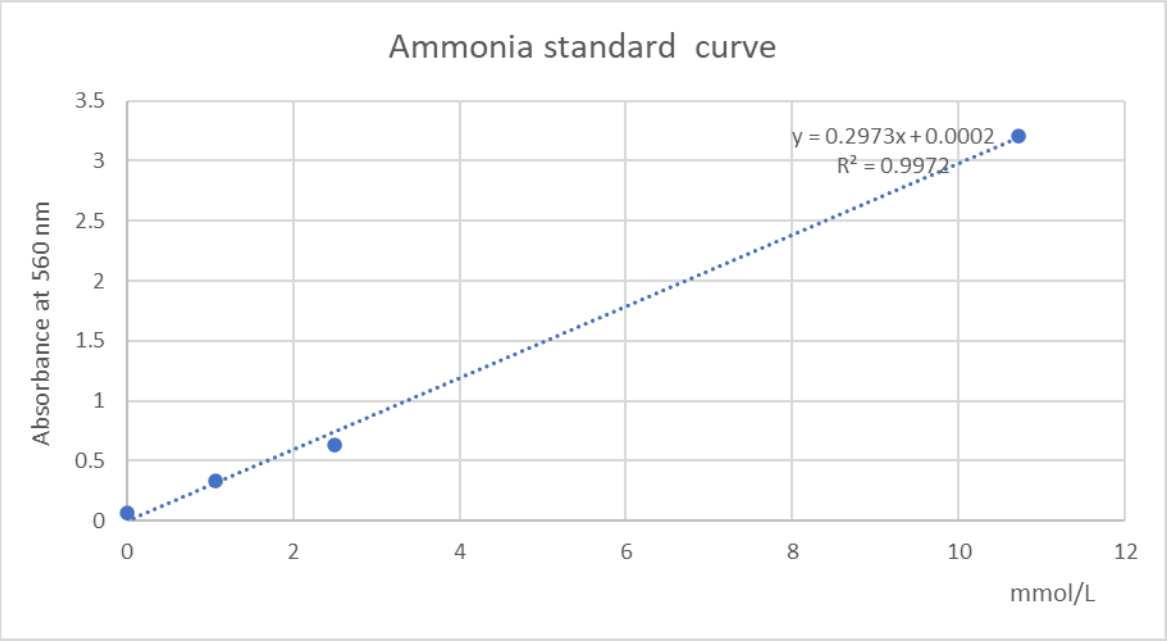

Figure B.3 Ammonia standard curve

APPENDIX C

RESULTS

1) Plant samples

Figure C1. *Saccharum officinarum* Linn. (A) tree (B) root of Khon Kaen 3 variety (C) root of U-Thong variety (D) root of Black Sugar Cane

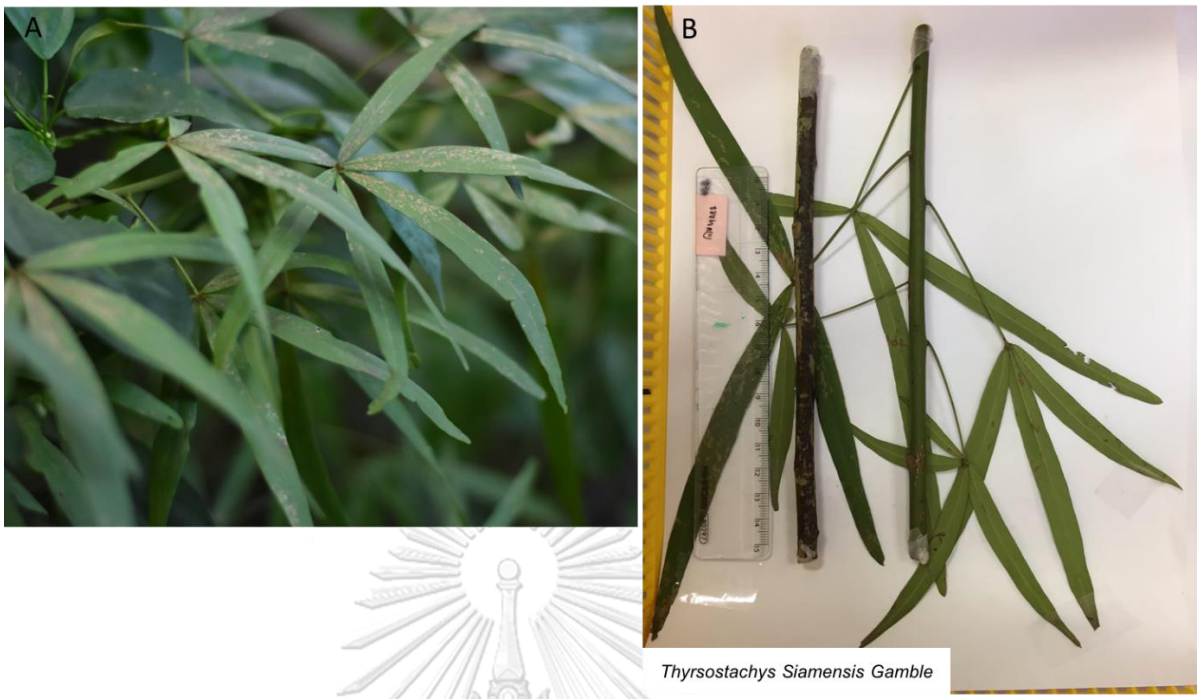

Figure C2. *Thyrsostachys siamensis* Gamble (A) tree (B) stem and leave

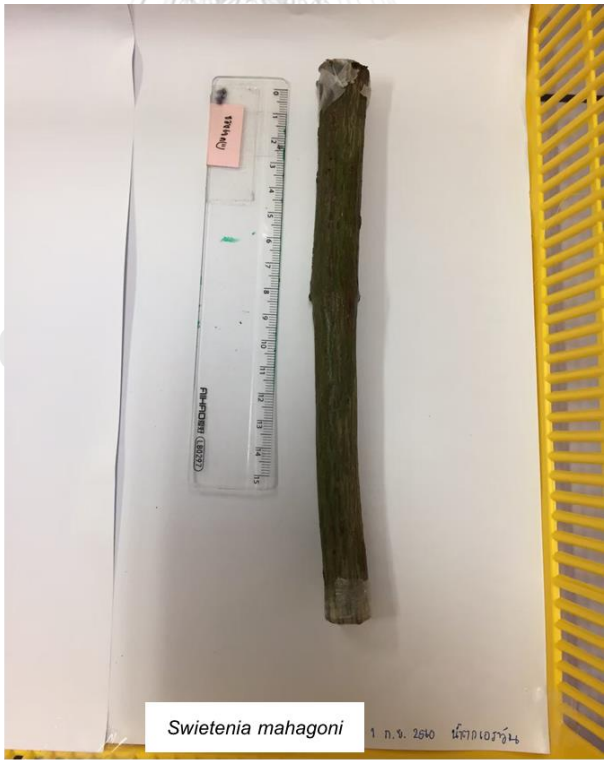

Figure C3. Stem of *Swietenia mahagoni* (L.) Jacq.

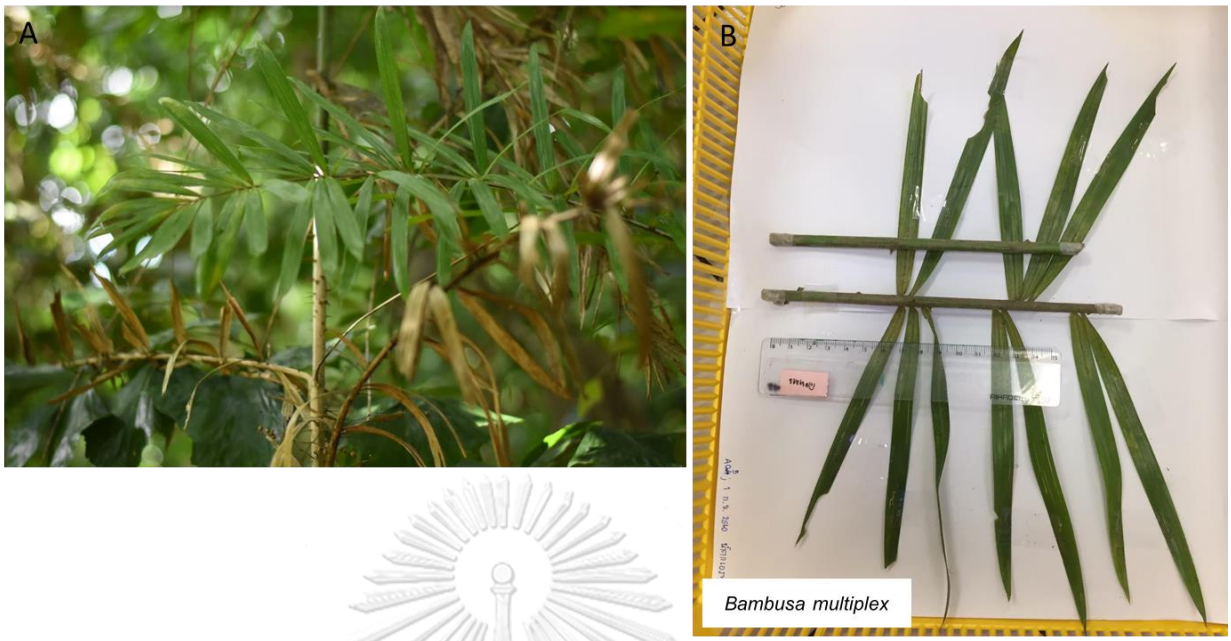

Figure C4 *Bambusa multiplex* (Lour.) Raeusch. ex Schult.f. (A) tree (B) stem and leave

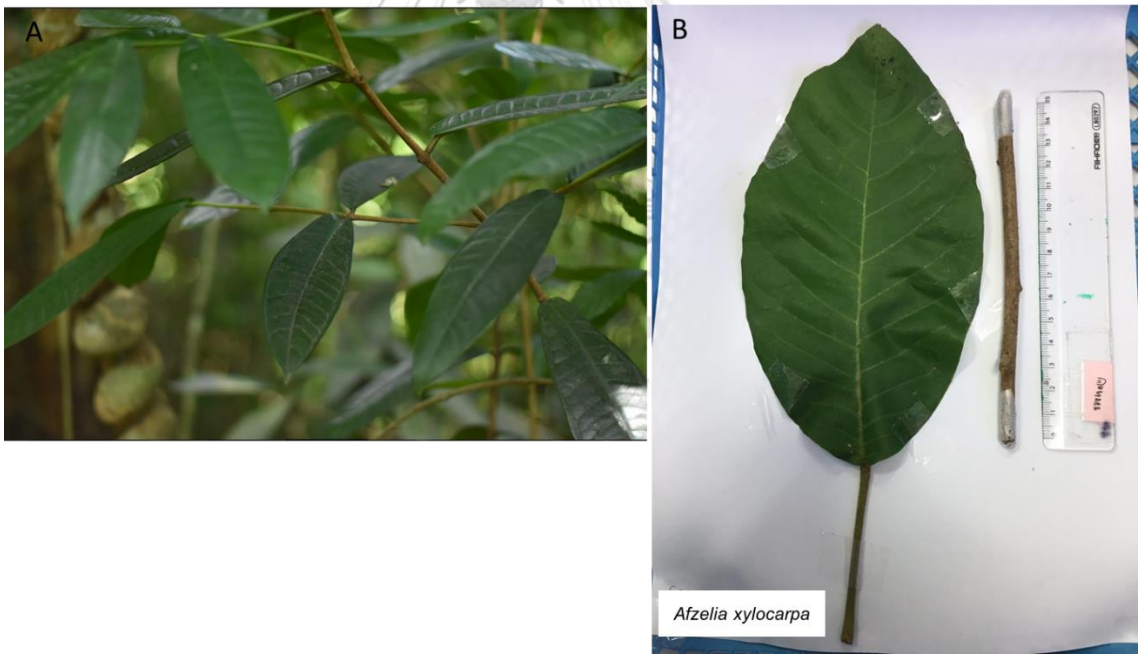

Figure C5. *Afzelia xylocarpa* (Kurz) Craib (A) tree (B) stem and leave

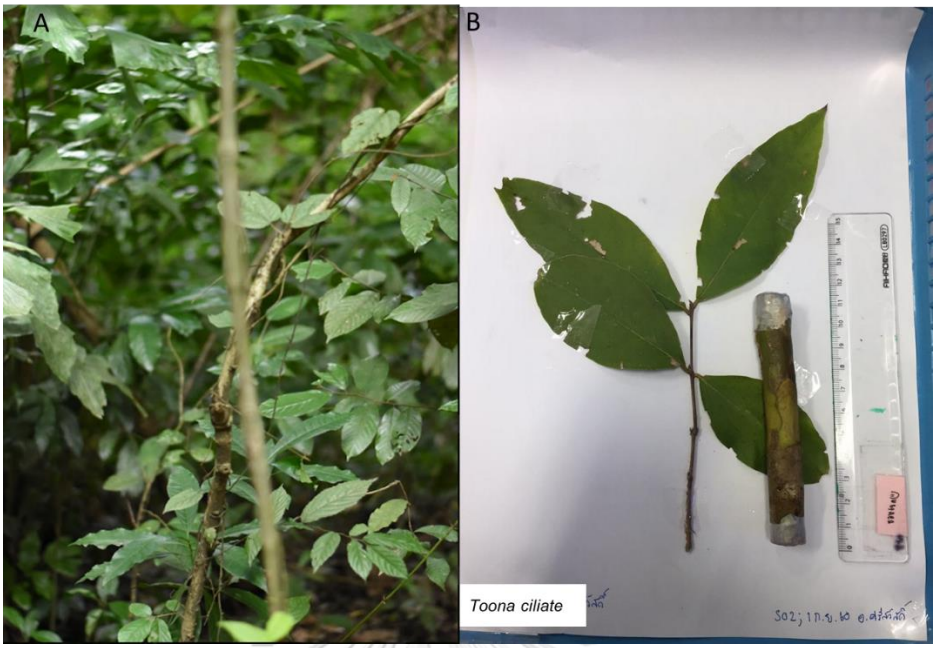

Figure C6. *Toona ciliate* M. Roem. (A) tree (B) stem and leave

Figure C7 Stem and leave of *Crateva religiosa* G.Forst.

Figure C8 *Phyllanthus emblica* L. (A) tree (B) stem and leave

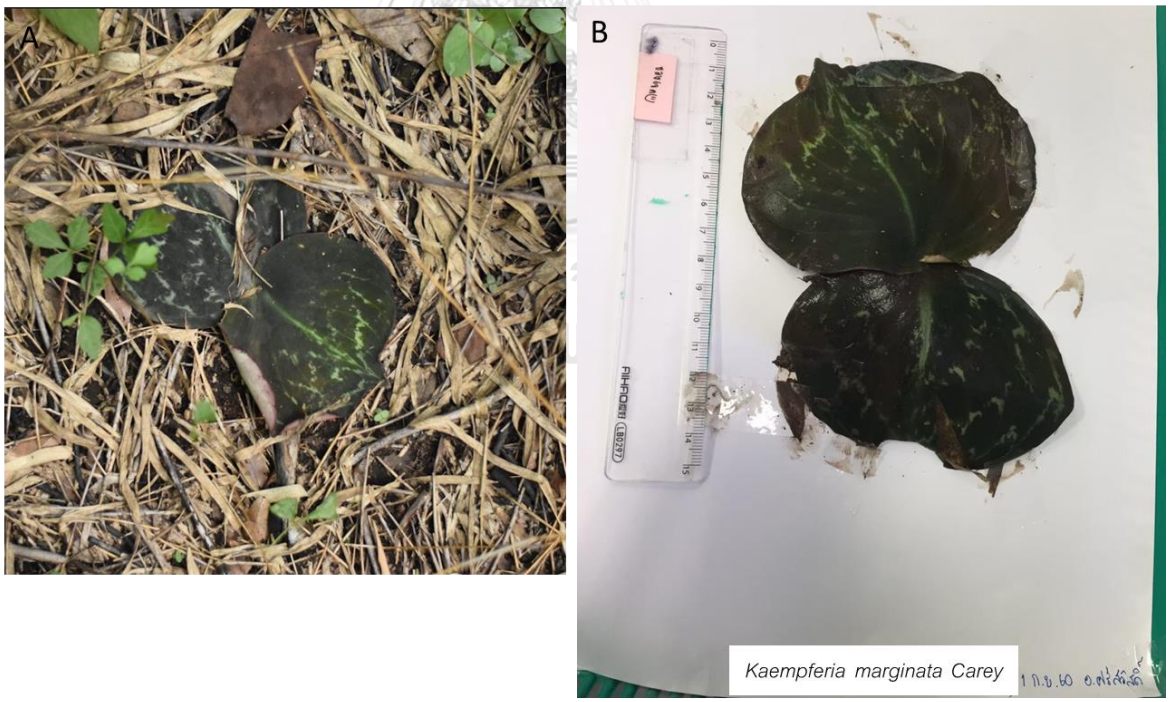

Figure C9. *Kaempferia marginata* Carey (A) tree (B) stem and leave

2) Dendrogram illustration

2.1) Grouping of bacterial strains isolated from root of sugarcane based in dendrogram illustration, was constructed by IBM SPSS statistics software (Version 22)

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ctose raffinose ribose sorbitol trehalose arabinose fructose galactose cellub
iose xylose sucrose cellform
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  /MEASURE=SEUCLID
  /ID=code
  /PRINT SCHEDULE
  /PLOT DENDROGRAM VICICLE.
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Cluster

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Case Processing Summary[a,b]

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a. Squared Euclidean Distance used

b. Average Linkage (Between Groups)

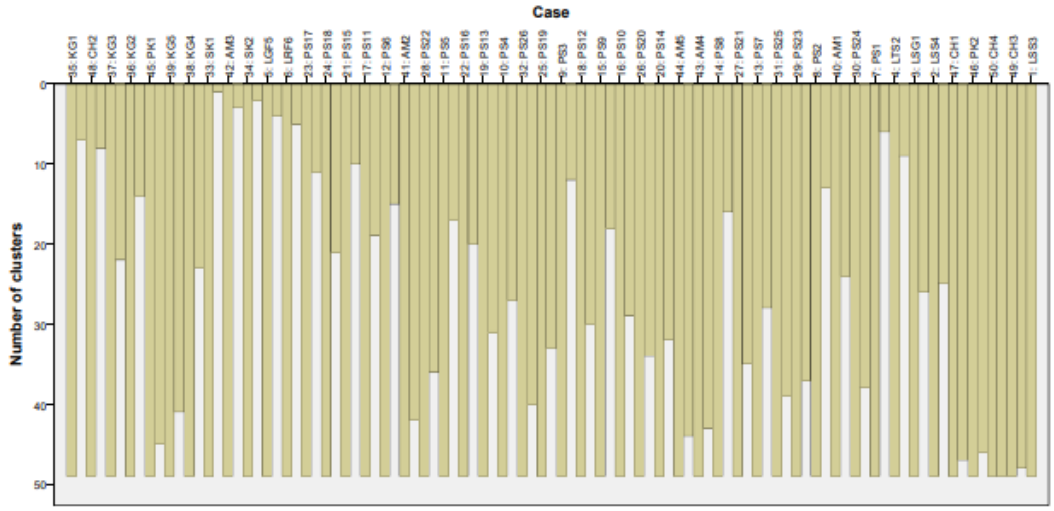
Average Linkage (Between Groups)

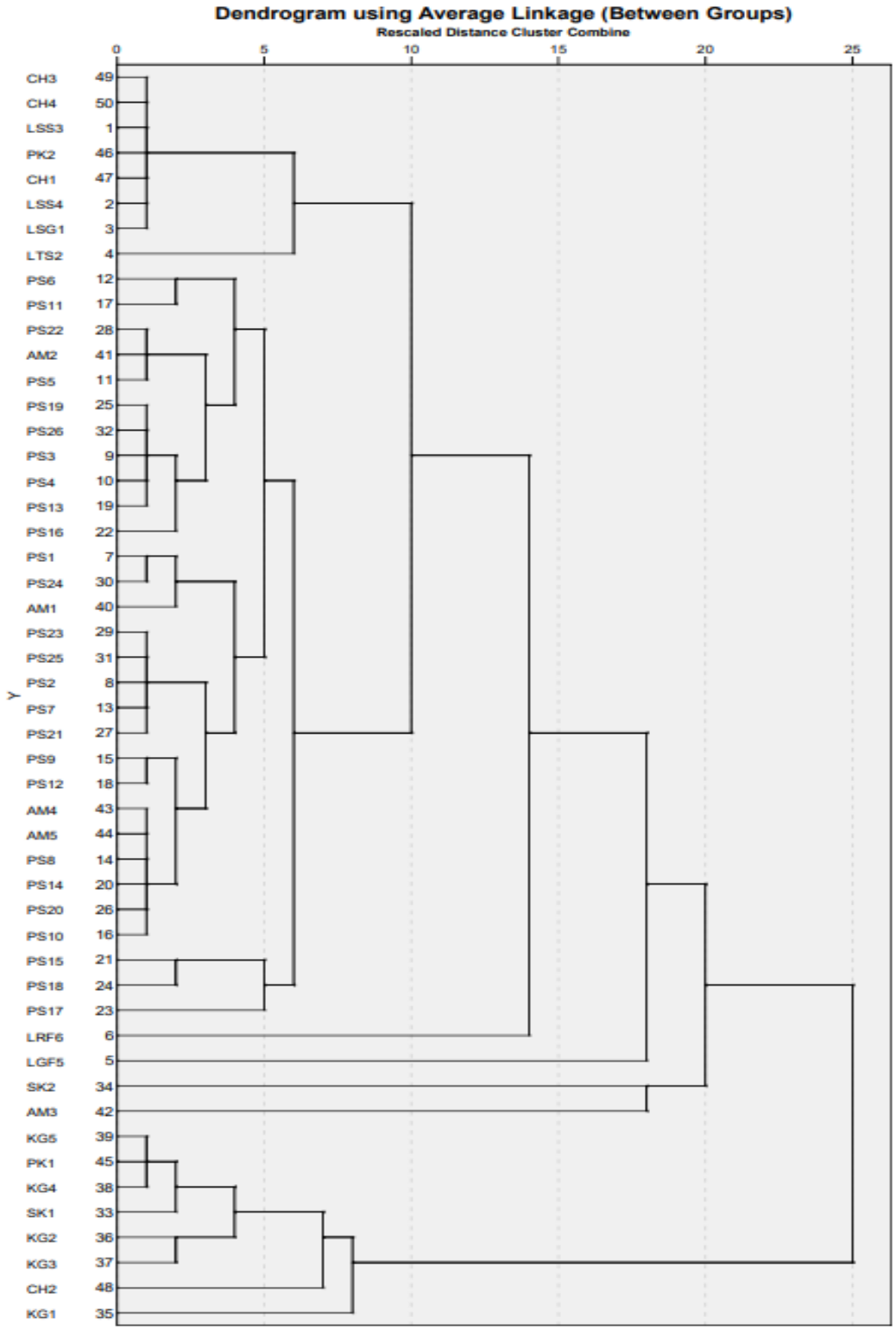
Agglomeration Schedule

Stage	Cluster Combined		Coefficients	Stage Cluster First Appears		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	49	50	.000	0	0	2
2	1	49	.000	0	1	4
3	46	47	.000	0	0	4
4	1	46	.000	2	3	25
5	39	45	.000	0	0	9
6	43	44	.000	0	0	7
7	14	43	.000	0	6	18
8	28	41	.000	0	0	14
9	38	39	.000	0	5	27
10	25	32	.000	0	0	17
11	29	31	.000	0	0	13
12	7	30	.000	0	0	26
13	8	29	.000	0	11	22
14	11	28	.000	0	8	33
15	13	27	.000	0	0	22
16	20	26	.000	0	0	18
17	9	25	.000	0	10	23
18	14	20	.000	7	16	21
19	10	19	.000	0	0	23
20	15	18	.000	0	0	32
21	14	16	.000	18	0	32
22	8	13	.000	13	15	34
23	9	10	.000	17	19	30
24	2	3	.000	0	0	25
25	1	2	.000	4	24	41
26	7	40	1.000	12	0	37
27	33	38	1.000	0	9	36
28	36	37	1.000	0	0	36
29	21	24	1.000	0	0	39
30	9	22	1.000	23	0	33
31	12	17	1.000	0	0	35
32	14	15	1.000	21	20	34
33	9	11	1.167	30	14	35
34	8	14	1.250	22	32	37
35	9	12	1.611	33	31	38
36	33	36	1.750	27	28	42
37	7	8	1.795	26	34	38

Agglomeration Schedule

Stage	Cluster Combined		Coefficients	Stage Cluster First Appears		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
38	7	9	2.125	37	35	40
39	21	23	2.500	29	0	40
40	7	21	2.852	38	39	44
41	1	4	3.000	25	0	44
42	33	48	3.333	36	0	43
43	33	35	3.714	42	0	49
44	1	7	4.817	41	40	45
45	1	6	6.632	44	0	46
46	1	5	8.769	45	0	48
47	34	42	9.000	0	0	48
48	1	34	10.075	46	47	49
49	1	33	12.595	48	43	0

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10
15
20
25
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CH3 49
CH4 50
LSS3 1
PK2 46
CH1 47
LSS4 2
LSG1 3
LTS2 4
PS6 12
PS11 17
PS22 28
AM2 41
PS5 11
PS19 25
PS26 32
PS3 9
PS4 10
PS13 19
PS16 22
PS1 7
PS24 30
AM1 40
PS23 29
PS25 31
PS2 8
PS7 13
PS21 27
PS9 15
PS12 18
AM4 43
AM5 44
PS8 14
PS14 20
PS20 26
PS10 16
PS15 21
PS18 24
PS17 23
LRF6 6
LGF5 5
SK2 34
AM3 42
KG5 39
PK1 45
KG4 38
SK1 33
KG2 36
KG3 37
CH2 48
KG1 35

2.2) Grouping of bacterial strains isolated from plants in Kanchanaburi province based in dendrogram illustration, was constructed by IBM SPSS statistics software (Version 22)

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ctose raffinose ribose sorbitol trehalose arabinose fructose galactose cellub
iose xylose sucrose cellform
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Cluster

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Case Processing Summary[a,b]

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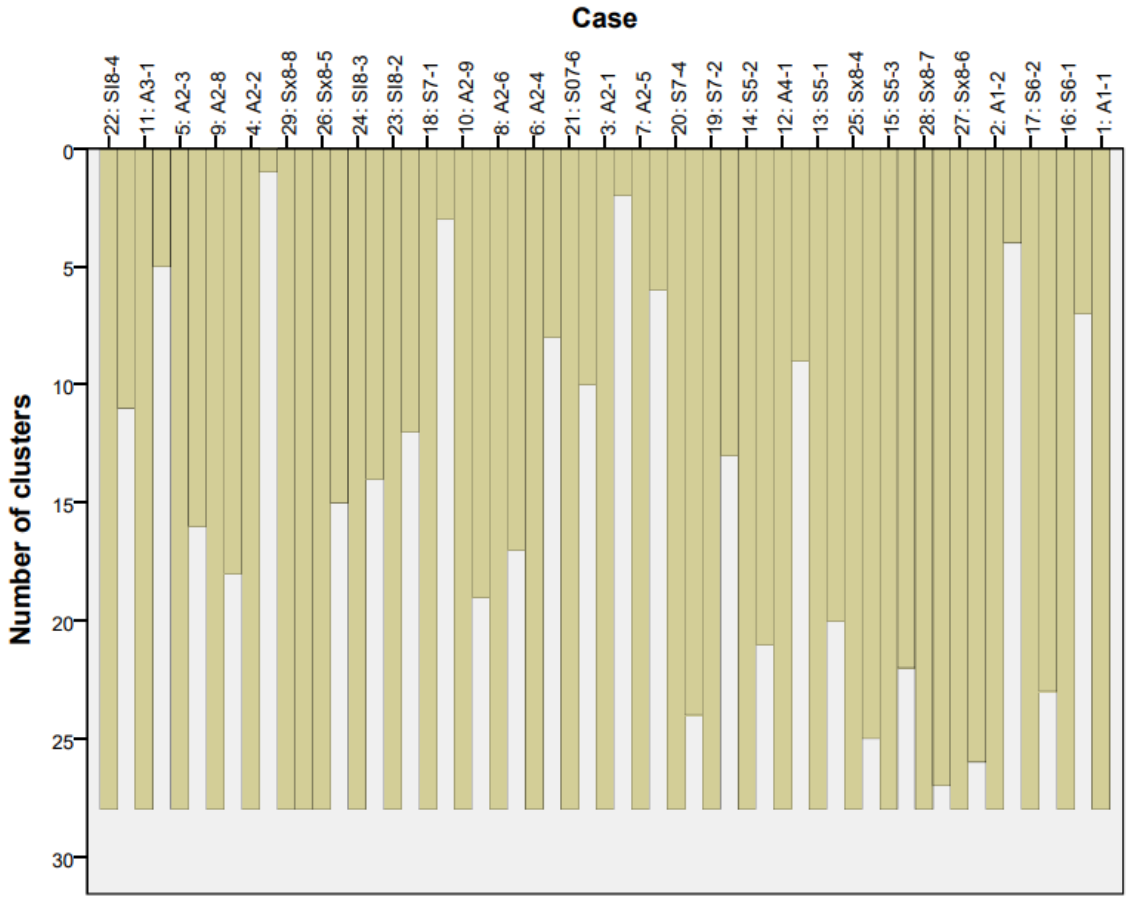
a. Squared Euclidean Distance used

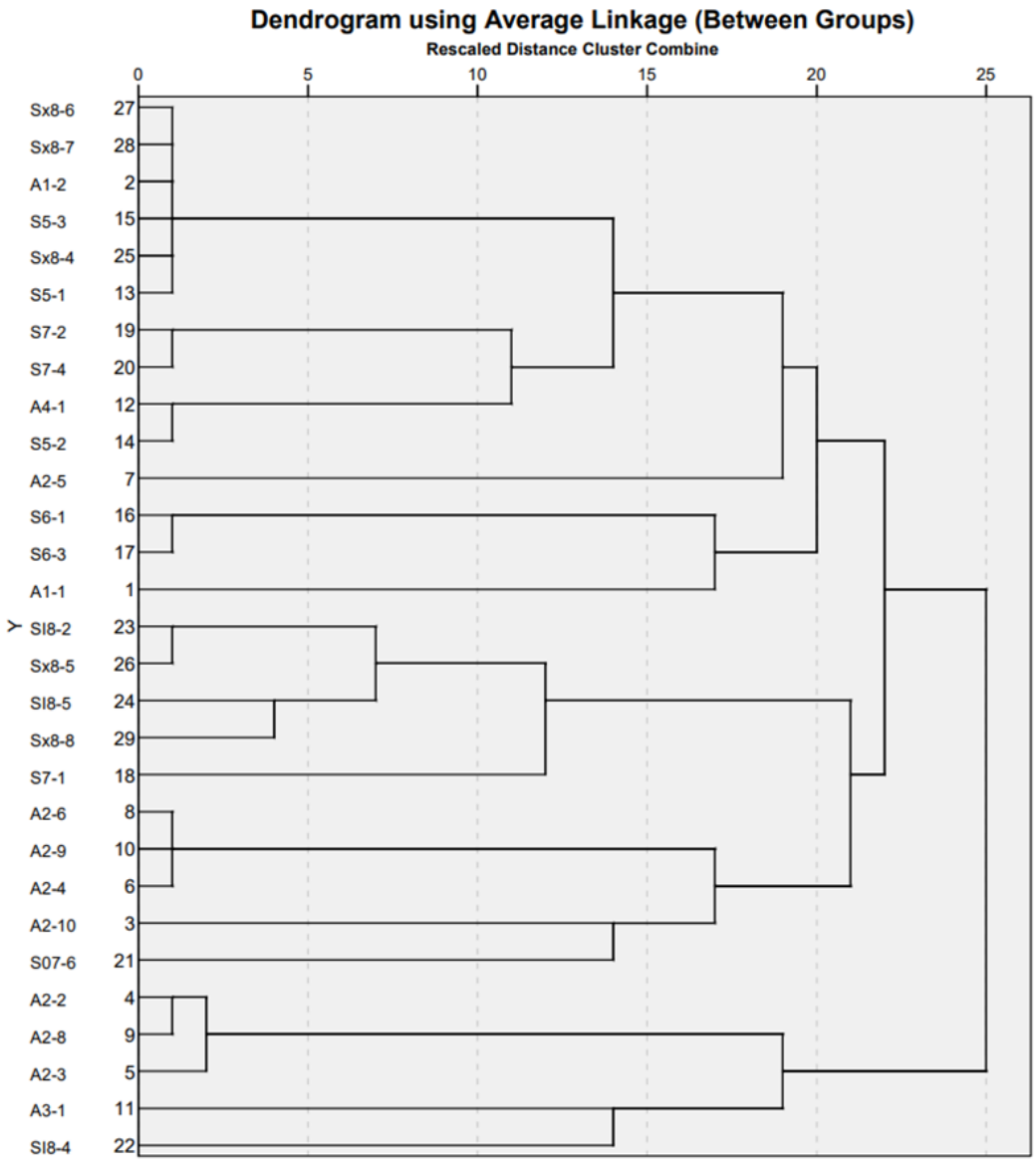
b. Average Linkage (Between Groups)

Average Linkage (Between Groups)



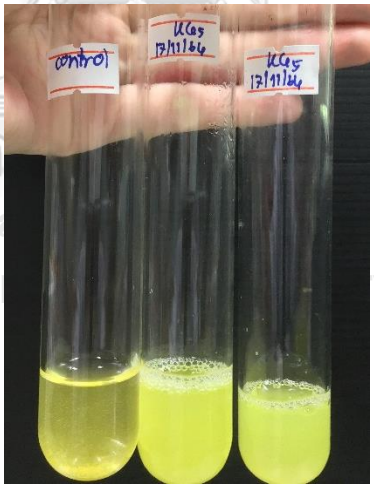
Agglomeration Schedule

Stage	Cluster Combined		Coefficients	Stage Cluster First Appears		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	26	29	.000	0	0	14
2	27	28	.000	0	0	3
3	2	27	.000	0	2	7
4	15	25	.000	0	0	7
5	19	20	.000	0	0	16
6	16	17	.000	0	0	22
7	2	15	.000	3	4	9
8	12	14	.000	0	0	16
9	2	13	.000	7	0	20
10	8	10	.000	0	0	12
11	4	9	.000	0	0	13
12	6	8	.000	0	10	21
13	4	5	1.000	11	0	24
14	24	26	2.000	0	1	15
15	23	24	4.000	0	14	17
16	12	19	6.000	8	5	20
17	18	23	7.500	0	15	26
18	11	22	8.000	0	0	24
19	3	21	8.000	0	0	21
20	2	12	8.000	9	16	23
21	3	6	9.000	19	12	26
22	1	16	10.000	0	6	25
23	2	7	10.000	20	0	25
24	4	11	11.333	13	18	28
25	1	2	11.667	22	23	27
26	3	18	11.720	21	17	27
27	1	3	12.700	25	26	28
28	1	4	14.033	27	24	0


Dendrogram using Average Linkage (Between Groups)
Rescaled Distance Cluster Combine
0
5
10
15
20
25
Y
Sx8-6 27
Sx8-7 28
A1-2 2
S5-3 15
Sx8-4 25
S5-1 13
S7-2 19
S7-4 20
A4-1 12
S5-2 14
A2-5 7
S6-1 16
S6-3 17
A1-1 1
SI8-2 23
Sx8-5 26
SI8-5 24
Sx8-8 29
S7-1 18
A2-6 8
A2-9 10
A2-4 6
A2-10 3
S07-6 21
A2-2 4
A2-8 9
A2-3 5
A3-1 11
SI8-4 22

3) Nitrogen fixation

Strain no.	OD_{560}			Average
	1	2	3	
LSG1	4.97	4.97	4.96	4.966667
LSS3	11.3	11.5	11.4	11.4
LRF6	5.32	5.26	5.27	5.283333
PS25	4.43	4.45	4.48	4.453333
SK2	6.7	6.32	6.62	6.546667
KG1	7.3	7.32	7.35	7.323333
AM2	8.5	8.38	7.98	8.286667
AM4	4.32	4.5	4.4	4.406667
CH4	4.6	5.05	4.7	4.783333



4) P and Zn solubilization of

4.1 P solubilization of bacterial strains

Strain no.	Clear zone diameter (cm)	Colony diameter (cm)	Strain no.	Clear zone diameter (cm)	Colony diameter (cm)
CH1	1.5	0.7	AM1	1	0.85
CH2	1.01	0.55	AM2	1	0.7
CH3	1.6	0.7	AM3	1.5	0.7
CH4	1.6	075	AM4	1.95	0.6
SK1	1	0.8	AM5	1	0.65
SK2	-	1.27	PS1	0.95	0.65
PK1	-	0.57	PS2	1.1	0.7
PK2	1.5	0.65	PS3	2	0.5
KG1	1.94	0.5	PS4	1.95	0.5
KG2	1.8	1.3	PS5	2.05	0.55
KG3	-	1.22	PS6	2.2	0.5
KG4	1.58	0.8	PS7	2	0.55
KG5	-	1.3	PS8	0.95	0.55
LSG1	1.6	0.6	PS9	2.5	0.7
LTS2	1.7	0.65	PS10	0.85	0.55
LSS3	1.2	0.7	PS11	2.1	0.65
LSS4	1.6	0.7	PS12	1.75	0.65
LGF5	1	0.6	PS13	2.3	0.65
LRF6	1	0.65	PS14	0.75	0.65

4.1 P solubilization of bacterial strains (continued)

Strain no.	Clear zone diameter (cm)	Colony diameter(cm)	Strain no.	Clear zone diameter (cm)	Colony diameter (cm)
PS15	2.1	0.5	A2-9	1.5	1.1
PS16	1.1	0.6	A2-10	1.55	1.15
PS17	1.5	0.65	A3-1	1.3	0.65
PS18	1.8	0.7	S4-1	1.3	1.0
PS19	0.85	0.5	S5-1	-	1.2
PS20	1	0.75	S5-2	1.5	0.8
PS21	1.7	0.6	S5-3	1.7	0.8
PS22	1.9	0.75	S6-1	-	0.7
PS23	1.85	0.85	S6-3	-	0.55
PS24	2.1	0.7	S7-2	1.35	1.2
PS25	1.85	0.7	S7-4	1.15	0.9
PS26	1.95	0.75	S7-6	1.95	1.65
A1-1	1.6	1.45	SI8-2	0.8	0.7
A1-2	0.95	0.8	SI8-4	1.35	1.15
A2-2	1.3	0.95	SI8-5	1.75	1.15
A2-3	1.65	1.3	Sx8-4	0.8	0.7
A2-4	2.3	1.1	Sx8-5	3.0	1.2
A2-5	2.3	1.25	Sx8-6	1	0.9
A2-6	1.0	0.8	Sx8-7	-	0.75
A2-8	-	0.7	Sx8-8	2	1.4

4.2 Zn solubilization of bacterial strains

Strain no.	Clear zone diameter (cm)	Colony diameter (cm)	Strain no.	Clear zone diameter (cm)	Colony diameter (cm)
CH1	2.8	0.55	AM1	1.7	0.55
CH2	1.33	0.55	AM2	1.85	0.6
CH3	2.7	0.5	AM3	2.2	0.6
CH4	2.7	0.65	AM4	2.05	0.5
SK1	1.68	0.6	AM5	2.05	0.6
SK2	-	-	PS1	1.6	0.6
PK1	-	0.65	PS2	1.75	0.6
PK2	2.9	0.6	PS3	1.95	0.6
KG1	3.4	0.6	PS4	1.7	0.5
KG2	1.34	0.55	PS5	1.7	0.5
KG3	1.64	0.5	PS6	1.85	0.55
KG4	0.79	0.5	PS7	1.7	0.5
KG5	2.28	0.65	PS8	1.9	0.5
LSG1	2.1	0.6	PS9	2.1	0.5
LTS2	3.5	0.65	PS10	1.95	0.5
LSS3	2.5	0.55	PS11	1.625	0.6
LSS4	2.3	0.5	PS12	1.8	0.5
LGF5	1.8	0.6	PS13	1.875	0.55
LRF6	1.9	0.65	PS14	2.1	0.6

4.2 Zn solubilization of bacterial strains (continued)

Strain no.	Clear zone diameter (cm)	Colony diameter (cm)	Strain no.	Clear zone diameter (cm)	Colony diameter (cm)
PS15	1.9	0.5	A2-9	2.05	0.6
PS16	1.9	0.5	A2-10	0.9	0.6
PS17	1.875	0.5	A3-1	1.8	0.65
PS18	2.05	0.6	S4-1	2.45	0.7
PS19	1.95	0.5	S5-1	1.9	0.85
PS20	2.15	0.6	S5-2	1.8	1.0
PS21	1.85	0.5	S5-3	1.9	0.8
PS22	2.05	0.6	S6-1	-	0.6
PS23	1.9	0.55	S6-3	-	0.65
PS24	2.25	0.6	S7-2	1.6	0.75
PS25	2.05	0.6	S7-4	2.3	0.7
PS26	2.05	0.6	S7-6	-	0.65
A1-1	2.2	0.85	Sl8-2	-	0.66
A1-2	1.9	0.8	Sl8-4	2.45	0.7
A2-2	1.1	6.5	Sl8-5	7.2	1.2
A2-3	1.2	0.8	Sx8-4	2.1	0.95
A2-4	3.0	0.65	Sx8-5	-	0.75
A2-5	1.0	2.25	Sx8-6	2.2	0.35
A2-6	2.7	0.7	Sx8-7	-	0.55
A2-8	1.3	0.95	Sx8-8	1.25	0.65

5) IAA production of bacterial strains

Strain no.	OD_{560}			Strain no.	OD_{560}		
	1	2	3		1	2	3
SK1	0.440	0.429	0.444	AM3	0.233	0.237	0.256
SK2	0.231	0.251	0.253	A1-2	0.199	0.199	0.194
PK1	0.353	0.331	0.344	S5-1	0.209	0.210	0.209
KG1	0.269	0.250	0.238	S5-2	0.221	0.194	0.192
KG2	0.263	0.243	0.272	S6-3	0.084	0.092	0.087
KG3	0.336	0.347	0.358	Sl8-5	0.233	0.225	0.227
KG4	0.478	0.445	0.471	Sx8-6	0.170	0.187	0.167
KG5	0.614	0.612	0.592	Sx8-8	0.407	0.407	0.406

6) Each growth parameters of 'Khao Dawk Mali 105' rice

Treatment	1	2	3	4	5	6	7	8	9	10	Average
Total length (cm)											
10µg/mL IAA	26.5	28.6	31.3	32.6	27.5	30.1	32.4	32.4	28.9	27.8	**29.81**
Control	15.2	16.5	12.6	17.3	14.6	17.5	14.3	15.1	17.4	14.6	**15.51**
SK2	19.5	18.4	17.5	20	21.3	19.8	22.2	20.6	18.9	20.4	**19.86**
PS25	18.4	19.7	18.7	16.3	19.8	16.2	17.9	19.1	17.3	18.8	**18.22**
LGS1	19.6	19.4	17.3	16.5	17.6	19.6	15.9	18.1	16.7	19.8	**18.05**
Root length (cm)											
10µg/mL IAA	6.1	6.2	6.4	5.8	5.9	6.3	6.1	6.2	6.3	6.3	**6.16**
Control	3.1	2.9	2.8	2.9	3.3	3.1	3.5	3.5	2.9	3.5	**3.14**
SK2	3.5	3.3	3.4	3.7	3.6	3.4	3.4	3.7	3.4	3.5	**3.49**
PS25	2.9	3.2	3.1	2.8	3.3	3.3	3.3	3.1	3.3	3.4	**3.17**
LGS1	3.3	3.1	2.8	3.2	2.9	3.3	3.2	3.1	3.2	2.8	**3.09**

6) Each growth parameters of 'Khao Dawk Mali 105' rice (continued)

Treatment	1	2	3	4	5	6	7	8	9	10	Average
Shoot length (cm)											
10µg/mL IAA	11.6	12.1	11.6	11.9	11.5	12.2	12.3	12.2	11.7	12.5	**11.96**
Control	7.7	7.1	6.7	6.9	7.3	7.5	7.2	7.1	7.5	7.5	**7.25**
SK2	11.2	10.8	10.5	11.2	10.7	11.1	10.9	11.6	10.9	11.5	**11.04**
PS25	9.6	10.4	10.2	9.8	11.1	10.1	9.1	10.4	10.2	10.5	**10.14**
LGS1	11.2	11.2	11.3	11.4	11.2	10.8	11.4	11.2	11.5	10.9	**11.21**
Number of lateral roots											
10µg/mL IAA	7	9	9	7	8	10	10	10	9	9	**9**
Control	8	7	7	8	8	6	9	7	6	6	**7**
SK2	9	8	7	10	8	9	10	7	7	8	**8**
PS25	10	8	9	8	7	9	9	8	9	10	**9**
LGS1	15	14	15	16	13	14	16	15	14	15	**15**
Shoot fresh weight (g)											
10µg/mL IAA	0.06	0.058	0.055	0.065	0.056	0.064	0.063	0.062	0.057	0.061	**0.0601**
Control	0.02	0.025	0.019	0.03	0.017	0.018	0.022	0.023	0.019	0.022	**0.0215**
SK2	0.04	0.05	0.036	0.041	0.047	0.044	0.039	0.048	0.044	0.041	**0.043**
PS25	0.03	0.031	0.029	0.025	0.033	0.028	0.035	0.033	0.028	0.031	**0.0303**
LGS1	0.04	0.041	0.044	0.039	0.038	0.043	0.041	0.039	0.037	0.04	**0.0402**
Root fresh weight (g)											
10µg/mL IAA	0.037	0.033	0.035	0.041	0.037	0.036	0.033	0.043	0.036	0.035	**0.037**
Control	0.01	0.015	0.009	0.007	0.012	0.011	0.009	0.015	0.011	0.012	**0.011**
SK2	0.031	0.034	0.029	0.033	0.031	0.038	0.024	0.032	0.032	0.03	**0.0314**
PS25	0.033	0.032	0.04	0.031	0.039	0.029	0.031	0.03	0.028	0.033	**0.0326**
LGS1	0.033	0.036	0.038	0.037	0.032	0.034	0.035	0.041	0.038	0.033	**0.0357**

6) Each growth parameters of 'Khao Dawk Mali 105' rice (continued)

Treatment	1	2	3	4	5	6	7	8	9	10	Average
Shoot dry weight (g)											
10µg/mL IAA	0.0061	0.0062	0.0056	0.0057	0.0054	0.0062	0.0062	0.0059	0.0056	0.0065	**0.00594**
Control	0.0020	0.0024	0.0024	0.002	0.0022	0.0021	0.0019	0.0018	0.0023	0.0022	**0.00213**
SK2	0.0037	0.0036	0.0038	0.0039	0.0036	0.0042	0.0041	0.0036	0.0038	0.0037	**0.0038**
PS25	0.0039	0.0042	0.0041	0.0037	0.0037	0.0036	0.0036	0.0038	0.0038	0.0037	**0.00381**
LGS1	0.0048	0.0052	0.0051	0.0047	0.0048	0.0048	0.0048	0.0054	0.0049	0.0053	**0.00498**
Root dry weight (g)											
10µg/mL IAA	0.0051	0.0049	0.0048	0.0052	0.0054	0.0048	0.0048	0.0054	0.0053	0.0055	**0.0051**
Control	0.0009	0.0012	0.0011	0.00098	0.0008	0.0009	0.0011	0.0007	0.0012	0.0007	**0.0010**
SK2	0.0026	0.0027	0.0026	0.0029	0.003	0.0029	0.0027	0.003	0.0026	0.0028	**0.00278**
PS25	0.0026	0.0024	0.0024	0.0026	0.0028	0.0029	0.0029	0.0026	0.0025	0.0025	**0.00262**
LGS1	0.004	0.0045	0.0044	0.0035	0.0037	0.0042	0.0044	0.0039	0.0041	0.0041	**0.00408**

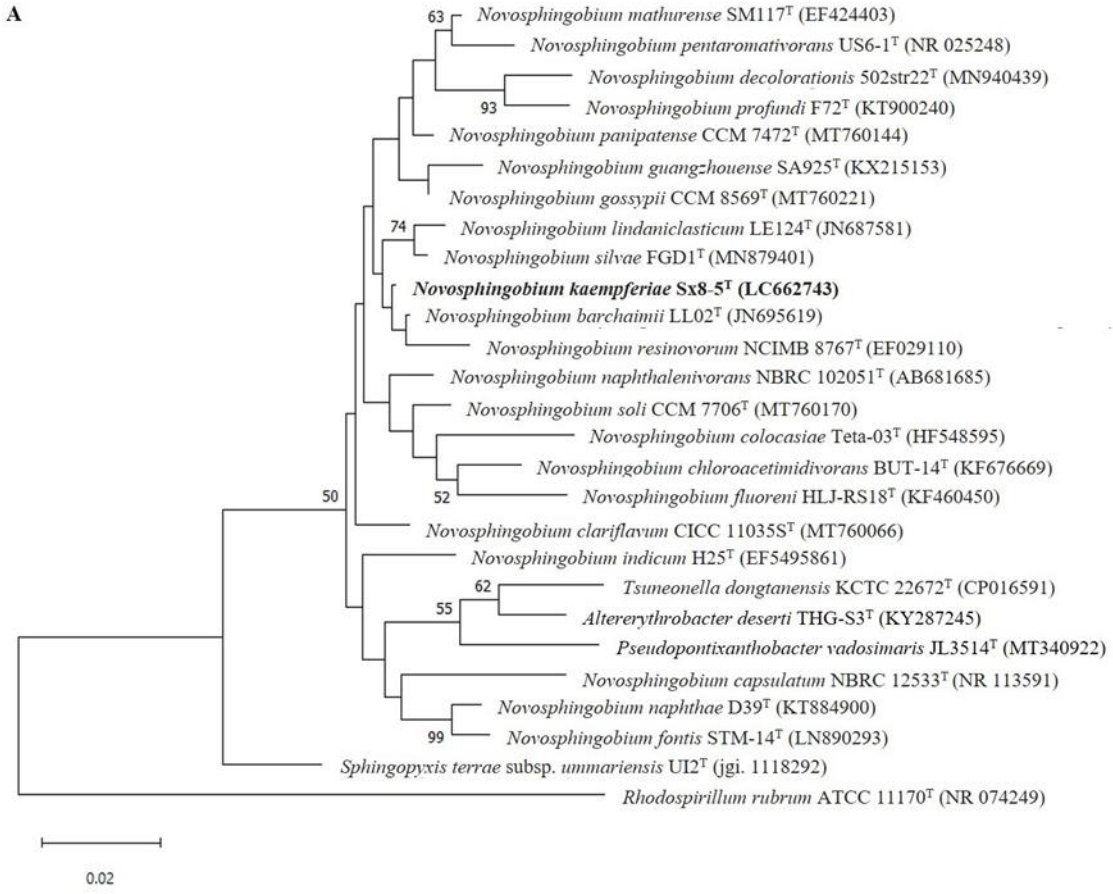
7) Each growth parameters of ‘‘RD6’ rice

Treatment	1	2	3	4	5	6	7	8	9	10	Average
Root length (cm)											
Sx8-8	5	5.8	6.2	3.8	4.6	5.1	4.7	6	3.9	3.2	**4.83**
S5-1	4.3	4.1	3	2.6	3.2	1.8	4.6	3.2	2.9	2.7	**3.24**
10 µg/mL IAA	10	10.5	12	10.5	8	10.2	9	8.5	8.2	9.5	**9.64**
30 µg/mL IAA	5.3	4.6	5.1	4.2	3.2	4	4.6	5.6	4.5	4.2	**4.53**
50 µg/mL IAA	4.2	4	3.8	5.1	3.5	3.4	3.6	3.6	4.2	3.9	**3.93**
Control	2.6	3	2.4	2.3	3.5	2.7	2.5	2.6	2.7	2.5	**2.68**
Shoot length (cm)											
Sx8-8	20	21	19	17	18	19	21.5	17	17	17.5	**18.7**
S5-1	13.6	13.9	13.8	13.7	13.7	13.75	13.9	13.9	13.6	13.7	**13.76**
10 µg/mL IAA	18	17.1	19	17.5	18.5	17.8	17.5	17.5	19	18	**17.99**
30 µg/mL IAA	17.6	17.5	19	17.4	18	18	17.8	17.5	18.3	17.8	**17.89**
50 µg/mL IAA	11	9	10	11.5	11	12.7	9.5	10	10.5	10	**10.52**
Control	9.2	9	9	9.5	9.8	10	9.6	9.8	9.6	9.2	**9.47**
Number of lateral roots											
Sx8-8	34	57	30	72	46	66	39	28	61	42	**47.5**
S5-1	35	36	37	34	36	36	34	36	35	39	**35.8**
10 µg/mL IAA	30	46	50	56	60	29	65	43	70	32	**48.1**
30 µg/mL IAA	50	52	44	36	55	62	56	48	32	70	**50.5**
50 µg/mL IAA	25	36	25	34	15	39	21	34	36	39	**30.4**
Control	18	17	17	25	19	26	29	15	11	18	**19.5**
Root fresh weight (g)											
Sx8-8	0.0342	0.0371	0.065	0.083	0.0231	0.0723	0.0341	0.0275	0.0576	0.057	**0.04909**
S5-1	0.076	0.045	0.063	0.043	0.0644	0.0863	0.078	0.0514	0.0443	0.0305	**0.05819**

7) Each growth parameters of 'RD6' rice (continued)

Treatment	1	2	3	4	5	6	7	8	9	10	Average
Root fresh weight (g)											
10 µg/mL IAA	0.071	0.0459	0.0565	0.0522	0.0489	0.065	0.0769	0.0786	0.0609	0.075	**0.06309**
30 µg/mL IAA	0.0326	0.0437	0.056	0.0614	0.0766	0.0658	0.065	0.0761	0.051	0.044	**0.05722**
50 µg/mL IAA	0.029	0.0554	0.0399	0.0607	0.0534	0.0767	0.0631	0.0309	0.043	0.032	**0.04841**
Control	0.0378	0.037	0.0343	0.0325	0.0346	0.0482	0.0423	0.033	0.036	0.047	**0.03827**
Shoot fresh weight (g)											
Sx8-8	0.0572	0.0412	0.082	0.0286	0.058	0.034	0.036	0.0812	0.056	0.0353	**0.05095**
S5-1	0.0478	0.0551	0.0705	0.056	0.07	0.0704	0.0433	0.045	0.0516	0.061	**0.05707**
10 µg/mL IAA	0.07	0.2379	0.1	0.05	0.078	0.12	0.096	0.25	0.13	0.2141	**0.1346**
30 µg/mL IAA	0.065	0.064	0.0672	0.056	0.0651	0.0714	0.0408	0.0422	0.055	0.051	**0.05777**
50 µg/mL IAA	0.0404	0.0664	0.0561	0.069	0.0389	0.0606	0.0486	0.0697	0.056	0.067	**0.05727**
Control	0.12	0.056	0.017	0.0593	0.12	0.0465	0.098	0.013	0.02	0.034	**0.05838**
Root dry weight (g)											
Sx8-8	0.0046	0.003	0.0029	0.0025	0.0028	0.003	0.0033	0.003	0.0035	0.0022	**0.00308**
S5-1	0.0038	0.0064	0.0045	0.0025	0.0039	0.0054	0.0045	0.0092	0.009	0.0076	**0.00568**
10 µg/mL IAA	0.0029	0.0017	0.0026	0.0032	0.0043	0.0032	0.0049	0.0041	0.0027	0.0034	**0.0033**
30 µg/mL IAA	0.0034	0.0036	0.0031	0.0038	0.0039	0.0038	0.0041	0.0048	0.0034	0.0042	**0.00381**
50 µg/mL IAA	0.0031	0.0041	0.0036	0.0044	0.0037	0.0042	0.0044	0.0043	0.0032	0.0041	**0.00391**
Control	0.0026	0.0033	0.0032	0.0024	0.0037	0.0033	0.0034	0.0027	0.0031	0.0029	**0.00306**
Shoot dry weight (g)											
Sx8-8	0.0068	0.006	0.0043	0.0041	0.0042	0.0045	0.0054	0.0044	0.0034	0.0046	**0.00477**
S5-1	0.0097	0.0059	0.0055	0.0054	0.0069	0.0072	0.007	0.0066	0.0088	0.0079	**0.00709**
10 µg/mL IAA	0.0065	0.0057	0.0053	0.0047	0.0074	0.009	0.0086	0.0056	0.0031	0.0067	**0.00626**
30 µg/mL IAA	0.0053	0.0077	0.0064	0.0062	0.0071	0.0096	0.0067	0.009	0.0067	0.0051	**0.00698**
50 µg/mL IAA	0.0068	0.0046	0.0076	0.0092	0.0086	0.0063	0.0062	0.008	0.0049	0.0053	**0.00675**
Control	0.0066	0.0056	0.0085	0.006	0.0077	0.0069	0.0076	0.0049	0.0054	0.0064	**0.00656**

8) Phylogenetic tree reconstructed by the maximum-likelihood (ML) (A), and maximum-parsimony (MP) (B) using 16S rRNA gene sequences of strain Sx8-5^{T} and related type strains.



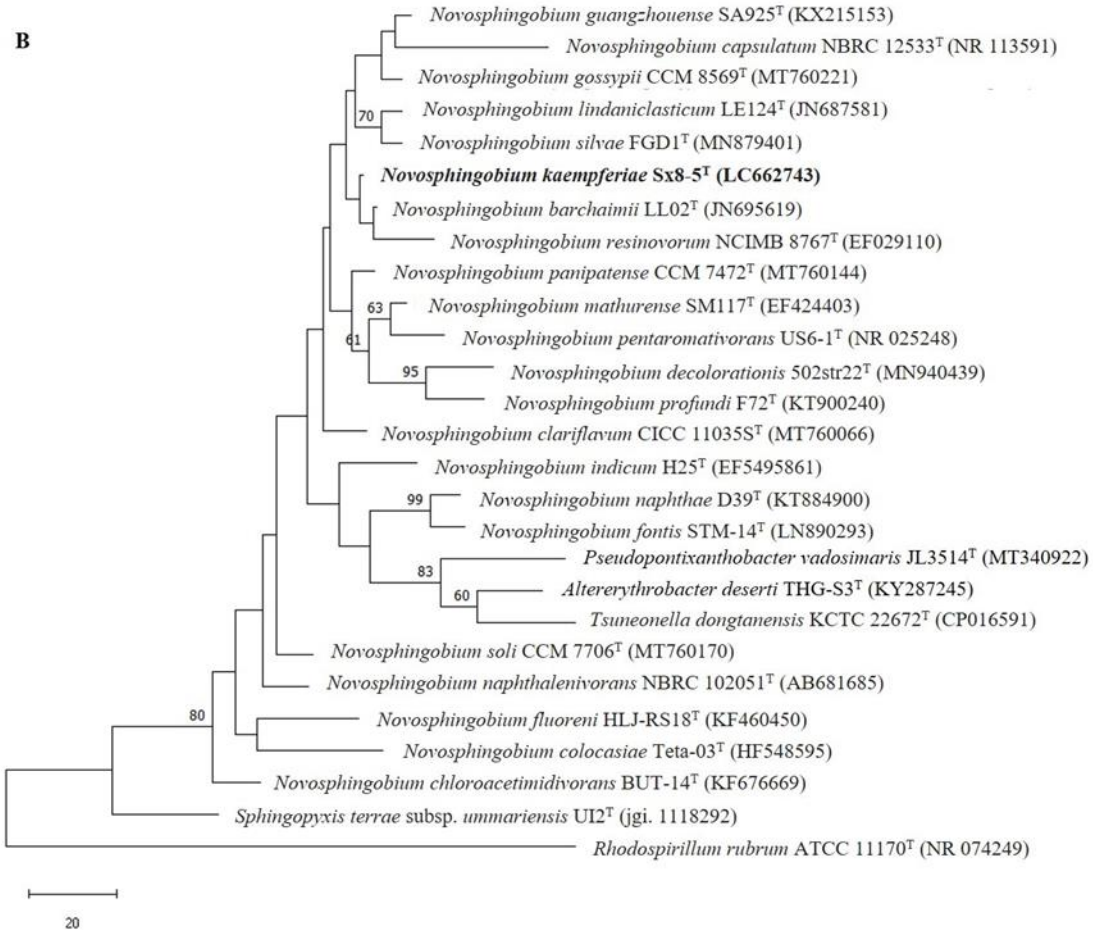
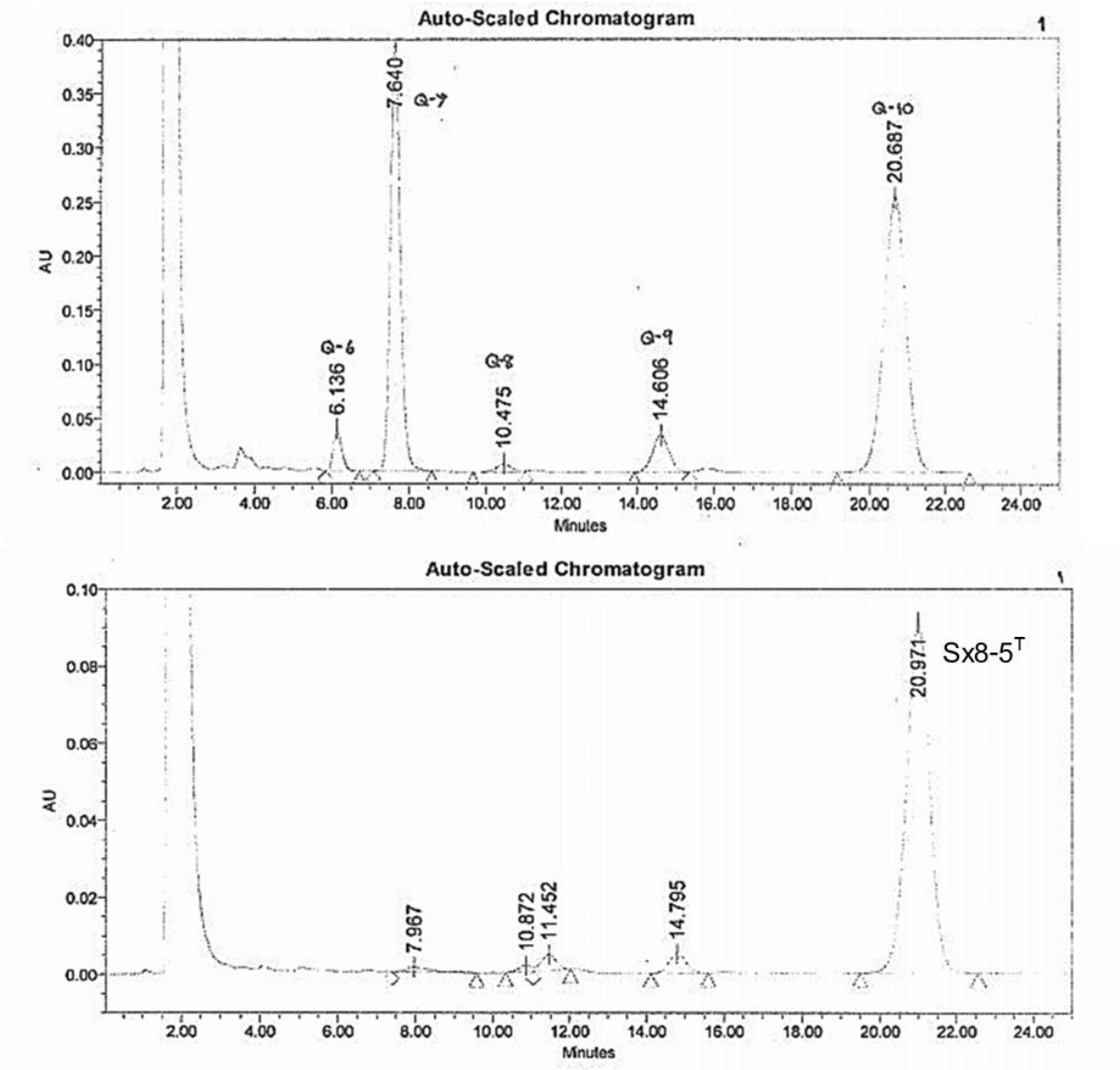


Figure C.1 Phylogenetic tree reconstructed by the maximum-likelihood (ML) (A), and maximum-parsimony (MP) (B) using 16S rRNA gene sequences of strain Sx8-5^{T} and related type strains. *Rhodospirillum rubrum* ATCC 11170^{T} (NR 074249) was used as an outgroup. Bootstrap values (≥50%) based on 1,000 replicates are shown at the branch nodes. Bar 0.02 and 20 substitutions per nucleotide position for ML and MP, respectively.

9) Chromatogram of ubiquinone Q10



VITA

NAME	Kanchana Sitlaothaworn
DATE OF BIRTH	29 October 1982
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	Faculty of Science, Chulalongkorn University, Thailand, 10330
HOME ADDRESS	112/287 Soi Ekachai 18, Ekachai Road, Khet Chom Thong, District Bangkok
PUBLICATION	- Sitlaothaworn, K., Budsabun, T., Dechkla, M., Yukphan, P., Tanasupawat, S. and Savarajara, A. (2022) Identification and plant growth-promoting activity of endophytic bacteria from sugarcane roots and genome analysis of Gluconacetobacter strain PS25. ScienceAsia 48, 1-8. - Sitlaothaworn, K., Budsabun, T., Booncharoen, A., Panphut, W., Savarajara, A. and Tanasupawat, S. (2022) Diversity of plant growth-promoting endophytic bacteria, genome analysis of strain Sx8-8 and Its rice germination promoting activity. Microbiology (Accepted). Sitlaothaworn, K., Yukphan, P., Budsabun, T., Charoenyingcharoen, P., Phongsopitanun, W., Savarajara, A. and Tanasupawat, S. (2022) Novosphingobium kaempferiae sp. nov., a phosphate-solubilizing bacterium isolated from stem of Kaempferia marginata Carey. International Journal of Systematic and Evolutionary Microbiology (Accepted).
PRESENTATION	Sitlaothaworn, K., Budsabun, T., Tanasupawat, S. and Savarajara, A. Characterization and application of indole-3-acetic acid-producing endophytic bacteria for in vitro promotion of seed germination. Thai Society for Biotechnology International Conference Online; April 29, 2022, Bangkok, Thailand (Poster AB-P-05).