ISOLATION OF PLANT-GROWTH-PROMOTING EPIPHYTIC BACTERIA FROM ORGANIC RICE PLANT

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หัวข้อวิทยานิพนธ์	การคัดแยกเชื้อแบคทีเรียอิพิไฟต์ที่ส่งเสริมการเจริญของต้นพืชจากต้นข้าวที่ปลูกด้วยวิธีอินทรีย์
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บทคัดย่อ

Plant-growth-promoting bacteria (PGPB) เป็นเชื้อแบคทีเรียที่มีความสามารถในการส่งเสริมการเจริญเติบโตของต้นพืช ในการศึกษาครั้งนี้สามารถแยกเชื้ออิพิไฟต์จากข้าวที่ปลูกด้วยวิธีอินทรีย์ จากจังหวัด กรุงเทพฯ ชลบุรี สระบุรี และสุพรรณบุรี ทั้งหมด 113 ไอโซเลต ลักษณะทางสัณฐานวิทยาเบื้องต้นเป็นแบคทีเรียแกรมบวก 68 ไอโซเลต และแบคทีเรียแกรมลบ 45 ไอโซเลต พบเซลล์ที่เป็นรูปร่างท่อนมากที่สุดคือ 106 ไอโซเลต และ 7 ไอโซเลต เป็นเชื้อรูปร่างกลม ในการศึกษาด้านความหลากหลายของเชื้อแบคทีเรียโดยเทียบชิ้นส่วนยีน 16S rRNA ที่สกัดได้จากเชื้อกับฐานข้อมูลพบว่าสามารถจัดจำแนกเชื้อออกเป็น 3 ไฟลัม ได้แก่ ไฟลัม*Firmicutes* (55%) ไฟลัม *Proteobacteria* (38%) และ ไฟลัม *Bacteroidetes* (7%) และทำการศึกษากิจกรรมการส่งเสริมการเจริญเติบโตของต้นพืช ได้แก่ การตรึงไนโตรเจน การละลายฟอสเฟส การสร้างสารซิเดอร์โรฟอร์ การสร้างเอนไซม์ ACC deaminase และการสร้างฮอร์โมน IAA จากเชื้ออิพิไฟต์ทุกตัว จากการศึกษาพบว่ามีเชื้อ 7 ไอโซเลตที่แสดงกิจกรรมมากกว่า 3 กิจกรรม จึงได้ทำการคัดเลือกมาเพื่อศึกษาต่อในขั้นตอนของ Seedling assay พบว่าไม่มีเชื้อไอโซเลตใดที่สามารถส่งเสริมการเจิญเติบโตของต้นพืชได้เลย นอกจากนั้นยังได้ทำการศึกษาความสามารถในการยับยั้งเชื้อก่อโรคในข้าว *X. oryzae* และ *P. grisea* พบว่ามี 24 ไอโซเลตในจีนัส *Bacillus Burkholderia Chitinophaga* และ *Pseudomonas* ที่มีความสามารถในการยับยั้งเชื้อก่อโรคพืชได้ โดยพบเปอร์เซ็นต์การยั้บยั้งการเจริญเติบโตของเชื้อก่อโรคตั้งแต่ 10 ถึง 100% จากนั้นได้ทำการศึกษาความสามารถในการผลิตเอนไซม์ช่วยที่ช่วยย่อยผนังเซลล์ พบว่ามีเชื้อ 2 ไอโซเลต ที่สามารถสร้างเอนไซม์ไคติเนส (Chitinase) ในขณะที่มีเชื้อ 19 ไอโซเลตที่สามารถสร้าง เอนไซม์โปรติเอส (Protease) และ 5 ไอโซเลต ที่ไม่พบกิจกรรมการสร้างเอนไซม์ใดเลย และได้คัดเลือกเชื้ออิพิไฟต์ที่มีความสามารถในการยับยั้งการเจริญของ

เชื้อก่อโรคไปศึกษาต่อในขั้นตอนของ Well Diffusion และ Disc Diffusion พบว่าไม่มีเชื้อไอโซเลตใดแสดงการยับยั้งเชื้อก่อโรคข้าวในการทดลองขั้นนี้ ทั้งนี้ผลการทดลองที่ได้ยังแสดงให้เห็นว่าเชื้ออิพิไฟต์มีความสามารถในการยับยั้งการเจริญของเชื้อก่อโรคได้ และอาจจะสามารถนำไปใช้เป็นสารยั้บยั้งทางชีวภาพเพื่อควบคุมโรคพืชต่อไปได้

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Abstract

Plant-growth-promoting bacteria (PGPB) are known to enhance plant growth by various direct and indirect mechanisms. One-hundred-and thirteen epiphytic bacteria were isolated from rice plants collected from organic rice farms in Bangkok, Chonburi, Saraburi and Suphanburi, Thailand. Sixty-eight and forty-five isolates were Gram-stained-positive and Gram-stained-negative, respectively. 106 and 7 isolates were observed as rods and cocci, respectively. According to their 16S rRNA gene sequences, bacteria were classified into *Firmicutes* (55%), *Proteobacteria* (38%) and *Bacteroidetes* (7%). Plant-growth-promoting activities including nitrogen fixation, ACC-deaminase production, siderophore production, phosphate solubilization and IAA production were tested in all bacteria. Seven isolates containing the highest number of activities were selected for seedling assay. However, positive effects by these bacteria were not observed under the tested conditions. Epiphytic bacteria were also tested for their antagonistic activities against *X. oryzae* and *P. grisea*, the causative agents of leaf blight and leaf blast in rice, respectively. Based on the dual-culture method, twenty-four isolates that belonged to genera *Bacillus*, *Burkholderia*, *Chitinophaga* and *Pseudomonas* were able to inhibit the pathogens. The percentages of pathogen growth inhibition ranged from 10 to 100%. Chitinase and protease activities were detected in two and 19 isolates, respectively. Five

isolates were tested negative for both enzymes. Antagonistic bacteria were further tested with well and disc diffusion methods. However, the results obtained from this experiment were all negative. The results obtained from this study indicated that some isolates may be utilized as biocontrol agents against the leaf blast and leaf blight diseases in rice.

Keywords : Antagonistic activities, Biocontrol agent, Epiphytic bacteria, Plant-growth-promoting activities, Rice

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Suchitra Aphimeteetamrong

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Chapter 1
Introduction

1.1 Research Motivation

Bacteria have been found associated with other living organisms including plants, animals and humans. Plant surfaces are generally colonized by diverse groups of bacteria that are recognized as epiphytic bacteria. Because of their close-relationship with the plant host, several strains of epiphytic bacteria were demonstrated for their direct and indirect plant-growth-promoting activities and were described as plant-growth-promoting bacteria (PGPB). Directly, they increased the bioavailability of nitrogen, phosphorus and iron through nitrogen fixation, phosphate solubilization and siderophore production. Additionally, they modulated the levels of phytohormones including auxin and ethylene through the production of indole-3-acetic acid and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. Indirectly, they inhibited the growth of phytopathogenic bacteria and fungi through the production of antibiotics or lytic enzymes. In agriculture, crop production heavily relied on chemical fertilizers and pesticides. However, because chemical substances imposed their toxic effects on humans and environments, the use of PGPB was considered an alternative practice for yield improvement and crop protection. Rice (*Oryza sativa* L.) was one of the staple crops for the world's population especially in Asian countries. In Thailand, more than 50% of the total agricultural area is dedicated for rice production. Rice was used for domestic consumption as well as international export. Similar to other crops, rice production depended on the use of various chemical fertilizers and pesticides. In order to provide an alternative way for improving rice production, this study aimed to isolate and study the diversity of epiphytic bacteria from roots, stems and leaves of rice plants. Their roles as PGPB were determined based upon the presence of both direct and indirect plant-growth-promoting activities. Potential isolates obtained from this study would be further studied and applied in agriculture as biofertilizers and biocontrol agents.

1.2 Objectives of the study

2.1 To study the diversity of epiphytic bacteria that were isolated from rice plants.

2.2 To examine plant-growth-promoting activities of epiphytic bacteria.

2.3 To investigate plant-growth-promoting effects on rice seedlings *in vitro*.

1.3 Scopes of the study

Epiphytic bacteria were isolated from leaves, stems and roots of rice plants that were collected from organic farms in Bangkok, Chonburi, Saraburi and Suphanburi provinces. Characterization of all bacteria was based on morphological characteristics and their partial 16S rRNA gene sequences. All isolates were tested for plant-growth-promoting activities including nitrogen fixation, phosphate solubilization, siderophore production, ACC-deaminase, biosynthesis of IAA. Isolates that displayed the highest number of activities were further studied on rice seedlings under various conditions. Antagonism against *Xanthomonas oryzae* and *Pyricularia grisea* were also studied.

1.4 Benefits of the study

From this study, the understanding of the diversity and plant-growth-promoting benefits of epiphytic bacteria on rice plants were acquired. The results obtained in this study also formed the basis for further application of epiphytic bacteria as biofertilizers and biological control agents that would lead to the reduction of chemical utilization in agriculture.

Chapter 2
Theory and Literature Reviews

2.1 Rice

Rice (*Oryza* spp.) is a member in the family Poaceae. Out of 22 wild species, *Oryza glaberrima* and *Oryza sativa* are the only two cultivated species (Trinkley and Fick, 2003). The former is grown in West Africa while the latter is common in the rest of the world (Jones *et al.*, 2001). Over half of the world's population, mostly in Asia, consumes rice. It is one of the leading food crops in the world. Rice grains contain a number of nutrients, such as, carbohydrates, proteins, vitamins and minerals (USDA Nutrient Database, 2016). More than 80% of rice production are served directly for human consumption (International Rice Research Institute, 2016). Some of them are also processed into other products, such as, cereals, snacks, brewed beverages, flour, etc. There are many different conditions and systems for rice cultivation. Generally, rice-growing environments are irrigated, rain-fed shallow, upland and flood-prone (Beighley, 2010). Over 70% of rice are grown on irrigated land and rain-fed lowland (International Rice Research Institute, 2016). It grows best in temperate and tropical climates. Ninety percent of the world's production of rice are from Asia because of the favorably warm and humid climates (Tran, 1997). Rice is one of the most important crops in Thailand (Leturque and Wiggins, 2011) which takes over 50% of agricultural land (Office of Agricultural Economics, 2015). Several categories of rice are exported. These include jasmine rice (Thai Hom-Mali rice), white rice, glutinous rice and parboiled rice (Co and Boosarawongse, 2007).

2.2 Epiphytic bacteria and 16S rRNA gene characterization

A large number of microorganisms are recognized as epiphytic bacteria because of their ability to colonize on surfaces of various plants organs during their life cycle. Phyllobacteria and rhizobacteria are subgroups of epiphytic bacteria that are associated with leaves and roots, respectively (Gnanamanickam and Immanuel, 2007). Several factors that influence bacterial population sizes as well as diversity were described.

(Lindow and Brandl, 2003). One of them was the plant host species. For example, the number of epiphytic bacteria on cucumbers and common beans were found higher than that on maize, oats, orchardgrasses and peas (O'Brien and Lindow, 1988 ; Kinkel *et al.*, 2000). Physicochemical environments of different plant surfaces also influenced bacterial colonization. Floral-colonizing bacteria were found different from those found in roots. Additionally, bacteria that produced pigments were more commonly found on leaves than roots (Lindow and Brandl, 2003). To study the diversity of epiphytic bacteria, bacterial characterization is performed based on the sequences of the 16S rRNA gene. In bacteria, 30S rRNA is transcribed from rRNA genes that are arranged as an operon. Subsequently, it is cleaved into 16S, 23S and 5S rRNA molecules by RNaseIII (Maidak, 1997). Among these three types of rRNAs, the 16S rRNA gene sequence was found the most specific to bacterial species (Woese, 1987). Its function has not changed over time. The approximately 1,500 bp length of the 16S rRNA gene facilitated fast sequencing and is large enough for taxonomic purposes (Janda and Abbott, 2007). As a result, the 16S rRNA gene becomes the most common gene for the study of bacterial phylogeny and taxonomy (Chakravorty *et al.*, 2007). The gene contained conserved and variable regions. Conserved regions were not species-specific and display high similarities between bacteria. In contrast, variable regions or hypervariable regions only display a high level of similarity at the species level (Van de Peer *et al.*, 1996). There are nine variable regions (V1-V9) scattering in the 16S rRNA gene. Among them, variable regions V2 (nucleotides 137–242), V3 (nucleotides 433-497) and V6 (nucleotides 986–1043) were found with high heterogeneity which make them suitable for the analysis of bacterial taxonomy (Chakravorty *et al.*, 2007).On the basis of the 16S rRNA gene sequences, epiphytic bacteria were characterized as members of various bacterial genera, such as, *Bacillus*, *Enterobacter*, Klebsiella, *Pseudomonas*, *Penibacillus*, *Microbacterium*, etc. Among these bacteria, some of them were reported for their direct plant-growth-promoting activities, and many of them were involved in plant pathogens suppression (Mercier and Lindow, 2000 ; Akhgar *et al.*, 2014 ; Majeed *et al.*, 2015).

2.3 Plant-growth-promoting bacteria

Plant-growth-promoting bacteria (PGPB) are beneficial bacteria (Esitken *et al.*, 2010). They can colonize various parts of plants, and some of them were shown to exhibit a symbiotic relationship with the host plants (Glick, 2012). These bacteria are able to survive, multiply and compete well with other microbes on the plants (Kloepper and Schroth, 1978). PGPB have abilities to increase plant growth and yields (Kumar *et al.*, 2012). Strains in genera *Acinetobacter*, *Agrobacterium*, *Azospirillium*, *Azotobacter*, *Bacillus*, *Burkholdria*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, *Serrotia* and *Streptomyces* are commercially available as biofertilizers (Glick, 2012). They directly promote plant growth by increasing the availability of nutrients, such as, nitrogen, phosphorus and iron. Many of them produce or modulate the level of phytohormones including indole-3- acetic acid (IAA), cytokinins, gibberellins and ethylene. PGPB also indirectly promote plant growth by synthesizing antibiotics, lytic enzymes, and fungicidal compounds to inhibit phytopathogenic microorganisms (Ahmad *et al.*, 2008; Glick, 2012 ; Majeed *et al.*, 2015).

2.4 Nitrogen fixation

Nitrogen is required for plant growth .(California Fertilizer Foundation, 2016). It is one of the fundamental components found in various biomolecules (Huber and Thompson, 2007 ; McCauley *et al.*, 2003 ; Walworth, 2013). Nitrogen is abundantly available in the form of nitrogen gas (N_2) that constitutes to approximately 78% of the earth's atmosphere. However, the gaseous state of nitrogen is not biologically available for plant growth (Glick, 2012). Several bacteria were reported for their nitrogen-fixing activities which transformed nitrogen gas to ammonium (NH_4^+) and nitrate (NO_3^-) forms that can be absorbed by plant roots (Daroub and Snyder, 2007). Nitrogen-fixing bacteria can be divided into two groups. Bacteria in the family *Rhizobiaceae* form symbiotic relationships with leguminous plants and non-leguminous trees (Ahemad and Khan, 2012 ; Zahran, 2001). The other group comprises of free-living bacteria in various genera including *Anabaena*, *Nostoc*, *Azospirillum*, *Azotobacter*, *Gluconoacetobacter* and *Azocarus* (Bhattacharyya and Jha, 2012). Nitrogenase is the key enzyme that is involved

in nitrogen fixation (Kim and Rees, 1994). It consists of two components: the tetrameric MoFe protein that comprises of two α and β subunits, the dimeric Fe protein that contains two identical subunits. These two proteins bind with the iron-molybdenum cofactor (FeMoco). The nitrogenase complex is encoded by *nif* genes which are generally arranged in a cluster of approximately 20 -24 kb with seven operons (Glick, 2012). *nifD* and *nifK* genes code for the Mo protein subunits and *nifH* codes for the Fe protein. Other *nif* genes are required for the assembly of nitrogenase. *nifB*, *nifQ*, *nifE*, *nifN*, *nifX*, *nifU*, *nifS*, *nifS*, *nifV*, *nifY* and *nifH* are involved in the synthesis of FeMoCo. Additionally, *nifS* and *nifU* and *nifW* and *nifZ* are involved in the assembly of iron-sulfur clusters and maturation of the nitrogenase components (Franche *et al.*, 2010).

2.5 Phosphate solubilization

Phosphorus (P) is essential for plant growth as one of the macroelements (Armstrong, 1999). It is present in both organic and inorganic forms in soil (Daroub and Snyder, 2007). Phosphorus plays several important roles in plants including the energy transformation and photosynthesis (Armstrong, 1999). The soluble forms of phosphorus that plants can absorb are the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) forms (Glass, 1989). However, phosphorus utilization by plants is generally limited because phosphorus is precipitated as metal-cation complexes and fixed in the soil (Gamalero and Glick, 2011). A number of bacteria were reported for their phosphate-solubilizing activities. They produced organic acid including gluconic and carboxylic acids that solubilized phosphate compounds. Additionally, acid phosphatase and phytase enzymes were found involved in the process (Souza *et al.*, 2015). Members in genera *Pseudomonas*, *Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium* and *Erwinia* showed the ability to solubilize insoluble inorganic-phosphate compounds, such as, dicalcium phosphate, tricalcium phosphate, hydroxyapatite and rock phosphate (Rodríguez and Fraga, 1999).

2.6 Indole-3-acetic acid production

Phytohormones are organic compounds which are produced in higher plants and involved in plant growth and development. They are also required for plant responses to environment conditions (Baca and Elmerich, 2003 ; Glick, 2012). Indole-3-acetic acid (IAA) is the major auxin in plants (Leveau and Lindow, 2005). IAA controls many important plant physiological processes including cell division, cell growth tissue differentiation and responses to light and gravity. It stimulates root elongation and increases the number of root hairs and lateral roots that is related to the nutrient uptake (Teale *et al.*, 2006). IAA was found produced and released by a number of microorganisms that were isolated from various plant species (Patten and Glick, 1996). Several IAA-producing bacteria were shown to significantly promote plant growth (Ambawade and Pathade, 2013 ; Mohite, 2013 ; Reetha *et al.*, 2014 ; Sharma and Rai, 2015).

There are several pathways for IAA synthesis. The pathway can be divided into two groups which are tryptophan-dependent and tryptophan-independent pathways (Baca and Elmerich, 2003). Tryptophan is the primary precursor for the biosynthesis of IAA in plants and microorganisms including plant-growth-promoting bacteria (Monteiro *et al.*, 1988 ; Ambawade and Pathade, 2013). Members in genera *Agrobacterium*, *Azospirillum*, *Bradyrhizobium*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Pseudomonas*, *Rhizobium* and *Synechocystis* were shown to employ the tryptophan-dependent pathway. The tryptophan-independent pathway were found more common in *Azospirilla*, cyanobacteria and plants (Baca and Elmerich, 2003 ; Ahemad and Kibret, 2014).

2.7 ACC deaminase production

Ethylene is a gaseous phytohormone. Ethylene is important in growth of various plant organs, such as, roots, stems, leaves, flowers and fruits. Additionally, it is involved in all stages of plant development (Goodlass and Smith, 1979). Several stress conditions may cause an overproduction of ethylene. These stresses include diseases, flood, drought, salinity and organic and inorganic toxic compounds. A high level of ethylene

inhibits root and shoot elongation and promotes leaf epinasty (Huang *et al.*, 2013). The precursor for the biosynthesis of ethylene is 1-aminocyclopropane-1- carboxylate (ACC) (Glick, 2012). Some PGPB are capable of lowering the level of ethylene in plants by producing ACC deaminase which cleaves ACC into ammonia and α-ketobutyrate (Ahemad and Kibret, 2014 ; Souza *et al.*, 2015). Previous reports showed that ACC-deaminase was found in bacteria that belonged to various genera, such as, *Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* (Ahemad and Kibret, 2014).

2.8 Siderophore production

Iron (Fe) is an essential micronutrient required by plants (Ahmed and Holmström, 2014). It is involved in many important biological processes, such as, photosynthesis and cellular respiration. Iron is also involved in chlorophyll production and used as the cofactor of various enzymes (Litwin and Calderwood, 1993 ; Kobayashi and Nishizawa, 2012). The lack of iron causes leaves to turn yellow-white and interveinal chlorosis (Uchida, 2000). Generally, iron is found in the form of ferric ($Fe3^{+}$) ion that is likely to form insoluble hydroxides and oxyhydroxides (Rajkumar *et al.*, 2010). The insolubility of Fe^{3+} causes the low biological availability of iron for assimilation by plants (Ma, 2005).

Siderophores are water-soluble molecules with low molecular mass (200-2,000 Da). They have a specificity and affinity for chelating Fe^{3+}. Siderophores can be produced by plants and microorganisms (Schwyn and Neilands, 1987 ; Ahemad and Kibret, 2014). Various studies reported that bacterial strains associated with plants produced siderophores (Souza *et al.*, 2015). Siderophores are released from bacterial cells, bind with Fe^{3+} in the environment and form Fe^{3+}-siderophore complexes. It is specifically transported through the outer membrane receptors into cells. Subsequently, Fe^{3+} in Fe^{3+}-siderophore complex is reduced to Fe^{2+} (Ali and Vidhale, 2013 ; Ahemad and Kibret, 2014). Siderophore receptors are divided into homologous and heterologous groups. Homologous receptors allow only siderophores that are produced by the same organism. For example, in *Escherichia coli*, the FepA receptor is used for transferring

ferric-enterobactin that is synthesized by the bacterium. Heterologous receptors recognize siderophores from different organisms. An example is the FhuA receptor in *E. coli* that is able to recognize siderophores produced by fungi (Helm and Chakraborty, 2003 ; Khan *et al*., 2009).

2.9 Antagonistic activities against plant pathogens

A number of bacteria were reported for their antagonistic effect against plant pathogens in several previous reports. For example, members in genera *Bacillus*, *Klebsiella*, *Microbacterium* and *Paenibacillus* were isolated from Korean rice cultivars and displayed antagonistic activities against two soil pathogenic fungi including *Fusarium oxysporum* and *Rhizoctonia solani in vitro*. Another study showed that inoculation of rice seeds with *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus subtilis* and *Brevibacillus brevis* decreased the incidence of the bacterial blight disease that was caused by *X. oryzae* pv. *oryzae*. (Chithrashree *et al*., 2011 ; Ji *et al*., 2014). Another example showed that a strain of *Pseudomonas fluorescens* that was isolated from rice rhizospheres exhibited a strong antibacterial activity against *X. oryzae* pv. *oryzae*. Additionally, the strain showed approximately 60% of the disease suppression under glass-house conditions. The biocontrol agent 2, 4- diacetylphloroglucinol (DAPG) produced by this bacterium was responsible for the biological suppression of the pathogen (Velusamy *et al*., 2013). Ohike *et al*. (2013) reported the presence of a strong antifungal activity by *Bacillus* sp. associated with chestnut tree leaves. The bacterium efficiently inhibited the growth of *F. oxysporum* and *R. solani*. The characterization of the antibiotic agent produced by this bacterium was shown to be iturin A.

Growth suppression of plant pathogens by antagonistic bacteria may be achieved through various mechanisms. Pathogens maybe directly inhibited by substances which are produced by bacteria, such as, diffusible antibiotics, volatile organic compounds (VOC), toxins and biosurfactants (Berg, 2009 ; Prashar *et al*., 2013). Antibiotics are extracellular secondary metabolites (Prashar *et al*., 2013). They are effective at low concentrations and highly specific to the target. Several bacteria including members in genera *Agrobacterium*, *Bacillus, Serratia* and *Streptomyces* were reported as antibiotic

producers (Stein, 2005). Another group is fluorescent *Pseudomonas* species. Phenazines, DAPG, pyoluteorin, pyrrolnitrin, lipopeptides and hydrogen cyanide were examples of antibiotic agents synthesized by this group of bacteria (Nowak-Thompson *et al.*, 1994 ; Haas and Keel, 2003). Alternatively, inhibition of pathogens may occur through the production of hydrolytic enzymes that lyse the pathogen cell walls. These include chitinases, cellulases, β-1, 3 glucanases, proteases, and lipases (Glick, 2012). Bacteria containing antimicrobial peptides (AMPs) also showed the antagonistic effect. These peptide molecules destroy the fungal cell wall or form holes in cellular membranes (Ren *et al.*, 2013 ; Zhao *et al.*, 2013). The competition for nutrients and niches is another mechanism for controlling pathogens. Pathogenic and non-pathogenic microorganisms that colonize on plants compete for limited nutrients. Rapid colonization of non-pathogenic bacteria potentially slow or stop the growth of pathogens on plants (Jones *et al.*, 2001).

Recently, several studies reported that there were a number of bacteria that contained plant-growth-promoting activities. Strains of *P. fluorescens* isolated from canola grown under salt-stressed conditions were able to produce ACC deaminase and utilize ACC as the sole nitrogen source. Additionally, the strains were tested positive for IAA production (Akhgar *et al.*, 2014). Ambawade and Pathade (2013) reported that *Stenotrophomonas maltophilia* isolated from banana roots exhibited a high level of IAA production. Additionally, *B. subtilis* and *P. fluorescens* isolated from plant rhizosphere were shown to produce IAA. The bacteria were inoculated on onion seeds to study their plant growth promotion in pot culture. Higher shoot and root length and shoot and root biomass were observed on inoculated plants compared to the non-inoculated control group (Reetha *et al.*, 2014). *Burkholderia* and *Enterobacter* isolated from rice roots were shown to display high levels of siderophore production (Souza *et al.*, 2013, Souza *et al.*, 2014). Moreover, bacteria isolated from rice plants were reported for their potential in nitrogen-fixing activity as shown by the presence of *nifH* genes. They were characterized as members in genera *Bacillus*, *Klebsiella*, *Paenibacillus* and *Microbacterium*. Several of them were reported for their plant-growth-promoting activities including auxin and siderophore production and phosphate solubilization. The strains improved plant growth

as determined by the increase in height and dry weight of inoculated plants. They also exhibited antagonistic effects against fungal pathogens including *F. oxysporum* and *R. solani* (Majeed *et al.*, 2015). Ji *et al.* (2014) isolated bacteria from wheat plants. They were tested for their plant growth promotion *in vitro* and were identified as *Bacillus* sp. and *Stenotrophomonas* sp. Phosphate solubilization was observed in *Bacillus* sp. while IAA synthesis and nitrogen fixation were found in *Stenotrophomonas* sp. Additionally, all strains were able to significantly increase shoot and root length, and shoot and root biomass compared to the control group. A recent study reported that *Enterobacter cloaceae* AJS-15 isolated from rhizospheric soil of Kapok bush (*Aerva javanica*) displayed the ACC-deaminase activity. The strain was able to grow under stress conditions. It produced IAA and solubilized the inorganic phosphate compound. Wheat growth was increased upon the inoculation with this bacterium. Antagonistic effects against bacterial and fungal pathogens including *E. coli*, *Erwinia Carotovora*, *Fusarium graminearum* and *F. oxysporum* were also observed (Singh and Jha, 2015).

2.10 Rice diseases

One of the major concerns in rice production is the crop loss caused by plant diseases. Leaf blight and leaf blast are two of the most destructive diseases in rice production. They are caused by *X. oryzae* pv. *oryzae* and *P. grisea*, respectively. The left blight disease was first recognized in rice plants that were grown in Japan. Since then, it has been reported in many countries around the world (European and Mediterranean Plant Protection Organization, 2007 ; Gnanamanickan, 2009). The severity of the disease depends on the crop's stage at the infection time. Leaf blight is a vascular disease which causes the systemic infection of rice (Mew, 1987). Tillering is the most susceptible stage for *X. oryzae* infection which leads to the yield loss up to 20-40%. The pathogen can spread by wind, rain, irrigation and from plant to plant. It enters the host through wounds or hydathodes and systemically spreads inside the plant through xylem vessels (Ou, 1985 ; Noda and Kaku, 1999 ; Jones *et al.*, 2001). The disease symptoms occur as small, yellow, water-soaked spots at the tip and the margin of the leaf (Mew, 1987 ; Mew *et al.*, 1993). These spots later spread along the veins. Tissues become chlorotic

and subsequently necrotic, tannish-grey to white lesions which may grow on both or just one side of the leaf (Mew, 1991 ; Niño-Liu *et al*., 2006). Colonies of *X. oryzae* are round and convex with a smooth surface and the yellow color of xanthomonadin, the characteristic pigment of the genus (Bradbury, 1984). Cells are able to produce capsular extracellular polysaccharide (EPS). *X. oryzae* is obligately aerobic. The optimal temperature for growth is between 25 °C to 30 °C.

The blast disease was first found in Asia and is now observed in over 85 countries. The disease caused significant yield losses in rice production in almost every rice-growing country. The disease is caused by *P. grisea*. The yield loss by the disease can be as high as 70-80% of the total production (Ou, 1985). The fungus spreads from crop to crop and can infect the crop at all stages (Plantwise, 2012). All above-ground parts of rice plants including leaf sheaths, nodes, the neck of rice panicles and roots can be affected by the disease. The fungus rapidly produces many spores when it is on a rice plant. Air, wind or rain may carry spores onto other surrounding plants (Sesma and Osbourn, 2004 ; Plantwise, 2012). Most infections occur on the leaves. Symptoms occur as white to gray spots with darker borders in the beginning. The spots become diamond-shaped and pale brown in the middle and dark brown around the borders. The lesion size is generally 1-1.5 cm long and 0.3-0.5 cm wide (Commonwealth of Australia, 2015). The panicles that are infected eventually turn white and die before reaching maturity (Kuyek, 2000 ; Castejón-Muñoz, 2008). *P. oryzae* is *Ascomycete* fungus. Mycelia are highly branched, septateand superficial. They bear conidia at the tip or the side of conidiophores. Conidia shapes are pyriform, 20×10 µm and have two to three septa. The conidiophores were found to be slender, straight, gray, grayish black or dark brown. High humidity causes the increase in sporulation. The optimum temperature for growth ranges from 25 °C to 30°C (Gashaw *et al*., 2014).

Chapter 3
Research methodology

3.1 Rice samples and isolation of bacteria

Rice plants were collected from organic rice farms in Bangkok, Chonburi, Saraburi and Suphanburi, Thailand. The samples were thoroughly washed under running tap water. Leaves, stems and roots were cut into small pieces. The samples were put in glass bottles containing sterile distilled water and shaken at 180 rpm for 30 minutes on a rotary shaker. Serial dilutions of bacterial suspension were prepared up to the 10^{-6} concentration. One hundred µl of diluted bacterial suspension at 10^{-5} and 10^{-6} concentrations were plated on nutrient agar (NA, HIMedia) and tryptone soya agar (TSA, TM MEDIA) and incubated at 30 °C for seven days. All isolates with different colony appearances were purified by restreaking on NA. Bacterial stocks were maintained in 25% (v/v) glycerol at -80 °C.

3.2 Morphological characterization of bacteria

All isolates were Gram stained. Bacteria were smeared and heat-fixed on the glass slide. Crystal violet was stained over the smear and gently rinsed off with water after one minute. The slide was flooded with iodine solution for one minute and rinsed off with water again. Safanin was applied for one minute after decolorization by 95% ethanol. The dye was washed off, and the slide was left until dry. Bacterial cells were examined under oil immersion (100x) using the bright field microscope. Gram-stained-positive and Gram-stained-negative bacteria were stained blue/purple and pink/red, respectively. For morphological study, bacteria were grown on nutrient agar (NA) at 30 °C for 48 hours. Colony size, shape, color, margin and opacity were examined.

3.3 DNA extraction, amplification and 16S rRNA gene sequencing

Genomic DNA of epiphytic bacteria was extracted using Presto™ Mini gDNA Bacteria kit (Geneaid, Taiwan) according to the manufacturer's protocol. The nearly complete 16S rRNA gene fragment was amplified using polymerase chain reaction (PCR). Reactions contained deionized water, primers (0.25 µm), dNTPs (0.2mM), MgCl2 (2mM), PCR buffer and *Taq* DNA polymerase. The 41F (5'-GCTCAGATTGAACGCTGGCG-3') and 1492R (5'- TACGGYTACCTTGTTACGACTT-3') universal primers were used (Mao *et al.*, 2012; Hongoh *et al.*, 2003). PCR products were analyzed using agarose gel electrophoresis. The PCR products were purified using Gel/PCR Purification Kit (Favorgen Biotech Corp, Taiwan) according to the manufacturer's protocol and subsequently used for sequencing with the same primers. Pair-wise alignment analysis was performed using the EzBioCloud database (http://www.ezbiocloud.net/).

3.4 Nitrogen fixation

One loop of epiphytic bacteria was resuspended in one ml of glucose-nitrogen-free broth (Ranganayaki *et al.*, 1981). The supernatant was discarded after centrifuged at 5,000 rpm for 15 minutes. Additional 500 µl of glucose-nitrogen-free broth were used for resuspension of the bacterial cells. Two µl of the bacterial suspension were spotted on glucose-nitrogen-free agar. The positive control group was the inoculation of bacterial suspension on glucose-nitrogen-free agar containing 3 mM $(NH_4)_2SO_4$. All plates were incubated at 30 °C for four days. Positive results were determined by the growth of bacteria on glucose-nitrogen-free agar and compared to the control plate.

3.5 Phosphate solubilization

Epiphytic bacteria were inoculated on Pikovskaya's medium (PVK) and National Botanical Research Institute's phosphate growth medium (NBRIP). All plates were incubated at 30 °C for four days. The presence of the clear zone around bacterial colonies was an indication of the phosphate-solubilizing activity.

3.6 Siderophore production

Epiphytic bacteria were grown on chrome azurol S (CAS) agar (Schwyn and Neilands, 1987) and incubated at 30 °C for 48 hours. Siderophore production was identified by the presence of the orange halo around the bacteria.

3.7 ACC deaminase production

One loop of epiphytic bacteria was resuspended in one ml of Dworkin and Foster (DF) salt minimal medium (Dworkin and Foster, 1958) and centrifuged at 5,000 rpm for 15 minutes. The supernatant was discarded. Bacterial cells were resuspended in additional 500µl of DF salt minimal medium. Two µl of bacterial suspension were spotted on DF salt minimal agar that was supplemented with 2 mM 1-aminocyclopropane-1-carboxylic acid. Inoculation of bacterial suspension on DF salt minimal agar and DF salt minimal agar containing 2 mM $(NH_4)_2SO_4$ were used as negative and positive controls, respectively. All plates were incubated at 30 °C for four days. Bacteria that were able to grow on DF salt minimal agar supplemented with ACC as the sole nitrogen source were identified as positive result.

3.8 IAA production

Bacteria were grown in nutrient broth (NB, HIMedia) supplemented with 1% (w/v) tryptophan and incubated at 30 °C for 48 hours on a shaker incubator. Bacterial suspension was centrifuged at 2,500 rpm for 5 minutes. IAA production was determined using a colorimetric technique. One-hundred µl of Salkowski's reagent was added to an equal amount of the supernatant (Ehmann, 1977). The presence of the pink color indicated a positive reaction for IAA production.

3.9 Preparation of bacterial inocula and rice seeds for seedling treatment

Epiphytic bacteria were grown on NA at 30 °C for 24 hours. Bacterial cells were suspended in a glass tube containing 10 ml of sterilized water. The suspension was mixed well using the vortex. The bacterial concentration was adjusted to McFarland standard No. 0.5. Sterilized rice seeds were germinated on water agar plates that

contained 0.4% (w/v) agar. Plates were kept under dark conditions for three days. For surface-sterilization, seeds of Khow Dok Mali 105 rice cultivar were submerged in 95% ethanol for 2 minutes. Rice seeds were further sterilized by incubating in 0.48% (v/v) NaClO solution for 30 minutes and then washed with sterilized distilled water for 15 minutes three times. Seedlings were coated with epiphytic bacterial by submerging them in bacterial suspension for 15 minutes. The control groups were submerged in sterilized water.

3.10 Seedling assay

Five seedlings coated with bacterial suspension were transferred to each test glass bottles. Water-agar medium containing 0.4% (w/v) agar was used for IAA producing bacteria, while water agar supplied with 0.72% (w/v) NaCl was used for ACC-deaminase positive bacteria. Hoagland medium and modified Hoagland medium supplied with 5% (w/v) $Ca_3(PO_4)_2$ were used for siderophore-producing and phosphate-solubilizing bacteria, respectively. Hoagland medium without the nitrogen source was used for nitrogen-fixing bacteria. All bottles were incubated at 25 °C and 16 hours of light and 8 hours of dark conditions. All seedlings were removed from the medium after 15 days. Each test was done in three replicates. All seedlings were dried at 70 °C for 48 hours and dry weight was measured. Growth indicators of all experimental groups were averaged. Plant growth promotion by epiphytic bacteria was determined by comparing all growth indicators of the experimental groups with those of the control groups. The statistical analysis was done by ANOVA using Tukey method. Fold increases of growth were calculated as follows.

$$\text{Fold increase of fresh/ dry weight} = \frac{\text{Average fresh/dry weight of experimental groups}}{\text{Average fresh/dry weight of control groups}}$$

3.11 Antagonistic activity against *X.oryzae* by dual culture method

X. oryzae was inoculated on NA and incubated at 30 °C for 48 hours. Epiphytic bacteria were grown on NA plates at 30 °C for seven days. The pathogen was streaked perpendicularly on the same plate as epiphytic bacteria. The antagonistic activity was determined after further incubation at 30 °C for three days. A positive result was

determined by the presence of the inhibition zone. NA plates streaked with *X. oryzae* only were use as control.

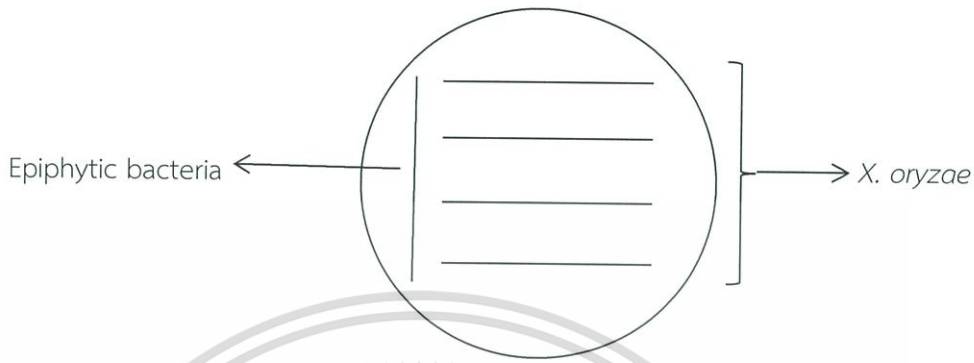

Figure 3.1 Diagram of the test of antagonistic effects against *X. oryzae*.

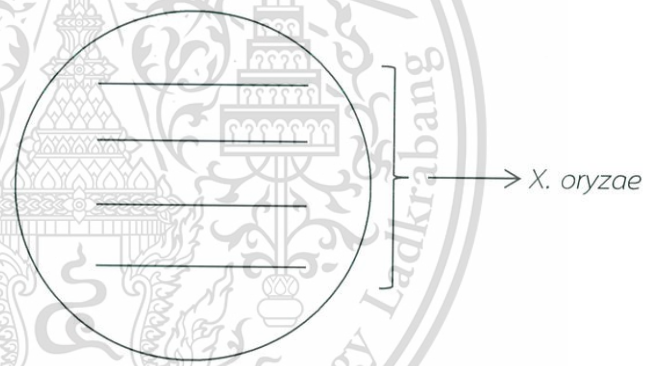

Figure 3.2 Diagram of the control plate for the test of antagonistic effects against *X. oryzae*.

3.12 Antagonistic activity against *P. grisea* by dual culture method

P. grisea was grown on potato dextrose agar (PDA, SRL). After seven days, mycelium plugs of *P. grisea* were punctured using a cork borer and placed on new PDA plates. Epiphytic bacteria were streaked 2.5 cm away from the mycelium plug on the other side of the medium. The PDA plates were cultured at 30 °C for 14 days. The

antagonistic activity was determined by the presence of the zone of inhibition between epiphytic bacteria and the fungus. *P. grisea* grown on PDA plates was used as controls.

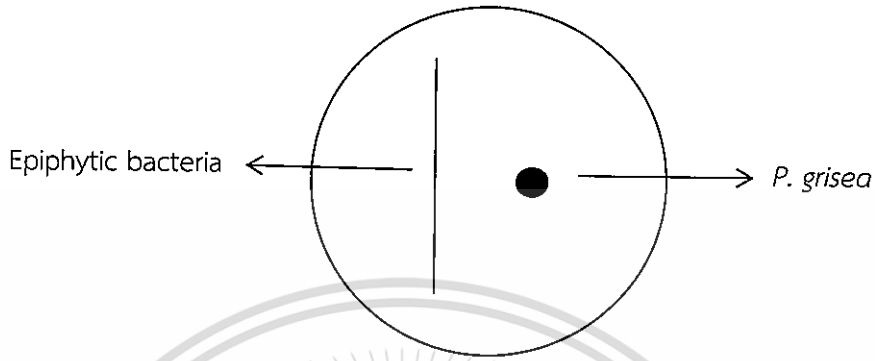

Figure 3.3 Diagram of the test of antagonistic effects against *P. grisea*.

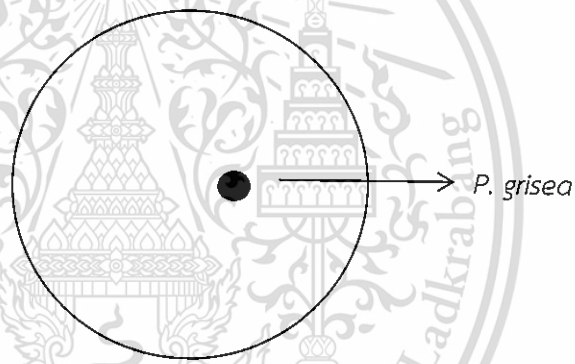

Figure 3.4 Diagram of the control plate for the test of antagonistic effects against *P. grisea*.

Percentage of pathogen growth inhibition can be calculated according to the formula below. All tests were performed in triplicate.

$$\text{Percentage of pathogen growth} = \frac{\text{Pathogen growth on medium inoculated with epiphytic bacteria}}{\text{Average pathogen growth on control plates}} \times 100$$

Percentage of pathogen growth inhibition = 100 - percentage of pathogen growth

3.13 Chitinase activity assay

NA medium containing 1% colloidal chitin was prepared for the chitinase activity test. Epiphytic bacteria were inoculated on medium. All plates were incubated at 30 °C for 48 hours. Positive results were determined by the presence of the clear zone around bacterial colonies.

3.14 Protease activity assay

Epiphytic bacteria were grown on skim-milk agar for the protease activity test and incubated at 30 °C. After 48 hours, clear zones were observed around bacteria that produced protease.

3.15 Well diffusion assay

Epiphytic bacteria were grown in 50 ml of NB at 30 °C for seven days on an incubator shaker. One ml of bacterial suspension was centrifuged at 13,000 rpm for 30 minutes. A five-mm cork borer was used to prepare four wells on a Petri plate containing 20 ml of NA medium. Twenty µl of the supernatant from bacterial cultures were added into the wells. On the other side of the plate, *X. oryzae* was streaked perpendicularly to the wells. All plates were incubated at 30 °C for three days.

On PDA plates, a single well was prepared using the cork borer. Twenty µl of the supernatant were added into the well. A mycelium plug of *P. grisea* was placed on the medium 1.5 cm away from the well. All plates were incubated at 30 °C for 7 days. Growths of the pathogens were recorded as described above.

3.16 Preparation of extracellular crude extracts

Epiphytic bacteria were grown in 50 ml of NB at 30 °C for seven days on a rotary shaker. Liquid-liquid extraction method was used to obtain the extracellular compounds from bacterial cultures. Ethyl acetate was used as the organic solvent to extract bioactive metabolites. Fifty ml of ethyl acetate and cell cultures were mixed together in a separating funnel and shaken for five minutes. The mixture separated into a lower phase (organic phase) and an upper phase (exhausted supernatant). Each culture was

extracted three times. The upper phase was collected. Ethyl acetate was evaporated using the rotary evaporator at 40 °C and 240 bar. Crude extracts were washed off the rotary bottle using methanol. The extracts were placed in sterilized brown bottles and left in the desiccator until the solvent completely evaporated. The weight of the extracts was also determined.

3.17 Disc diffusion method

Crude extracts were dissolved and diluted in absolute methanol. Sterilized paper discs no. 1 was impregnated with the extracts at 150 mg/µl and 300 mg/µl concentration. The discs were left at room temperature until the diluent completely evaporated. Four discs were placed onto Petri plates containing 20 ml of NA medium. *X. oryzae* was streaked perpendicularly to the discs. All plates were incubated at 30 °C for 72 hours. Mycelium plugs of *P. grisea* were placed on PDA plates. Discs loaded with crude-extract suspension were placed 1 and 1.5 cm away from the mycelium plug. Plates were incubated at 30 °C for 7 days. Discs impregnated with absolute methanol were used as controls. Growths of pathogens were recorded as described above.

Chapter 4
Main Results and Discussion

4.1 Bacterial isolation

A total of 113 epiphytic bacteria were isolated from leaves, stems and roots of rice plants collected from four organic rice farms in Thailand. The isolate codes were given by the isolated part and the rice source. The first digit represents the isolated parts which are roots (1), stems (2) and leaves (3). The second digit was the rice sources including Suphanburi province (0), Bangkok province (1), Chonburi province (2) and Saraburi province (3). The last two digits are the number of colonies when they were obtained from the medium.

Nine isolates were isolated from organic Hom-nil rice plants in Bangkok. Five of them were Gram-stained-positive while four of them were Gram-stained-negative. Nine isolates were rods. Thirty-seven bacteria were isolated from organic Rice-berry plants in Chonburi province. Twenty-one and 16 isolates were Gram-stained-positive and Gram-stained-negative, respectively. Thirty-four isolates were rods, and three isolates were cocci. From organic Rice-berry plants in Saraburi province, 35 bacterial isolates were obtained. Seventeen isolates were Gram-stained-positive and 18 isolates were Gram-stained-negative. Rod-shaped and coccus-shaped cells were recorded for 33 isolates and two isolates, respectively. Thirty-two isolates were isolated from organic Rice-berry plants in Suphanburi province. Twenty-five of them were Gram-stained-positive, and seven of them were Gram-stained-negative. Among these, 30 isolates were observed as rod-shaped. Two isolates were cocci (Table 4.1). Complete details on the morphology of each isolate are provided in Appendix Table B1.

Table 4.1 Epiphytic bacteria isolated from rice plants.

Rice cultivar/Source	Number of isolates			Gram reaction		Cell Shape		Total
	roots	stems	leaves	Positive	Negative	Rod	Coccus	
Hom- nil rice/ Bangkok	5	2	2	5	4	9	0	9
Rice berry/ Chonburi	11	9	17	21	16	34	3	37
Rice berry/ Saraburi	12	11	12	17	18	33	2	35
Rice berry/ Suphanburi	19	9	4	25	7	30	2	32
Total	47	31	35	68	45	106	7	113

4.2 Molecular characterization of epiphytic bacteria

Genomic DNA was extracted from all epiphytic bacteria. Nearly-complete 16S rRNA gene fragments were amplified by the polymerase chain reaction (PCR) method. PCR products were analyzed using agarose gel electrophoresis with 0.8% (w/v) agarose gel (Figure 4.1).

16S rRNA gene sequences of all isolated bacteria were used for pair-wise alignment analysis using the EzBioCloud database (http://www.ezbiocloud.net/). The sequences were compared with those of recognized species on the database. Thirty-four isolates showed 100% similarity while 79 isolates showed 98.50% to 99.99% similarity. Details of the pairwise-alignment analysis of all isolates are provided in Appendix Table B2. Bacteria were classified into three phyla including *Firmicutes*, *Proteobacterium* and *Bacteroidetes* and further divided into 24 genera (Appendix Table B3).

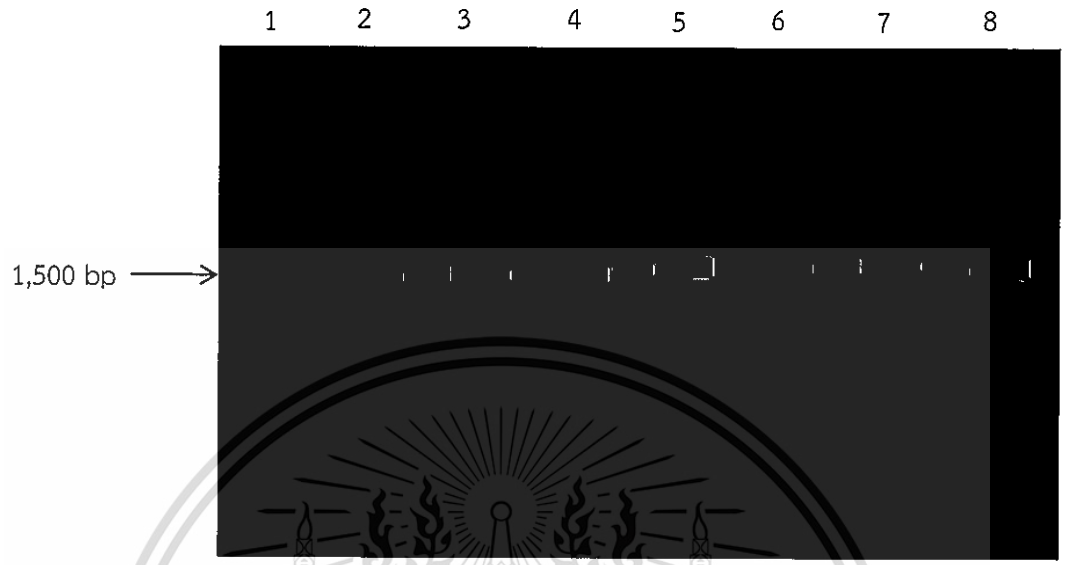

Figure 4.1 Nearly-complete 16S rRNA gene fragments (approximately 1,500 bp) were analyzed on agarose gel. Lane 1 is 1,500-bp DNA fragments and used as the standard. Lanes 2 to 8 showed the amplified 16S rRNA gene fragments of epiphytic bacteria.

These three phyla are the most common phyla that were found on plants. For example, Weinert *et al.* (2011) isolated bacteria from potato cultivars. The result showed that the most dominant phylum was *Proteobacteria* (46%) followed by *Firmicutes* (18%), *Actinobacteria* (11%), *Bacteroidetes* (7%) and *Acidobacteria* (3%). Osman *et al.* (2017) reported that the major proportion of bacteria that were isolated from rice rhizosphere and roots belonged to phyla *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. In *Arabidopsis thaliana*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* were associated with roots and leaves (Bodenhausen *et al.*, 2013). Additionally, these three phyla were also found in roots and stems of sugarcane (Beneduzi *et al.*, 2013).

Sixty-two isolates which constituted to 54.8 % of the total number of isolates were members in the phylum *Firmicutes*. They were members of four genera including *Bacillus* (51 isolates), *Staphylococcus* (six isolates), *Fictibacillus* (four isolates), and *Exiguobacterium* (one isolate). Forty-three isolates or 38.1% of the total number of

isolates belonged to the phylum *Proteobacteria*. These bacteria were classified as members of genera *Pseudomonas* (twelve isolates), *Acinetobacter* (six isolates), *Burkholderia* (four isolates), *Enterobacter* (four isolates), *Klebsiella* (three isolates), *Aeromonas* (two isolates) and *Chromobacterium* (two isolates). One isolate was recorded for each of the following genera: *Aquitalea*, *Brevundimonas*, *Citrobacter*, *Kinneretia*, *Pandoraea*, *Rahnella*, *Roseateles*, *Serratia*, *Vogesella* and *Xanthomonas*. Eight isolates were members in the phylum *Bacteroidetes* and made up to 7.1% of the total number of isolates. They were divided into three genera including *Chryseobacterium* (four isolates), *Chitinophaga* (three isolates) and *Sphingobacterium* (one isolate). The result obtained in the present study was consistent with previous studies on diversity of plant-associated bacteria. Xia *et al.* (2015) isolated bacteria from several plants and found that the most commonly found phylum was *Firmicutes* (44%) followed by *Proteobacteria* (34%) and *Bacteroidetes* (6%). In their study, members in the genus *Bacillus* were the most abundant. Several reports indicated that a number of strains in the genus showed *Bacillus* plant-growth-promoting characteristics (Beneduzi *et al.*, 2008 ; Yadav *et al.*, 2011 ; Souza *et al.*, 2015). Another study reported that *Bacillus* was the dominant genus (39%) among bacteria isolated from water in rice paddies (Reche and Fiuza, 2005). *Pseudomonas* was the second largest group that was found in the present study. Consistently, *Pseudomonas* was reported as the most abundant group of Gram-negative bacteria associated with rice seeds, roots and rhizosphere (Cottyn *et al.*, 2009 ; Naqanandini *et al.*, 2011 ; Kumar *et al.*, 2012). In other works, *Pseudomonas* was reported as one of the dominant genus of the spinach phyllosphere (Lopez-Velasco *et al.*, 2013). In the present study, *Bacteroidetes* was found as the minor group. Consistently, members in *Bacteroidetes* were reported for their lower abundance on leaves of angiosperms compared with gymnosperms (Redford *et al.*, 2010). Other previous reports also showed that *Bacteroidetes* comprised less than 5% of total bacterial community of soil, rhizosphere and several plants. Most of the strains were characterized as *Chryseobacterium* sp., *Flavobacterium* sp. and *Sphingobacterium* sp. (Farina *et al.*, 2011 ; Costa *et* al., 2012 ; Xia *et al.*, 2015).

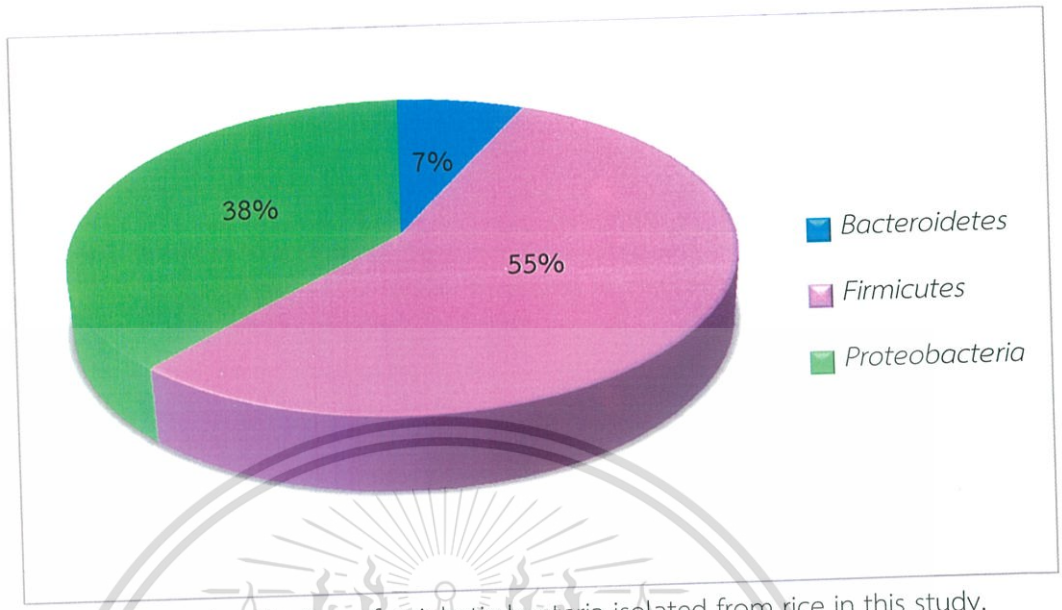

Figure 4.2 Phylum classification of epiphytic bacteria isolated from rice in this study.

In the present study, the numbers and compositions of epiphytic bacteria isolated from each part of the rice plants were different (Figure 4.3). Bacterial compositions in roots and stems were relatively similar and consisted of all three phyla. In contrast, only *Firmicutes* and *Proteobacteria* were isolated from leaves. Forty-seven bacteria which constituted to 42% of all bacteria were isolated from rice roots. The most abundant phylum was *Firmicutes* (53.2% of bacteria isolated from roots) followed by *Proteobacteria* (34%) and *Bacteroidetes* (12.7%). Thirty-one isolates were found from stems and made up to 27% of the total number of bacteria. They were members of phyla *Proteobacteria* (53% of bacteria isolated from stems), *Firmicutes* (40%) and *Bacteroidetes* (7%). From leaves, thirty-five bacterial isolates or 31% of all bacteria were obtained. These bacteria were members of phyla *Firmicutes* (71.4%) and *Proteobacteria* (28.5%). The discrepancy between roots, stems and leaves may reflect the distinction in the amount of nutrients available for epiphytic bacteria in these organs (Lindow and Brandl, 2003). Moreover, the influences of environmental factors on epiphytic bacterial populations have been previously observed. Epiphytic bacteria were likely removed or spread by wind and rainfall (Butterworth and McCartney, 1991 ; Soil Science Society of America, 2017 ; The Scientist, 2017). Bacteria on above-ground parts were more prone to UV irradiation than those on underground parts (Jacobs *et al.*, 2005 ; Gnanamanickam

and Immanuel, 2007). Additionally, leaf age is another factor that affected the population size. For example, the population sizes of *E. coli* O157:H7 and *Salmonella enterica* on young leaves were higher than those on older leaves of lettuce (Brandl and Amundson, 2008).

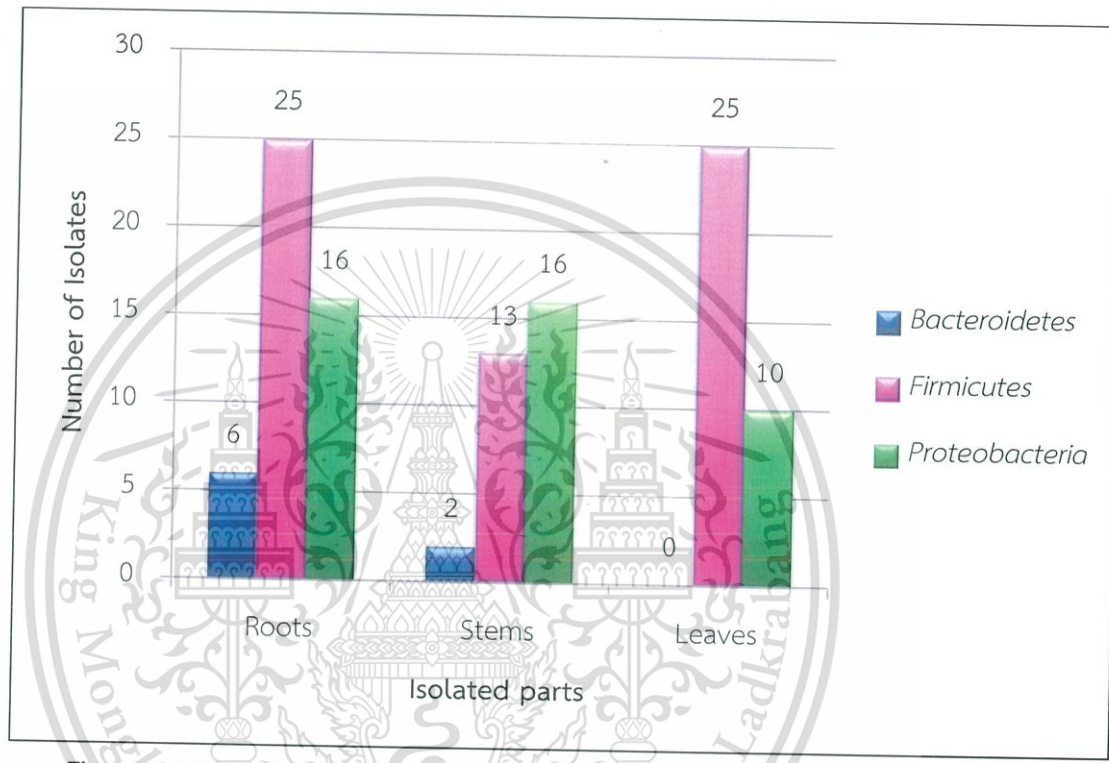

Figure 4.3 Diversity of epiphytic bacteria based on isolated parts.

4.3 Plant-growth-promoting activities

All epiphytic bacteria were tested for their plant-growth-promoting activities including nitrogen fixation, ACC deaminase production, siderophore production, phosphate solubilization and IAA production. 92 isolates showed at least one of the tested plant-growth-promoting activities (Appendix Table B4).

Thirty-six isolates showed the nitrogen-fixing activity. They were able to grow on glucose- nitrogen-free agar compared to the positive control group that were grown on glucose-nitrogen-free agar supplemented with $(NH_4)_2SO_4$ as the nitrogen source. In contrast, bacteria that were unable to fix nitrogen did not grow on the medium (Figure

4.4). Most of these bacteria (23 isolates) were the phylum *Firmicutes* (*Bacillus*, *Exiguobacterium*, *Fictibacillus* and *Staphylococcus*). The second-largest group (11 isolates) belonged to the phylum *Proteobacteria* (*Acinetobacter*, *Aeromonas*, *Brevundimonas*, *Klebsiella*, *Pandoraea* and *Pseudomonas*). Two isolates were in the phylum *Bacteroidetes* (*Chitinophaga* and *Chryseobacterium*). Similar to the current study, members of *Acinetobacter*, *Aeromonas*, *Bacillus*, *Chryseobacterium*, *Exiguobacterium*, *Klebsiella*, *Pseudomonas* and *Staphylococcus* were reported for their potential in nitrogen fixation (Orhan, 2016 ; Sulistiyani and Lisdiyanti, 2016 ; Yasmin *et al.*, 2016 ; Toribio-jiménez *et al.*, 2017). Additionally, the nitrogen-fixing activity was observed in isolates of genera *Chitinophaga*, *Fictibacillus* and *Pandoraea* for the first time.

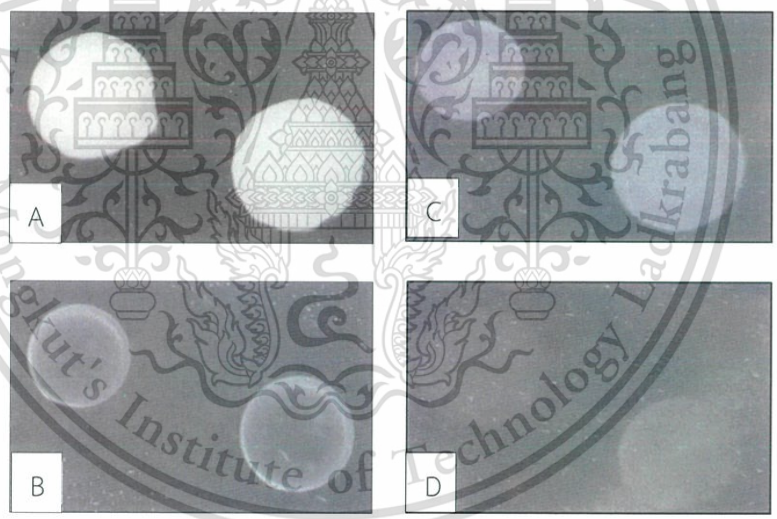

Figure 4.4 Bacteria were tested on glucose-nitrogen-free agar supplemented with $(NH_4)_2SO_4$ (A, C) and glucose-nitrogen-free agar (B, D). Isolate 1304 (A, B) showed the positive result, while isolate 2214 (C, D) showed the negative result.

Phosphate is an essential element for plant growth and development (Rodríguez and Fraga, 1999). PVK and NBRIP media were used to investigate the phosphate-solubilizing activity of epiphytic bacteria. A positive result can be determined by the presence of the clear zone that appeared around bacterial colonies (Figure 4.5). Ten isolates that belonged to genera *Bacillus*, *Burkholderia*, *Pseudomonas* and *Roseateles* were tested positive. Similar to the present study, several strains of *Bacillus*, *Burkholderia* and *Pseudomonas* were previously reported positive for phosphate solubilization (Nailwal *et al*., 2014 ; Orhan, 2016 ; Li *et al*., 2016 ; Toribio-jiménez *et al*, 2017). Consistently, bacteria were isolated from dipterocarps roots and soil in Indonesia were able to solubilize $Ca_3(PO_4)_2$ on NBRIP medium. They reported that *Burkholderia* sp. CK52 and *Roseateles* sp. CK15 were most efficient in phosphate solubilization (Sitepu *et al*., 2013).

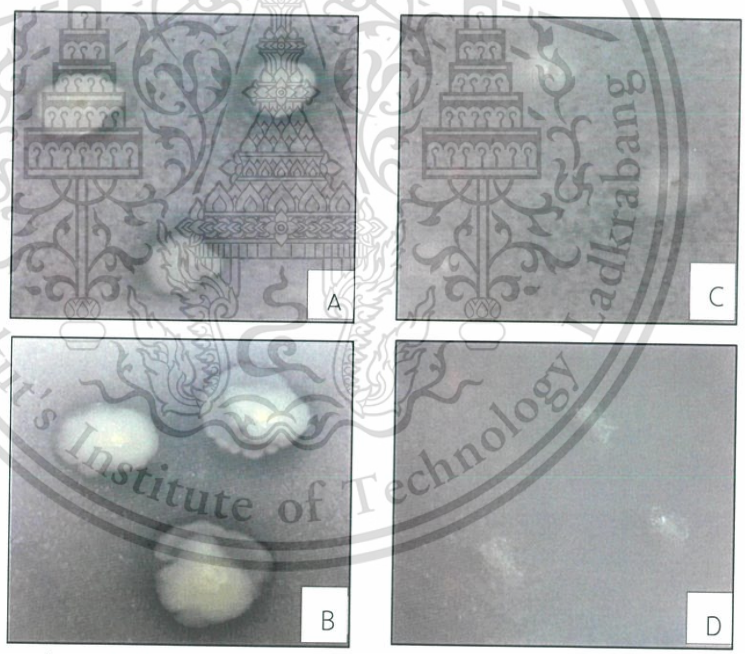

Figure 4.5 PVK (A, C) and NBRIP (B, D) media were used to test the phosphate-solubilizing activity. Isolates 2312 (A, B) showed the positive results on PVK and NBRIP media, respectively. In contrast, Isolates 1201 (C, D) were tested negative on PVK and NBRIP media, respectively.

Siderophores are iron-chelating compounds which are synthesized by many bacteria (Ali and Vidhale, 2013). From this study, 49 isolates were tested positive for siderophore production that can be indicated by the presence of the orange halo around bacterial colonies on CAS agar (Figure 4.6). The major siderophore-producing group was *Firmicutes* (31 isolates). These bacteria belonged to genera *Bacillus*, *Exiguobacterium*, *Fictibacillus* and *Staphylococcus*. The second largest group was *Proteobacteria* (15 isolates). They were members in genera *Acinetobacter*, *Aeromonas*, *Burkholderia*, *Kinneretia*, *Klebsiella*, *Pandoraea*, *Pseudomonas* and *Serratia*. Three isolates of the phylum *Bacteroidetes* were found positive. These bacteria were in genera *Chitinophaga*, *Chryseobacterium* and *Sphingobacterium*. The result obtained in this study was consistent with what was observed in other previous reports. Strains of genera *Acinetobacter*, *Burkholderia*, *Klebsiella*, *Pseudomonas*, *Staphylococcus* and *Serratia* were isolated from rhizosphere of several plants and able to produce siderophores (Rokhbakhsh-Zamin *et al.*, 2011 ; Maindad *et al.*, 2014 ; Toribio-jiménez *et al.*, 2017). *Exiguobacterium* sp. MH3 and *Chryseobacterium* sp. C138 associated with *Lemna minor* and rice rhizosphere, respectively, were found producing siderophores (Radzki *et al.*, 2013 ; Tang *et al.*, 2013). Moreover, *Bacillus* and *Sphingomonas* exhibited the production of siderophores and the highest production was obtained from *Bacillus* sp. pp02 (Li. *et al.*, 2016). In contrast to the present study, previous reports showed that strains of genera *Fictibacillus*, *Kinneretia* and *Pandoraea* were tested negative for siderophore production.

Overproduction of ethylene caused by stresses inhibits root and shoot elongation (Saraf *et al.*, 2010). ACC-deaminase cleaves the ethylene precursor ACC and subsequently decreases the ethylene level in plants (Onofre-Lemus *et al.*, 2009). All epiphytic bacteria in this study were grown on DF salt minimal agar supplemented with ACC as the sole nitrogen source. Bacteria were also grown on DF salt minimal agar and DF salt minimal agar containing 2 mM $(NH_4)_2SO_4$ and used as negative and positive controls, respectively. Only ACC-deaminase-producing bacteria were able to grow on the medium containing ACC and the positive control plate while isolates that were unable to produce ACC deaminase grew on the positive control plates only (Figure 4.7).

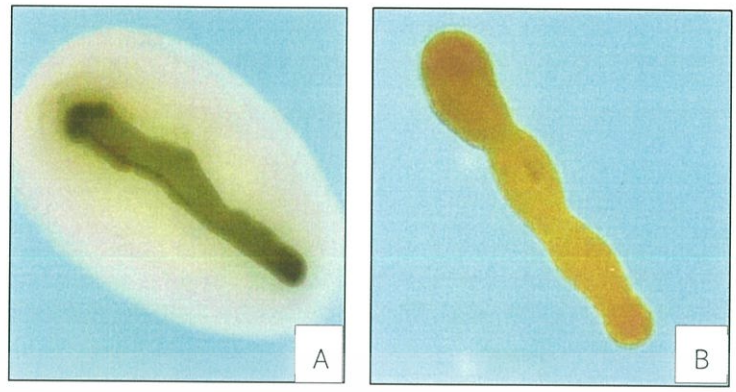

Figure 4.6 Bacteria were tested on CAS agar for siderophore production. Isolate 2310 (A) showed the positive result while isolate 2303 (B) showed the negative result.

In the present study only members in phyla *Proteobacteria* (14 isolates) and *Firmicutes* (17 isolates) displayed the positive result. These isolates belonged to genera *Acinetobacter*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Roseateles*, *Serratia*, *Bacillus* and *Exiguobacterium*. The results that were obtained from the current study was consistent with other reports. Members of *Proteobacteria* including *Acinetobacter* sp., *Burkholderia* sp., *Enterobacter* sp., *Pseudomonas* sp. and *Serratia* sp. sp. were isolated from plants roots and rhizosphere and able to utilize ACC by producing ACC deaminase (Huang *et al.*, 2013 ; Ali *et al.*, 2014 ; Miao *et al.*, 2015). Several strains of *Burkholderia* (Onofre-Lemus *et al.*, 2009) and *Pseudomonas* (Rashid *et al.*, 2012) were investigated for ACC deaminase production.The highest enzyme production was observed in *Burkholderia terricola* LMG 20594^{T} (Onofre-Lemus *et al.*, 2009 ; Rashid *et al.*, 2012). *Serratia* sp. SL-12 isolated from salt lake was reported for ACC deaminase production and was able to promote growth of wheat plants under salt stress conditions (Singh and Jha, 2015). This suggests that ACC-deaminase-producing bacteria may be used as biofertilizers to increase plant tolerance towards stress conditions. Moreover, bacteria in genera *Bacillus* and *Exiguobacterium* were tested positive in this study and another report. Halotolerant and halophilic strains of *Bacillus atrophaeus*, *Bacillus gibsonii*, *Bacillus* sp. and *Exiguobacterium aurantiacum* were reported as ACC-deaminase producers (Orhan, 2016). Different from this study, ACC-deaminase production by *Roseateles* was observed in this study for the first time.

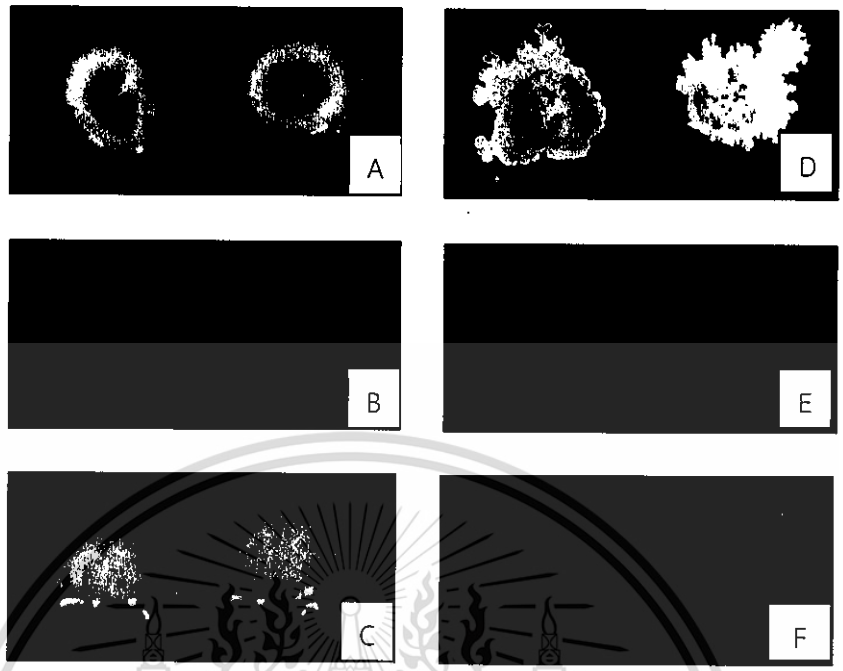

Figure 4.7 Bacteria were tested for their ACC deaminase production by using DF salt minimal agar containing $(NH_4)_2SO_4$ as the positive control plate (A,D), DF salt minimal agar as the negative control plate (B,E) and DF salt minimal agar containing 0.03% (w/v) ACC (C,F). Isolate 3212 (A, B, C) and 1316 (D, E, F) displayed the positive and negative results, respectively.

IAA is one of the major phytohormones that is required for proper growth and development in plants (Mohite, 2013). Several bacteria were reported producing IAA using L-tryptophan as the precursor (Reetha *et al.*, 2014). All epiphytic bacteria were grown in nutrient broth supplemented with 0.2% (w/v) L-tryptophan in order to test for IAA production. The supernatants of 19 isolates turned pink when added with Salkowski's reagent. This indicated the positive result of IAA production compared to IAA solution and non-inoculated medium (Figure 4.8). The predominant group (16 isolates) of IAA-producing bacteria was *Proteobacteria* that consisted of members in genera *Acinetobacter*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pandoraea*, *Pseudomonas* and *Rahnella*. Three isolates were members of *Firmicutes*. They belonged to genera *Bacillus*, and *Staphylococcus*. The result in this study was consistent with other studies on IAA-

producing bacteria. Bacteria in genera *Acinetobacter*, *Bacillus, Enterobacter*, *Klebsiella*, *Pseudomonas* and *Staphylococcus* obtained from roots of several plants were reported for IAA production (Rokhbakhsh-Zamin *et al*., 2011 ; Li *et al*., 2016 ; Orhan, 2016 ; Toribio-jiménez *et al*., 2017). In another report, strains of *Burkholderia cepacia*, *Bacillus amyloliquefaciens*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa* were able to produce IAA (Dinesh *et al*., 2015). Moreover, *Rahnella* sp. BIHB 783 isolated from soil was recorded for the production of IAA. *Burkholderia* sp. isolated from *Rhododendron arboretum* showed the positive result of IAA production. This study was the first time to report the production of IAA by a member in the genus *Pandoraea*.

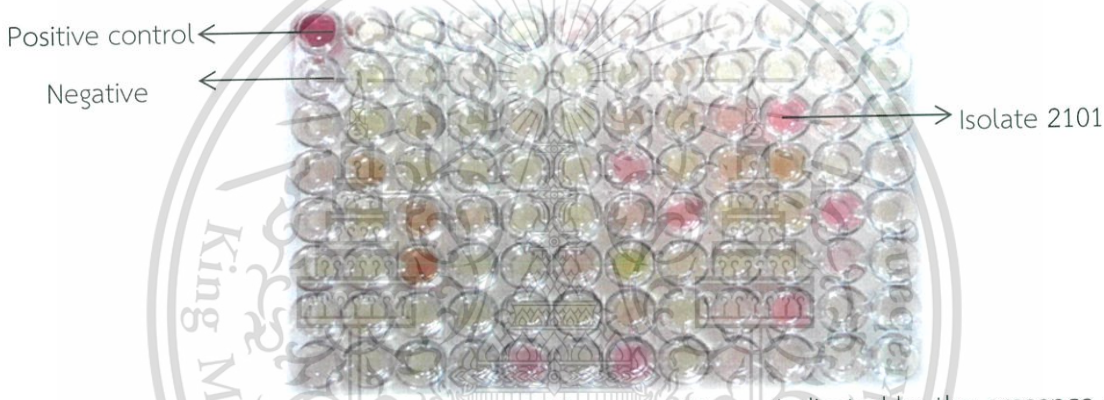

Figure 4.8 IAA production test. Isolate 2101 was positive as indicated by the presence of the pink color after Salkowski's reagent was added to the supernatant. The positive control was 1% (w/v) IAA. The negative control was non-inoculated NB medium.

4.4 Plant growth promotion by epiphytic bacteria

Seven epiphytic bacteria containing at least three plant-growth-promoting activities were selected for seedling assay (Table 4.2). Based on their partial 16S rRNA gene sequence analysis, they belonged to genera *Bacillus* (isolates 1308, 2211, 2312 and 3209), *Burkholderia* (isolate 2310), *Exiguobacterium* (isolate 1205) and *Pseudomonas* (isolate 3218). A previous report showed that bacteria containing maximum *in vitro* plant-growth-promoting activities displayed the best plant growth promotion on tea plants by increasing shoot and root length and plants biomass (Dutta *et al*., 2015). In the present study, rice seedlings were co-cultivated with the bacteria and grown under

various conditions in order to determine their effects on seedling biomass. Control groups were non-inoculated seedlings grown under similar conditions. Growth promotion by epiphytic bacteria was determined by comparing the dry weight of the bacteria-treated groups with the control groups and shown as the fold increase.

Table 4.2 Plant-growth-promoting bacteria that were selected for seedling assay.

Isolates	Plant-growth-promoting activities				
	Nitrogen fixation	Phosphate solubilization	Siderophore production	ACC deaminase production	IAA production
Exiguobacterium sp. 1205	+	-	+	+	-
Bacillus sp. 1308	+	-	+	+	-
Bacillus sp. 2211	-	-	+	+	+
Burkholderia sp. 2310	-	+	+	+	+
Bacillus sp. 2312	+	-	+	+	-
Bacillus sp. 3209	+	-	+	+	-
Pseudomonas sp. 3218	+	+	-	+	-

*Note

+ : Positive result

- : Negative result.

Nitrogen is one of the essential macronutrients that are required for plant growth (Franche *et al.*, 2010). In order to investigate the effect of nitrogen-fixing epiphytic bacteria on growth of rice seedlings, isolates 1205, 2312, 1308, 3209 and 3218 that were tested positive for nitrogen fixation were used in this test. The result showed that seedlings treated with isolate 1205 displayed the highest fold increase (1.00±0.05) and

was significantly different ($p<0.05$) from other groups (Figure 4.9). However, none of these tested groups showed the positive effect on plant growth.

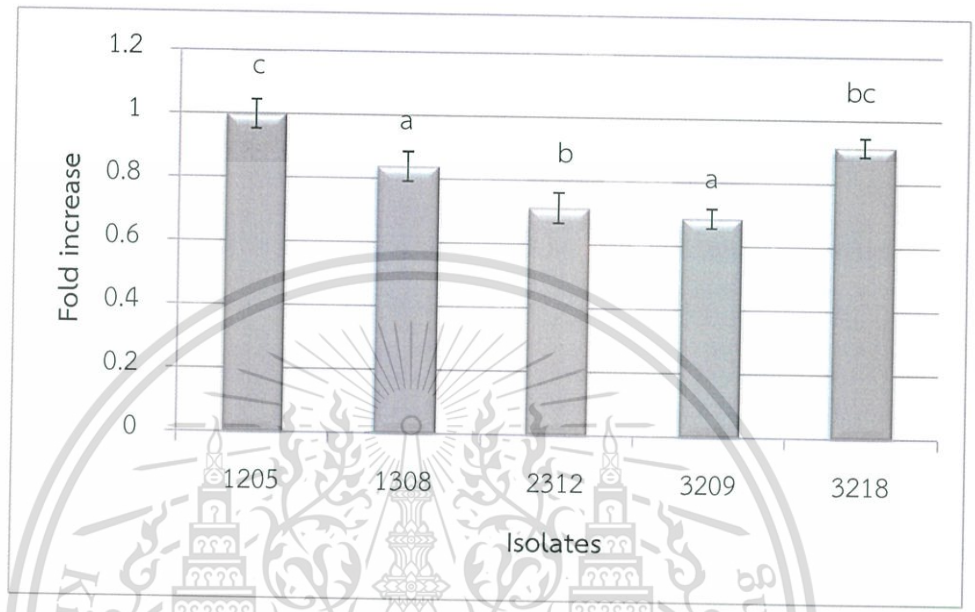

Figure 4.9 Fold increase of dry weight of rice seedlings treated with nitrogen-fixing bacteria. Different letters indicates statistically significant difference ($p<0.05$).

Similar to nitrogen, phosphorus is one of the important macroelements required for plant growth and development (Armstrong, 1999). Isolates 2310 and 3218 which displayed the phosphate-solubilizing activity were tested for their ability to promote growth of rice seedlings that were grown on modified Hoagland medium containing 5% (w/v) $Ca_3(PO_4)_2$. The result showed that both isolates did not promote plant growth as the fold increases of dry weight were lower than 1.00 (Figure 4.10). Additionally, the effects of both isolates on seedling dry weight were not significantly different ($p<0.05$).

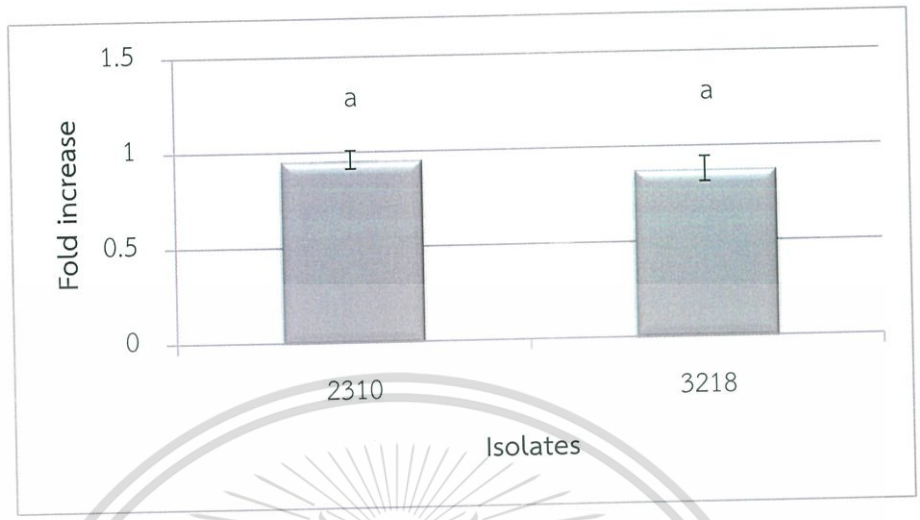

Figure 4.10 Fold increase of dry weight of rice seedlings treated with phosphate-solubilizing bacteria. Different letters indicates statistically significant difference ($p<0.05$).

Iron is important for many biological processes including chlorophyll synthesis. The lack of iron is the cause of leaf chlorosis (Uchida, 2000). Siderophores have high affinity towards Fe^{3+} and facilitate the uptake of Fe^{3+} by plants (Ahemad and Kibret, 2014). In this test, isolates 1205, 1308, 2211, 2310, 2312 and 3209 were selected because of their siderophore production. Seedlings that were inoculated with these isolates were grown on Hoagland medium that contain 0.1% (w/v) Fe-EDTA. However, positive effects by these bacteria were not observed under the tested conditions as determined by the fold increases that were less than 1.00 in all treated groups (Figure 4.11). The highest fold increase (0.96±0.06) was obtained in seedlings inoculated with isolate 1205.

Ethylene is important for plant growth and development but excessive levels of ethylene could inhibit plant root and shoot elongation. Overproduction of ethylene can be caused by stress conditions including salinity stress (Huang *et al.*, 2013). Because of the relationship between ethylene and salinity stress, ACC-deaminase-positive isolates including 1205, 1308, 2211, 2310, 2312, 3209 and 3218 were co-cultured with rice seedlings that were grown in water-agar medium supplemented with 0.72% (w/v) NaCl.

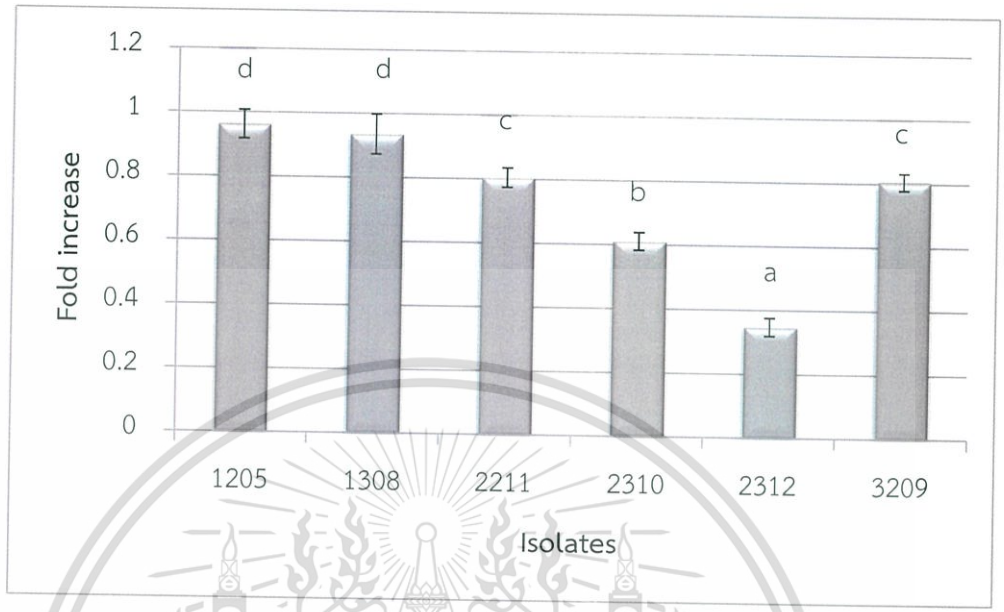

Figure 4.11 Fold increase of dry weight of rice seedlings treated with siderophore-producing bacteria. Different letters indicates statistically significant difference ($p<0.05$).

The result showed that the highest fold increase (0.97+0.02) was obtained from isolate 1205. This was significantly different from other treated groups (Figure 4.12). However, the fold increases of all bacteria-treated groups were lower than 1.00. This indicated that all isolates did not show a positive effect on seedling biomass under salinity stress.

IAA is one of the major phytohormones. It promotes cell elongation and increases the number of root hairs which directly enhances the nutrient uptake by plants (Ambawade and Pathade, 2013). For the effect of bacterial IAA on plant growth, isolates 2211 and 2310 that were tested positive for IAA production were selected. Inoculated seedlings were grown on water-agar medium. The average fold increases of dry weight of rice seedling inoculated with isolates 2211 and 2310 were 0.96±0.04 and 0.89±0.06, respectively (Figure 4.13). This result indicated that both isolates did not promote growth of the seedlings compared to the control groups.

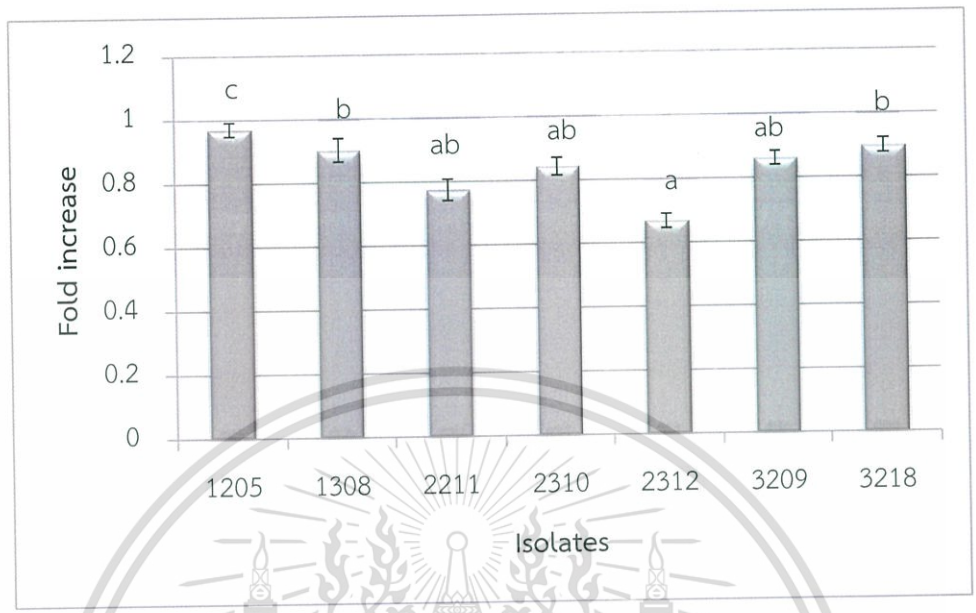

Figure 4.12 Fold increase of dry weight of rice seedlings treated with ACC-deaminase-producing bacteria. Different letters indicates statistically significant difference ($p<0.05$).

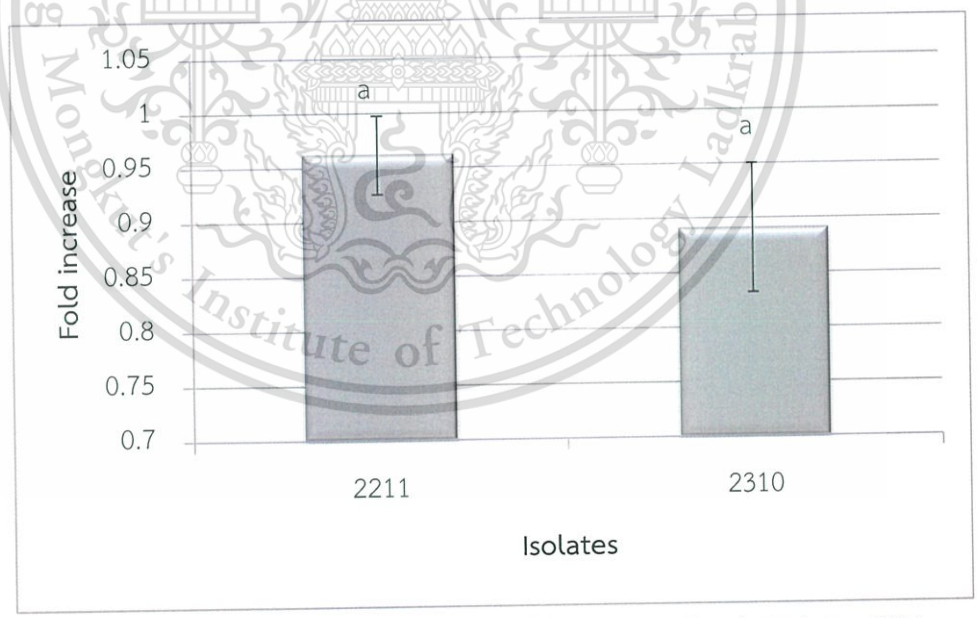

Figure 4.13 Fold increase of dry weight of rice seedlings treated with isolates 2211 and 2310 that produced IAA. Similar letters indicates statistically non-significant difference ($p<0.05$).

Seven selected isolates were tested for their growth promotion in rice seedlings under various conditions. However, the overall results did not indicate their positive effects on rice seedling. In contrast, several strains of other members in similar genera were reported to increase growth in various plants. A previous study showed that inoculation of *Bacillus pseudomycoides* SN29, *Burkholderia* sp. TT6 and *P. aeruginosa* KH45 on tea plants promoted root and shoot growth (Dutta *et al.*, 2015). Reetha *et al.* (2014) inoculated *P. fluorescens* and *B. subtilis* onto onion plants. The increases in shoot and root biomass were observed compared with the non-inoculated groups. The discrepancy between the results obtained in the present and previous studies may be related to the bacterial ability to colonize the plant host. A correlation between bacterial colonization and plant growth promotion were observed in previous studies. Five isolates including *Caulobacter* sp. FA 13, *Pantoea* sp. FF 34, *Sphinogobium* sp. FC 42, *Pseudomonas* sp. FB 12, and *Enterobacter* sp. FD17 were inoculated on maize seeds and improved the germination rate. The maximum germination rate increase was observed from strain FD17. The strain also increased the leaf area and plant biomass compared to the non-inoculated plants. Bacterial colonization efficiency was 2.93×10^{7} cells g^{-1} shoot fresh biomass. Maximum colonization of strain FD17 occurred in rhizosphere, roots and shoots. Additionally, strain FD17 was the dominant strain in rhizosphere and roots compared to other strains (Naveed *et al.*, 2013). Based on the previous and present results, the colonization ability potentially was another factor that influences growth promotion in rice seedlings by epiphytic bacteria. In order to confirm this speculation, additional tests on plant colonization by epiphytic bacteria in this study are needed.

4.5 Antagonistic activity of epiphytic bacteria against rice pathogens

All epiphytic bacteria were tested for their antagonistic effect against *X. oryzae*, the causative agent of the leaf blight disease in rice, *in vitro*. The pathogen was streaked on NA plates that were pre-cultured with epiphytic bacteria. The inhibition zone was the indicator of the antagonistic activity and observed with five bacterial isolates (Figure 4.14). They were *Bacillus* sp. 1012, *Burkholderia* sp. 2310, *Chitinophaga* sp. 1303,

Chitinophaga sp. 1310 and *Chitinophaga* sp. 1313. The highest percentage of pathogen inhibition was obtained from *Chitinophaga* sp. 1310 (36.5±3.84). This was significantly different ($p<0.05$) from other treated group (Figure 4.15).

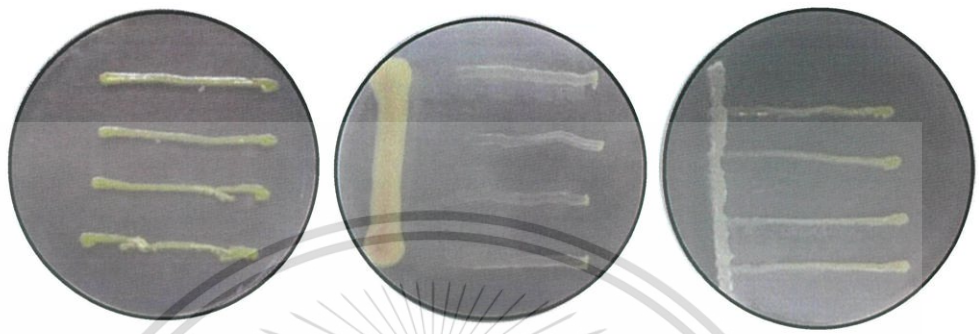

Figure 4.14 Biological control activities of epiphytic bacteria against *X. oryzae*

(A) *X. oryzae* on control plates without epiphytic bacteria.

(B) Isolate 1310 showed the positive result that was indicated by the presence of the inhibition zone.

(C) Isolate 1315 showed the negative result that was indicated by the absence of the inhibition zone.

Epiphytic bacteria were co-cultured with *P. grisea* that causes the leaf blast disease in order to test for their suppression of pathogen growth. The presence of the inhibition zone indicated the antagonistic activity of epiphytic bacteria (Figure 4.16). Twenty-three isolates were found positive for this test. They were members in genera *Bacillus* (16 isolates), *Burkholderia* (four isolates), *Chitinophaga* (two isolates) and *Pseudomonas* (one isolate). *Bacillus* sp.1021, *Bacillus* sp. 1110, *Bacillus* sp. 2211, *Pseudomonas* sp. 2302, *Bacillus* sp. 2306, *Bacillus* sp.3210, *Bacillus* sp. 3308 and *Bacillus* sp. 3310 displayed 100% pathogen growth inhibition. These strains were significantly different ($p<0.05$) from other treated group (Figure 4.17). Moreover, four isolates including *Bacillus* sp. 1012, *Burkholderia* sp. 2310, *Chitinophaga* sp. 1303 and *Chitinophaga* sp. 1313 were able to suppress both *X. oryzae* and *P. grisea* growth.

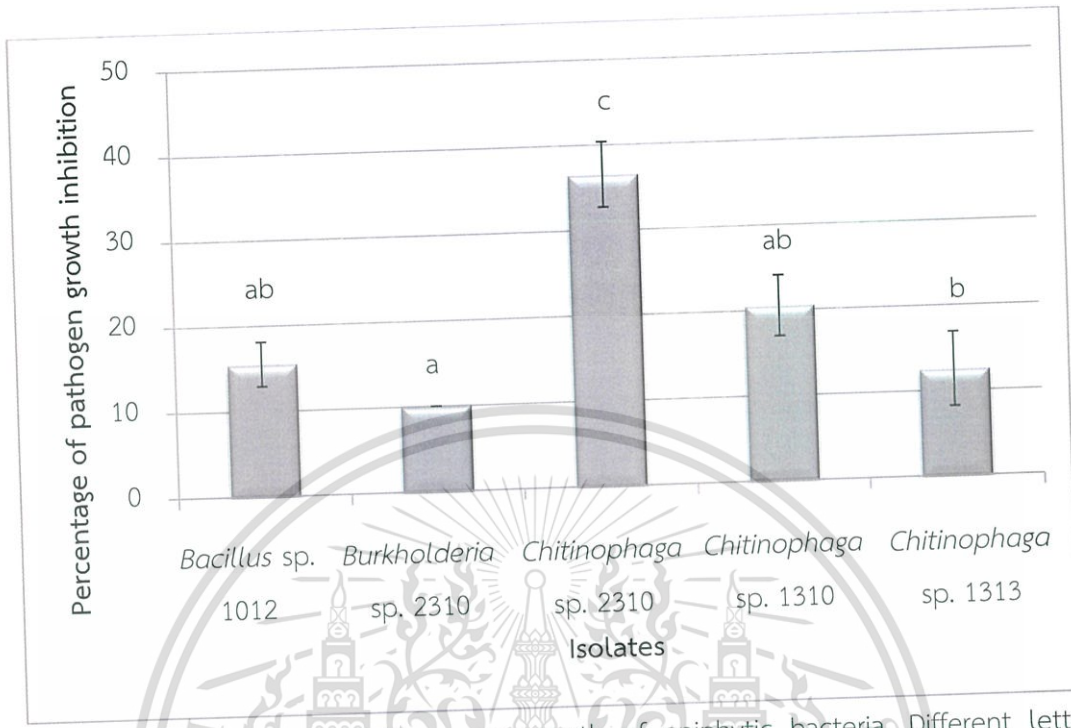

Figure 4.15 Percentage of *X. oryzae* growth of epiphytic bacteria. Different letters indicates statistically significant difference ($p<0.05$).

Figure 4.16 Biological control activities of epiphytic bacteria against *P. grisea*

(A) *P. grisea* on control plates without epiphytic bacteria.

(B) Isolate 1110 showed the positive result because of the presence of the inhibition zone.

(C) Isolate 2313 showed the negative result because of the absence of the inhibition

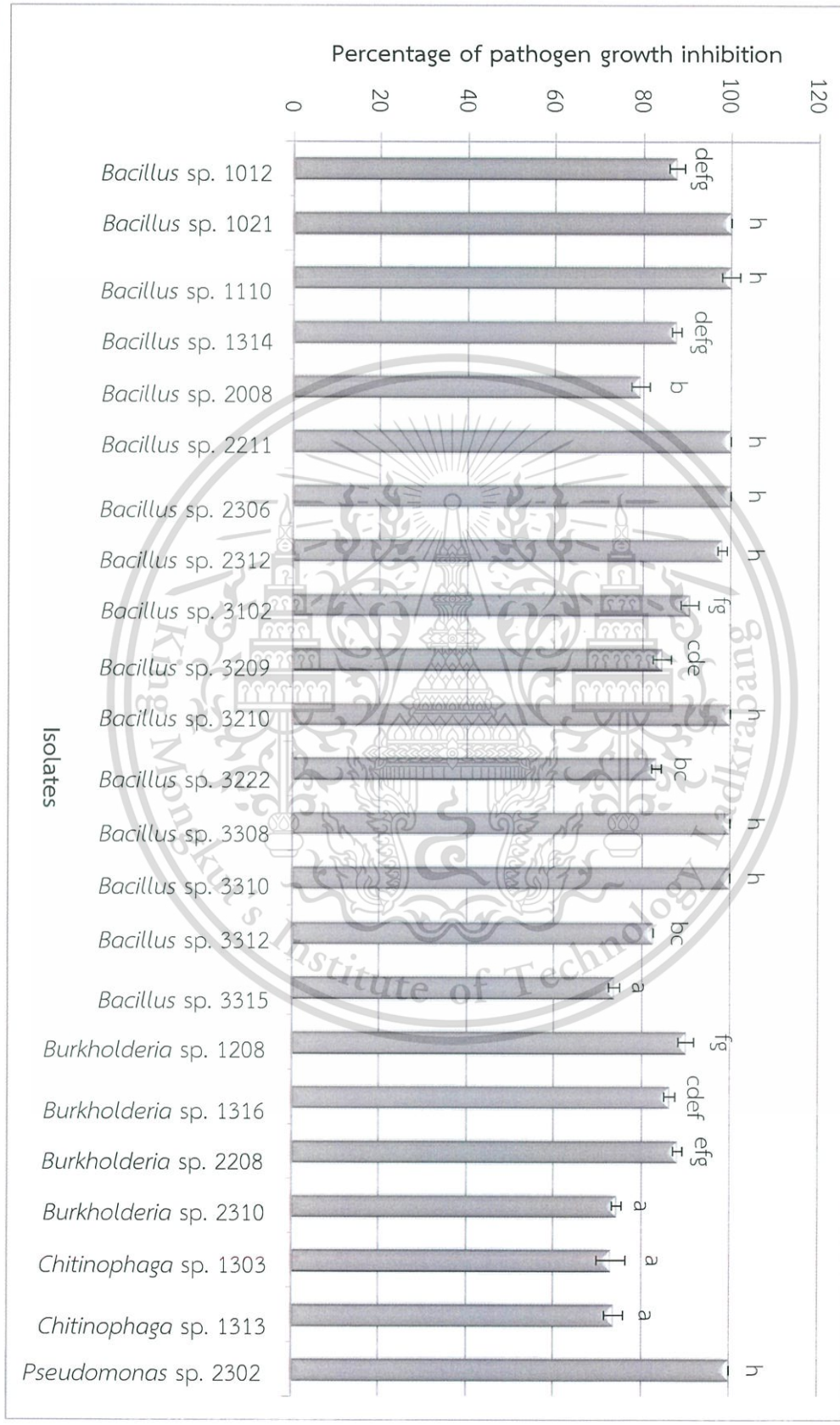

Figure 4.17 Percentage of *P. oryzae* growth of epiphytic bacteria. Different letters indicates statistically significant difference ($p<0.05$)

The results obtained from the current study were consistent with previous reports. Strains of *Bacillus* spp. displayed antagonistic activities against *X. oryzae* and *P. grisea*. *Bacillus* sp. Rh219 was reported for its antagonistic activity against *X. oryzae* pv. *oryzae*. The bacterium was inoculated onto rice plants and significantly improved the plant health by reducing the severity of the leaf blight disease and increasing shoot and root length as well as plant biomass compared with the control plants (Yasmin *et al.*, 2016). Another report showed that *Bacillus cereus and* Bacillus *subtilis* isolated from rice plants in China were able to suppress the growth of *P. grisea in vitro*. They were also efficient in controlling *P. grisea* under greenhouse conditions (Tokpah *et al.*, 2016). Members of the genus *Pseudomonas* have been studied and widely used for controlling plant diseases (Chung *et al.*, 2015). A study showed that bacteria isolated from soil and belonging to the genus *Pseudomonas* displayed over 50% inhibition of *P. grisea* spore germination (Ueno *et al.*, 2016). In contrast to the present study where none of *Pseudomonas* isolates were able to suppress *X. oryzae* growth, other *Pseudomonas* strains were reported for their suppression on *X. oryzae* (Rangarajan *et al.*, 2002). *Burkholderia* species were found in various types of environments (Coenye *et al.*, 2001 ; Coenye and Vandamme, 2003). Some strains were reported as plant pathogens but several other strains exhibited antagonistic activities against phytopathogens (Scuderi *et al.*, 2009). A strain of *Burkholderia* sp. was able to suppress the spore germination of *P. grisea* (Lemtukei *et al.*, 2017). The strain also displayed the production of fungicides against phytopathogenic fungi including *Alternaria alternata*, *Cochliobolus miyabeanus*, *Colletotrichum orbiculare*, *Corynespora cassiicola* and *Fusarium oxysporum* f. sp. *spinaciae*. However, there was no report on the inhibition of *X. oryzae* by members of the genus *Burkholderia*. This was different from the present study where *Burkholderia* sp. 2310 displayed growth suppression of the bacterial pathogen. *Chitinophaga* bacteria isolated in this study showed the antagonistic effects against *X. oryzae* and *P. grisea*. This is somewhat different from what was recently reported on a strain of the same genus that was isolated from soil samples collected in Okinawa. It showed an antifungal activity against *P. grisea*, and the inhibition

of *P. grisea* spore germination was over 50%. However, the inhibitory effect of this *Chitinophga* strain against pathogenic bacteria was not observed (Ueno *et al.*, 2016).

4.6 Chitinase and protease activities of antagonistic bacteria

Production of lytic enzymes is one of the mechanisms by which bacteria employ to suppress growth of other microorganisms. Chitinase is able to lyse fungal cell walls that are composed of chitin while protease degrades proteins that are constituents of both fungal and bacterial cell walls (Glick, 2012 ; Choudhary *et al.*, 2014). Twenty-four isolates of epiphytic bacteria that showed antagonistic effects against *X. oryzae* and *P. grisea* were tested for the activity of both chitinase and protease on NA medium containing colloidal chitin and skim-milk agar, respectively. Only isolates 1208 and 2208 of the genus *Burkholderia* were found positive for the chitinase activity as determined by the appearance of the clear zone around bacterial colonies (Figure 4.18). In contrast, 19 isolates were tested positive for the protease activity (Figure 4.19). These included isolates that belonged to genera *Bacillus* (eleven isolates), *Burkholderia* (four isolates), *Chitinophaga* (three isolates) and *Pseudomonas* (one isolate). The results of all tested isolates are shown in Table 4.3.

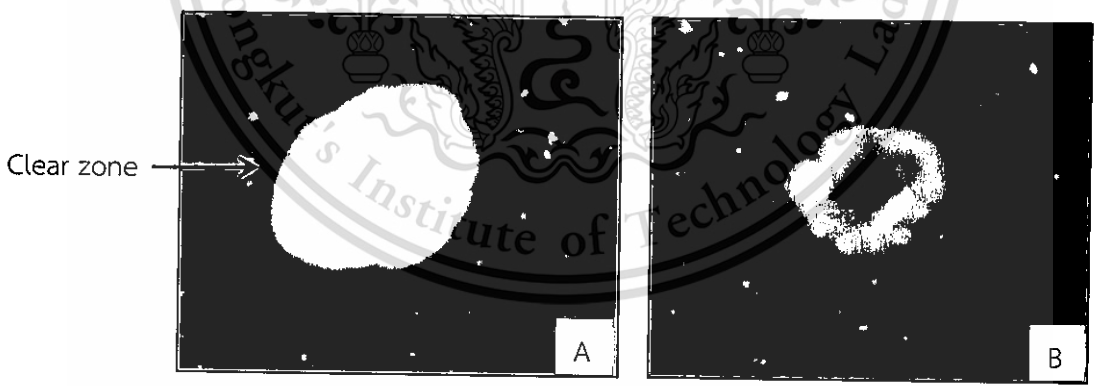

Figure 4.18 The chitinase activity was determined on NA medium that contained colloidal chitin. Isolate 2208 (A) was recorded as positive by the clear-zone formation (arrow) while isolate 1316 (B) was unable to form the clear zone and recorded as negative.

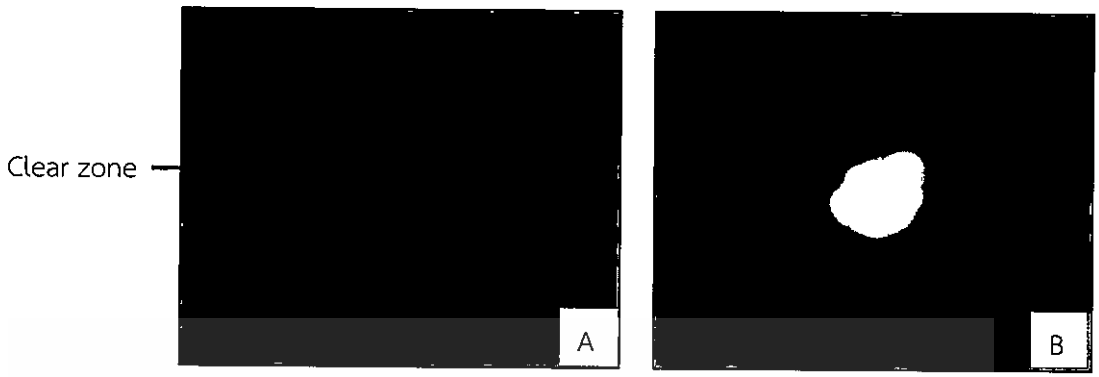

Figure 4.19 The protease activity was determined on skim-milk agar medium. Isolate 1012 (A) was recorded as positive by the presence of the clear zone (arrow) around the colony. Isolate 1314 (B) was recorded as negative because of the absence of the clear zone.

Some bacteria produced lytic enzymes, such as, chitinases, β-1, 3 glucanases, proteases, and lipases that can lyse cell walls of pathogens and suppress their growth (Glick, 2012).The result obtained in this study showed that the protease activity was more common among antagonistic isolates than the chitinase activity. This indicated that cell-wall proteins of the pathogens were potentially the major targets for growth suppression by these epiphytic bacteria. Chitinase was observed in two *Burkholderia* isolates that yielded negative results in the *in vitro* antagonism experiment against *P. grisea*. This result suggested that chitinase may not be involved in growth suppression of the fungus in the present study. Additionally, four antagonistic isolates did not produce either chitinase or protease. Similar results were also observed in some previous studies. In early report described the isolation of strains of *Bacillus* sp. from vinegar waste compost. They were able to suppress the growth of *F. oxysporum* f. sp. strains, *Fusarium graminearum*, *Fusarium moniliforme*, *Glomerella cingulate* and *R. solani*. However, some strains were able to produce protease and chitinase. (Lin *et al.*, 2014). Additionally, the result that obtained from other report showed that the percentage of biocontrol efficacy of some lytic enzyme producing strains were not significantly different from the strain that not producing any enzyme (Tokpah *et al.*, 2016). Taken together with previous reports, the results obtained in this study indicated that other

mechanisms may also be involved in the inhibition of *X. oryzae* and *P. grisea* by epiphytic bacteria.

Table 4.3 Enzymatic activities of epiphytic bacteria with antagonistic activity against *X. oryzae* and *P. grisea*.

Isolates	Antagonistic activities		Enzymatic activities	
	X. oryzae	*P. grisea.*	Chitinase	Protease
Bacillus sp. 1012	+	+	-	+
Bacillus sp. 1021	-	+	-	+
Bacillus sp. 1110	-	+	-	+
Bacillus sp. 1314	-	+	-	-
Bacillus sp. 2008	-	+	-	+
Bacillus sp. 2211	-	+	-	+
Bacillus sp. 2306	-	+	-	-
Bacillus sp. 2312	-	+	-	+
Bacillus sp. 3102	-	+	-	+
Bacillus sp. 3209	-	+	-	+
Bacillus sp. 3210	-	+	-	+
Bacillus sp. 3222	-	+	-	+
Bacillus sp. 3308	-	+	-	+
Bacillus sp. 3310	-	+	-	+
Bacillus sp. 3312	-	+	-	-
Bacillus sp. 3315	-	+	-	+
Burkholderia sp. 1208	-	+	+	+
Burkholderia sp. 1316	-	+	-	+
Burkholderia sp. 2208	-	+	+	+
Burkholderia sp. 2310	+	+	-	+
Chitinophaga sp. 1303	+	+	-	-
Chitinophaga sp.	+	-	-	-

Isolates	Antagonistic activities		Enzymatic activities	
	X. oryzae	*P. grisea.*	Chitinase	Protease
1310				
Chitinophaga sp. 1313	+	+	-	+
Pseudomonas sp. 2302	-	+	-	+

*Note

+ : Positive result

- : Negative result.

4.7 Extraction of bioactive compounds

Production of bioactive compounds is one of the mechanisms reported in antagonistic bacteria (Janardhan *et al.*, 2014). In order to investigate their bioactive-compound production, twenty-four antagonistic bacteria were grown in NB medium. The supernatants were used to determine the presence of bioactive compounds that inhibited growth of *X. oryzae* and *P. grisea* using the well diffusion assay. However, the supernatants of these isolates yielded negative results. This may be caused by the small amount of the supernatants used in the study that may not contain sufficient amounts of bioactive compounds to display the inhibitory effects. Alternatively, bioactive compounds were extracted from bacterial cultures in NB medium using the liquid-liquid extraction method. Ethyl acetate was used as the solvent. The extracts were further concentrated by evaporation and dissolved in a small amount of methanol. Paper discs impregnated with the extracts were tested for the inhibition of the pathogens. However, similar to the well diffusion assay, negative results were observed with the crude extracts obtained from all bacterial isolates.

The results in the present study showed that growth suppression of *X. oryzae* and *P. grisea* by epiphytic bacteria were more effective when bacterial cells were used because the culture supernatants and crude extracts yielded negative results. This was

also observed in a previous study. The efficiencies of culture filtrates and whole bacterial cells in suppressing growth of *Rhodotorula pilimanae*, *Penicillium digitatum*, *Penicillium expansum*, *Botrytis cinerea*, *Aspergillus flavus*, *Aspergillus ochraceus* and *Alternaria alternata* were compared using plate assay. The result showed that the inhibition zones obtained from the culture filtrates were much smaller than those of the whole bacterial cells. Additionally, the filtrates of some strains were unable to inhibit the pathogens (Scuderi *et al.*, 2009). There are several other mechanisms of bacteria to suppress the pathogen growth. Some mechanisms work well with the whole bacterial cell. These included nutrient competition and volatiles compound production. Nonpathogenic bacteria can colonize plant surfaces faster and acquire more nutrients than pathogens. This leads to the lack of resources to supply the growth of the pathogens (Glick, 2012). Alternatively, bacteria may synthesize volatiles compounds that suppress the pathogen growth (Glick, 2012 ; Yasmin *et al.*, 2016). *Burkholderia tropica* was reported for the production of volatile compounds that inhibited fungal pathogens including, *Colletotrichum gloesporioides*, *Fusarium culmorum*, *F. oxysporum* and *Sclerotium rolffsi*. Antagonistic bacteria in this study should be further tested for their nutrient competition as well as the production of volatile compounds.

The negative result in the disc diffusion assay did not exclude the production of bioactive compounds by epiphytic bacteria. It may reflect the inappropriate use of ethyl acetate as the solvent in this study. Previous reports showed different efficiencies of various types of solvent for extraction of bioactive compounds from living organisms. Bimakr *et al.* (2011) compared four different solvent systems that were used to extract bioactive flavonoids from spearmint leaves. These included methanol, pure ethanol, 70% (v/v) ethanol and petroleum ether. The result showed that the highest yield (267.3 mg/g) was obtained from methanol extraction. The yield obtained from 70% ethanol extraction (257.6 mg/g) was higher than pure ethanol extraction (218 mg/g). The lowest extraction yield was found with petroleum ether (30.4 mg/g). Similarly, several types of solvent were tested for extraction of antioxidant compounds from two edible mushrooms *Lentinus edodes* and *Volvariella volvacea*. The lowest yield was found in ethyl acetate extraction of *L. edodes* (6% of crude extract) and *V. volvacea* (1%). The

highest yield of *L. edodes* (49%) was obtained from butanol extraction while the highest yield of *V. volvacea* (85%) was obtained from water extraction (Cheung and Cheng, 2005). Bacon *et al.* (2016) extracted antibacterial compounds from chili peppers using different concentrations of methanol. Antibacterial activity was tested against 15 strains of *Salmonella enteric*, *E. coli* and *Listeria monocytogenes*. The crude extract from 95% methanol extraction showed the inhibition against 14 of 15 pathogen strains while the extract obtained from 70% methanol extraction inhibited only six strains. In another previous report, hexane, ethanol and water were used for antimicrobial extraction from algae (*Synechocystis* sp. and *Himanthalia elongate)*. The highest bioactive compound yield was obtained from water extraction. Consistently, it also showed the highest antimicrobial activity against *E. coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*. The second highest yield was obtained from ethanol extraction but it showed the lowest antimicrobial activity. This indicated that the extraction yields were not always correlated with the inhibition activity (Plaza *et al.*, 2010).

Chapter 5
Conclusions and Suggestions

5.1 Conclusions

One-hundred-and-thirteen epiphytic bacteria were isolated from leaves, stems and roots of rice plants collected from four organic rice farms in Bangkok, Chonburi, Saraburi and Suphanburi, Thailand. Forty-seven isolates were obtained from roots, 31 isolates were obtained from stems and 35 isolates were obtained from leaves. Sixty-eight and 45 isolates were Gram-stained-positive and Gram-stained-negative, respectively. 106 isolates were rod-shaped while seven isolates were coccus. Partial 16S rRNA gene sequences were used for bacterial identification and classification. Epiphytic bacteria were identified as members of phyla *Firmicutes* (55%), *Proteobacteria* (38%) and *Bacteroidetes* (7%). All three phyla were found in roots and stems while only *Firmicutes* and *Proteobacteria* were obtained from leaves. All isolates were tested for their plant-growth-promoting activities. Thirty-six isolates were found positive for the nitrogen-fixing activity on glucose-nitrogen-free agar. Ten isolates showed the positive result of phosphate solubilization by the presence of the clear zone on PVK and NBRIP media. Forty-nine bacteria were tested positive for siderophore production as indicated by the orange halo around the colony on CAS agar. Thirty-one bacteria produced ACC deaminase and were able to grow on DF salt minimal agar containing ACC. Nineteen isolates exhibited IAA production according to the change of the supernatant color from yellow to pink after Salkowski's reagent was added. Seven epiphytic bacteria containing at least three plant-growth-promoting activities were selected for seedling assay but the positive effect on rice seedling biomass was not observed under the tested conditions. All epiphytic bacteria were also tested for their antagonistic activities against rice pathogens *X. oryzae* and *P. grisea*. The result showed that twenty-four isolates were able to inhibit the pathogen growth. Five isolates were able to inhibited *X. oryzae* and Twenty-three isolates were found positive for *P. grisea* growth suppression. Moreover, four isolates which were *Bacillus* sp. 1012, *Burkholderia* sp. 2310, *Chitinophaga* sp. 1303 and *Chitinophaga* sp. 1313 were able to inhibit both pathogens. Inhibition percentage

ranged from 10% to 100%. The highest inhibition percentage (36.5%) against *X. oryza* was obtained from *Chitinophaga* sp. 1303 while *Bacillus* sp. isolates 1021, 1110, 2211, 2306, 3210, 3308, 3310 and *Pseudomonas* sp. 2302 exhibited 100% inhibition against *P. grisea* . Bacteria containing antagonistic activities were tested for their lytic enzyme production. Among this, two isolates displayed chitinase activity and 19 isolates were tested positive for protease activity. All twenty-four isolates were used for well and disc diffusion assay. However, no isolates exhibited the positive result on these tests.

5.2 Suggestions

5.2.1 In seedling assay, the use of a single epiphytic bacterium was very different from the natural conditions where a number of bacteria co-colonized the same plant part. For future research, effects of combined epiphytic bacterial isolates should be investigated.

5.2.2 The negative results obtained from well and disc diffusion assay may indicate the absence of bioactive compounds in bacterial cultures. However, ethyl acetate was the only solvent used for extraction of bioactive compounds. The results may alternatively suggest that ethyl acetate was not an appropriate solvent. To confirm this speculation, other solvent systems should be tested for extraction of bioactive compounds.

References

Ahemad, M. and Khan, M.S. 2012. "Effects of pesticides on plant growth promoting traits of *Mesorhizobium* strain MRC4." *Journal of the Saudi Society of Agricultural Sciences*. 11 : 63–71.

Ahemad, M. and Kibret, M. 2014. "Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective." *Journal of King Saud University-Science*. 26 : 1-20.

Ahmad, F. Ahmad I. and Khan, M.S. 2008. "Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities." *Microbiological Research*. 163 : 173-181.

Ahmed, E. and Holmström, S.J.M. 2014. "Siderophores in environmental research: roles and applications." *Microbial Biotechnology*. 7 : 196-208.

Akhgar, A.R. Arzanlou M. Bakker, P. A. H. M. and Hamidpour, M. 2014. "Characterization of 1 aminocyclopropane-1-carboxylate (ACC) deaminase-containing *Pseudomonas* spp. in the Rhizosphere of Salt-Stressed Canola." *Pedosphere*. 24(4) : 461-468.

Ali, S.S. and Vidhale, N.N. 2013. "Bacterial siderophore and their application: A review." *International Journal of Current Microbiology and Applied Sciences*. 12 : 303-312.

Ali, S.Z. Sandhya, V. and Rao, L.V. 2014. "Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp." *Annals of Microbiology*. 64(2): 493-502.

Ambawade, M.S. and Pathade, G.R. 2013. "Production of indole acetic Acid (IAA) by *Stenotrophomonas maltophilia* BE25 isolated from roots of banana (*Musa* spp)." *International Journal of Science and Research*. 4 : 2644-2650.

Armstrong, D.L., editor. 1999. **Better Crops with Plant Food**. Norcross : Potash and Phosphate Institute (PPI).

Baca, B.E. and Elmerich, C. 2003. "Microbial Production of Plant Hormones." 113-143. in Elmerich, C. and Newton, W.E. **Associative and endophytic nitrogen-fixing bacteria and cyanobacterial Associations**. Netherlands : Kluwer Academic.

Bacon, K. Boyer, R. Denbow, C. O'Keefe, S. Neilson, A' and Williams, R. 2016. "Evaluation of different solvents to extract antibacterial compounds from jalapeño peppers." *Food Science and Nutrition*. DOI: 10.1002/fsn3.423.

Beighley, D.H. 2010. "Growth and production of rice." 35-68. in Verheye, W. H. Soils, **Plant Growth and Crop Production.** 2. Paris : Eolss Publishers Company Limited.

Berg, G. 2009. "Plant – microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture." *Applied Microbiology and Biotechnology*. 84 : 11-18.

Bhattacharyya, P.N. and Jha, D.K. 2012. "Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture." *World Journal of Microbiology Biotechnology*. 28 : 1327–1350.

Beneduzi, A. Moreira, F. Costa, P.B. Vargas, L.K. Lisboa, B.B. Favreto, R. Baldani, J.I. and Passaglia, L.M.P. 2013. "Diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the South of Brazil." *Applied Soil Ecology*. 63 : 94-104.

Beneduzi, A. Peres, D. Vargas, L.K. Bodanese-Zanettini, M.H. and Passaglia, L.M.P. 2008. "Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil." *Applied Soil Ecology*. 39 : 311-320.

Bimakr, M. Rahman, R.A. Taip, F.S. Ganjiloo, A. Salleh, L.M. Selamat, J. Hamid, A. and Zaidul, I.S.M. 2011. "Comparison of different extraction methods for the extraction of major bioactive flavonoid compounds from spearmint (*Mentha spicata* L.) leaves." *Food and Bioproducts Processing*. 89 : 67-72.

Bodenhausen, N. Horton, M.W. and Bergelson, J. 2013. "Bacterial Communities Associated with the Leaves and the Roots of *Arabidopsis thaliana*." *PLOS ONE* 8(2) : e56329.

Bradbury, J.F. 1984. "Genus II. *Xanthomonas* Dowson." 199-210. in Krieg, N.R. and Holt, J.G. **Bergey's manual of systematic bacteriology.** Baltimore: Williams and Wilkins.

Brandl, M.T. and Amundson, R. 2008. "Leaf age as a risk factor in the contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*." *Applied and Environment Microbiology*. 74: 2298–2306.

Butterworth, J. and McCartney, H.A. 1991. "The dispersal of bacteria from leaf surfaces by water splash." *Journal of Applied Microbiology*. 71(6): 484-496.

California Fertilizer Foundation (CFF). 2016. **Plant nutrients – nitrogen.** [Online]. Available : https://www.learnaboutag.org/resources/fact/nitrogen.pdf.

Castejón-Muñoz, M. 2008. "The effect of temperature and relative humidity on the airbone concentration of *Pyricularia oryzae* spores and the development of rice blast in southern Spain." *Spanish Journal of Agricultural Research*. 6 : 61-69.

Chakravorty, S. Helb, D. Burday, M. Connell, N. and Alland, D. 2007. "A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria." *Journal of Microbiological Methods*. 69(2) : 330-339.

Chithrashree. Udayashankar, A.C. Nayaka, S.C. Reddy, M.S. and Srinivas, C. 2011. "Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*." *Biological Control*. 59 : 114-122.

Cheung, L.M. and Cheung, P.C.K. 2005. "Mushroom extracts with antioxidant activity against lipid peroxidation." *Food Chemistry*. 89(3) : 403-409.

Choudhary, B. Nagpure, A. and Gupta, R. 2014. "Fungal cell-wall lytic enzymes, antifungal metabolite(s) production, and characterization from *Streptomyces exfoliatus* MT9 for controlling fruit-rotting fungi." *Journal of Basic Mircobiology*. 54 : 1295-1309.

Chung, E.J. Hossain, T. Khan, A. Kim, K.H. Jeon, C.O. and Chung, Y.R. 2015. "*Bacillus oryzicola* sp. nov., an endophytic bacterium isolated from the roots of rice with antimicrobial, plant growth promoting, and systemic resistance inducing activities in rice." *The Plant Pathology Journal*. 31(2) : 152-164.

Co, H. C. and Boosarawongse, R. 2007. "Forecasting Thailand's rice export: Statistical techniques vs. artificial neural networks." *Computers and Industrial Engineering*. 53 : 610-627.

Coenye, T. Vandamme, P. Govan, J.R.W. and LiPuma, J.J. 2001. "Taxonomy and identification of the *Burkholderia cepacia* complex." *Journal of Systematic and Evolutionary Microbiology*. 50: 2135-2139.

Coenye, T. and Vandamme, P. 2003. "Diversity and significance of *Burkholderia* species occupying diverse ecological niches." *Environmental Microbiology* 5: 719-729.

Commonwealth of Australia. 2015. **National Diagnostic Protocol for *Pyricularia oryzae***. [Online]. Available : http://plantbiosecuritydiagnostics.net.au/wordpress/wp-content/uploads/2016/03/NDP-14-P-Rice-Blast-Magnaporthe-V2.pdf.

Costa, L.E.O. Queiroz, M.V. Borges, A.C. Moraes, C.A. and Araújo, E.F. 2012. " Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (*Phaseolus vulgaris*)." *Brazilian Journal of Microbiology*. 43(4) : 1562-1575.

Cottyn, B. Debode, J. Regalado, E. Mew, T.W. and Swings, J. 2009. "Phenotypic and genetic diversity of rice seed-associated bacteria and their role in pathogenicity and biological control." *Journal of Applied Microbiology*. 107 : 885-897.

Daroub, S. H. and Snyder, G. H. 2007. "The Chemistry of Plant Nutrients in Soil" 1-7. in Datnoff, L. E. Elmer W. and H. Huber D. M. **Mineral Nutrition and Plant Disease.** Minnesota : American Phytopathological Society.

Dworkin, M. and Foster, J. 1958. "Experiments with some microorganisms which utilize ethane and hydrogen." *Journal of Bacteriology*, 75: 592-601.

Dinesh, R. Anandaraj, M. Kumar, A. Bini, Y.K. Subila, K.P. and Aravind, R. 2015. "Isolation, characterization, and evaluation of multi-trait plant growth promoting rhizobacteria for their growth promoting and disease suppressing effects on ginger." *Microbiological Research*. 173: 34-43.

Dutta, J. Handique, P.J. and Thakur, D. 2015. "Assessment of culturable tea rhizobacteria isolated from tea estates of Assam, India for growth promotion in commercial tea cultivars." *Frontiers in Microbiology*. 6 : 1252. DOI: 10.3389/fmicb.2015.01252.

Ehmann, A. 1977. "The Van Urk-Salkowski reagent-a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives." *Journal of Chromatography*. 132 : 267-276.

Esitken, A. Yildiz, H.E. Ercisli, S. Donmez, F.M. Turan, M. and Gumes, A. 2010. "Effects of plant growth promoting bacteria (PGPB) on yield, growth and nutrient contents of organically grown strawberry." *Scientia Horticulturae*. 124: 62-66.

European and Mediterranean Plant Protection Organization (EPPO). 2007. ***Xanthomonas oryzae.*** [Online]. Available : http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2338.2007.01162.x/full.

Farina, R Beneduzi, A. Ambrosini, A. Campos, S.B. Lisboa, B.B. Wendisch, V. Vargas, L.K. and Passaglia, L.M.P. 2011. "Diversity of plant growth-promoting rhizobacteria communities associated with the stages of canola growth." *Applied Soil Ecology*. 55 : 44-52.

Franche, C. LindstrÖm, and K. Elmerich, C. 2010. Nitrogen-fixing bacteria asscociated with leguminous and non- leguminous plants. 35-39. in Dessaux, Y. Hinsinger, P. Lemanceau, P. **Rhizosphere: Achievements and Challenges**. Dordrecht : Springer Netherlands.

Felsenstein, J. 1985. "Confidence limits on phylogenies: An approach using the bootstrap." *Evolution*. 39 : 783-791.

Gamalero, E. and Glick, B.R. 2011. "Mechanisms Used by Plant Growth-Promoting Bacteria." 17-43. in Maheshwari, D.K. **Bacteria in Agrobiology: Plant Nutrient Management**. Berlin Heidelberg : Springer.

Gashaw, G. Alemu, T. and Tesfaye, K. 2014. "Morphological, physiological and biochemical studies on *Pyricularia grisea* isolates causing blast disease on finger millet in Ethiopia." *Journal of Applied Biosciences*. 74 : 6059-6071.

Glass, A.D.M. 1989. **Plant nutrition: An introduction to current concepts**. Boston : Jones and Bartlett.

Glick, B.R. 2012. "Plant growth-promoting bacteria: Mechanisms and applications." *Scientifica*. 2012 : DOI: 963401.

Gnanamanickam, S.S. and Immanuel, J.E. 2007. "Epiphytic bacteria, their ecology and functions." 131-153. in Gnanamanickam, S.S. **Plant-Associated Bacteria**. Dordrecht : Springer.

Gnanamanickan, S.S. 2009. **Biological Control of Rice Disease**. Dordrecht : Springer Netherlands.

Goodlass, G. and Smith, K.A. 1979. "Effect of ethylene on root extension and nodulation of pea (*Pisum sativum* L.) and white clover (*Trifolium repens* L.)." 387–395. in Lambers, H. **Plant and Soil**. 51(3). Boston : Martinus Nijhoff.

Haas. D, and Keel, C. 2003. "Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance of biological control of plant disease." *Annual Reviews of Phytopathology*. 41 : 117–153.

Helm, D. and Chakraborty, R. 2003. "Structures of siderophore receptors." 261-287. in Winkelmann, G. **Microbial Transport Systems**. Wiley Online Library.

Hongoh, Y. Yuzawa, H. Ohkuma, M. and Kudo, T. 2003. "Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment." *FEMS Microbiology Letters*. 221 : 299-304.

Huang, H. Huang, M. Fan, G. Liu, Z. Wang, J. Duan, Q. and Zhang, Q. 2013. "Isolation and characterization of 1-aminocyclopropane- 1-carboxylate (ACC) deaminase-containing plant growth-promoting rhizobacteria from carnation soil and roots." *African Journal of Microbiology Research*. 50 : 5664-5668.

Huber, D.M. and Thompson , I.A. 2007. "Nitrogen and plant disease." 41-43. in Datnoff, L. E. Elmer W. and H. Huber D. M. **Mineral Nutrition and Plant Disease**. Minnesota : American Phytopathological Society.

International Rice Research Institute (IRRI). 2016. **Trends in global rice consumption**. [Online]. Available : http://irri.org/rice-today/trends-in-global-rice-consumption.

Janardhan, A. Kumar, A.P. Viswanath, B. Saigopal, D.V.R. and Narasimha, G. 2014. "Production of bioactive compounds by *Actinomycetes* and their antioxidant properties." *Biotechnology Research International*. 2014 : DOI: 10.1155/2014/217030

Janda, J.M. and Abbott, S.L. 2007. "16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls." *Journal of clinical microbiology*. 45(9) : 2761-2764.

Jacobs, JL. Carroll, TL. And Sundin, GW. 2005. "The role of pigmentation, ultraviolet radiation tolerance, and leaf colonization strategies in the epiphytic survival of phyllosphere bacteria." *Microbial Ecology*. 49(1): 104-113.

Ji, S.H. Gururani, M.A. and Chun, S. 2014. "Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars." *Microbiological Research*. 169 : 83-98.

Jones, M. Yacouba, S. Nwilene, F. Beye, A. Dobo, M. and Jalloh A. 2001 "*Oryza glaberrima* x *Oryza sativa* interspecifics for Africa." 137-149. in Rockwood, W. G. **Rice Research and Production in the 21st Century**. New York : International Rice Research Institute.

Kinkel, L.L. Wilson, M. and Lindow, S.E. 2000. "Plant species and plant incubation conditions influence variability in epiphytic bacterial population Size." *Microbial Ecology*. 39 : 1-11.

Kim, J. and Rees, D.C. 1994. "Nitrogenase and biological nitrogen fixation." *Biochemistry Journal*. 33(2) : 389–397.

Kimura, M. 1980. "A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences." *Journal of Molecular Evolution*. 16 : 111–120.

Khan, M.S. Zaidi, A. Wani, P.A. and Oves, M. 2009. "Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils." *Environmental Chemistry Letters*. 7 : 1–19.

Kloepper, J.W. and Schroth, M.N. 1978. "Plant growth-promoting rhizobacteria on radishes." 879–882. in . Station de Pathologie Ve´ge´tale et de Phytobacte´riologie. **Proceedings of the 4th International Conference on Plant Pathogenic Bacteria**. 2. Angers : Institut national de la recherché agronomique (INRA).

Kobayashi, T. and Nishizawa, N.K. 2012. "Iron uptake, translocation, and regulation in higher plants." *Annual Reviews of Plant Biology*. 63 : 131-52.

Kumar G. P. Desai, S. Amalraj, L. D. Ahmed, M. H. and Reddy, G. 2012. "Plant growth promoting *Pseudomonas* spp. from diverse agro-ecosystems of India for

Sorghum bicolor L." *Journal of Fertilizers and Pesticides*. S7 : 001. DOI:10.4172/2155-6202.S7-001.

Kuyek, D. 2000. **Blast, biotech and big business: Implications of corporate strategies on rice research in Asia.** [Online]. Available : https://www.grain.org/article/entries/36-blast-biotech-and-big-business-implications-of-corporate-strategies-on-rice-research-in-asia.

Leveau, J.H.J. and Lindow, S.E. 2005. "Utilization of the plant hormone indole-3-acetic acid for growth by *Pseudomonas putida* strain 1290." *Applied and Environmental Microbiology*. 71(5) : 2365-2371.

Leturque, H. and Wiggins, S. 2011. "Thailand's progress in agriculture: Transition and sustained productivity growth." **Development Progress**. London : Overseas Development Institute.

Lemtukei, D. Tamura, T. Nguyen, Q.T. and Ueno, M. 2017. "Inhibitory activity of *Burkholderia* sp. isolated from soil in Gotsu City, Shimane, against *Magnaporthe oryzae*." Ad*vances in Microbiology*. 137-148.

Li, X. Geng, X. Xie, R. Fu, L. Jiang, J. Guo, L. and Sun, J. 2016. "The endophytic bacteria isolated from elephant grass (*Pennisetum purpureum* Schumach) promote plant growth and enhance salt tolerance of hybrid *Pennisetum*." *Biotechnology for Biofuels*. 9:190.

Lindow, S. E. and Brandl, M. T. 2003. "Microbiology of the phyllosphere." *American Society for Microbiology*. 69(4) : 1875-1883.

Lin, Y. Du, D. Si, C. Zhao, Q. Li, Z. and Li, P. 2014. "Potential biocontrol *Bacillus* sp. strains isolated by an improved method from vinegar waste compost exhibit antibiosis against fungal pathogens and promote growth of cucumbers." *Biological Control*. 71 : 7-15.

Litwin, C.M. and Calderwood, S.B. 1993. "Role of iron in regulation of virulence genes." *Clinical Microbiology Reviews*. 6 : 137-149.

Lopez-Velasco, G. Carder, P.A. Welbaum, G.E. and Ponder, M.A. 2013. "Diversity of the spinach (*Spinacia oleracea*) spermosphere and phyllosphere bacterial communities." *FEMS Microbiology Letters*. 346 : 146-154.

Ma, J.F. 2005. "Plant root responses to three abundant soil minerals: silicon, aluminum and iron." *Critical Reviews in Plant Sciences*. 24(4) : 267–281.

Mao, D. Zhou, Q. Chen, C. and Quan, Z. 2012. "Coverage evaluation of universal bacterial primers using the metagenomic datasets." *BMC Microbiology*. 12 : 66.

Maidak, B.L. Olsen, G.J. Larsen, N. Overbeek, R. McCaughey, M. J. and Woese, C. R. 1997. "The RDP (Ribosomal Database Project)." *Nucleic Acids Research*. 25 : 109–110.

Maindad, D.V. Kasture, V.M. Chaudhari, H. Dhavale, D.D. Chopade, B.A. and Sachdev, D.P. 2014. "Characterization and fungal inhibition activity of siderophore from wheat rhizosphere associated *Acinetobacter calcoaceticus* strain HIRFA$_{32}$." *Indian Journal of Microbiology*. 54(3): 315-322.

Majeed, A. Abbasi, M. K. Hameed, S. Imran, A. and Rahim, N. 2015. "Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion." *Frontiers in Microbiology*. 6 : 198.

McCauley, A. Jones, C. and Jacobsen, J. 2003. "**Plant nutrient functions and deficiency and toxicity symptoms.**" [Online]. Available : http://landresources.montana.edu/nm/documents/NM9.pdf.

Mercier, J. and Lindow S.E. 2000. "Role of leaf surface sugars in colonization of plants by bacterial epiphytes." *Applied and Environmental Microbiology*. 66 : 369-374.

Mew, T.W. 1987. "Current status and future prospects of research on bacterial blight of rice." *Annual Review of Phytopathology*. 25: 359-82.

Mew, T.W. 1991. "Rice Diseases." 187-236. in Luh B.S. **Rice**. Volume I. Production/Volume II. Utilization. Philadelphia : Springer US.

Mew, T. W. Alvarez, A. M. Leach, J. E. and Swings, J. 1993. "Focus on bacterial blight of rice." *Plant Disease*. 77 : 5-11.

Miao, G. Jian-jiao, Z. En-tao, W. Qian, C. Jing, X. and Jian-guang, S. 2015. "Multiphasic characterization of a plant growth promoting bacterial strain, *Burkholderia* sp. 7016 and its effect on tomato growth in the field." *Journal of Integrative Agriculture*. 14(9): 1855-1863.

Mohite, B. 2013. "Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth." *Journal of Soil Science and Plant Nutrition*. 13(3) : 638-649.

Monteiro, A.M. Crozier, A. and Sanberg, G. 1988. "The biosynthesis and conjugation of indole-3-acetic acid in germinating seeds and seedlings of *Dalbergia dolichopetala*." *Planta*. 174(4) : 561-568.

Nailwal, S. Anwar, M.S. Budhani, K.K. and Nailwal, T.K. 2014. "*Burkholderia* sp. from rhizosphere of *Rhododendron arboretum*: Isolation, identification and plant growth promoting (PGP) activities." *Journal of Applied and Natural Science*. 6(2): 473-479.

Naqanandini, S. Balachandar, D. and Kumar, K. 2011. "Diversity analysis of *Pseudomonas* in rice rhizosphere for multifaceted plant growth promotion." *Acta Microbiologica Et Immunologica Hungarica*. 58(4) : 247-258.

Naveed, M. Mitter, B. Yousaf, S. Pastar, M. Afzal, M. and Sessitsch, A. 2013. "The endophyte *Enterobacter* sp. FD17: a maize growth enhancer selected based on rigorous testing of plant beneficial traits and colonization characteristics." *Biology and Fertility of Soils*. 50 : 249-262.

Niño-Liu, D.O. Ronald, P.C. and Bogdanove, A.J. 2006. "*Xanthomonas oryzae* pathovars: model pathogens of a model crop." *Molecular Plant Pathology*. 7(5) : 303-324.

Noda, T. and Kaku, H. 1999. "Growth of *Xanthomonas oryzae* pv. *oryzae* in planta and in guttation fluid of rice." *Annals of the Phytopathological Society of Japan*. 65 : 9–14.

Nowak-Thompson, B. Gould, S.J. Kraus, J. and Loper, J.E. 1994. "Production of 2, 4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5." *Canadian Journal Microbiology*. 40 : 1064–1066.

O'Brien, R.D. and Lindow, S. E. 1988. "Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria." *Phytopathological*. 79(5) : 619-627.

Office of Agricultural Economics (OAE). 2015. **Agricultural statistics of Thailand: crop year 2015.** [Online]. Available : http://www.oae.go.th/download/download_journal/2559/yearbook58.pdf.

Ohike, T. Makuni, K. Okanami, M. and Ano, T. 2013. "Screening of endophytic bacteria against fungal plant pathogens." *Journal of Environmental Sciences*. 25. Suppl 1 : S122-S126.

Onofre-Lemus, J. Herna´ndez-Lucas, I. Girard, L. and Caballero-Mellado, J. 2009. "ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, a widespread trait in *Burkholderia* species, and its growth-promoting effect on tomato plants." *Applied and Environmental Microbiology*. 75(20) : 6581-6590.

Orhan, F. 2016. "Alleviation of salt stress by halotolerant and halophilic plant growth-promoting bacteria in wheat (*Triticum aestivum*)." *Brazilian journal of microbiology*. 47: 621-627.

Osman, J.R. Fernandes, G. and DuBow, M.S. 2017. "Bacterial diversity of the rhizosphere and nearby surface soil of rice (*Oryza sativa*) growing in the Camargue (France)." *Rhizosphere*. 3: 112-122.

Ou, S.H. 1985. **Rice disease.** 2nd ed. Commonwealth Agricultural Bureau International Mycological Institute, United Kingdom : Farham House.

Patten, C.L. and Glick, B.R. 1996. "Bacterial biosynthesis of indole-3- acetic acid." *Canadian Journal of Microbiology*. 42(3) : 207–220.

Plantwise. 2012. **Rice blast.** [Online]. Available : http://www.plantwise.org/KnowledgeBank/FactsheetForFarmers.aspx?pan=20147801396.

Plaza, M. Santoyo, S. Jamie, L. Reina, G. Herrero, M. Señoráns, F.J. and Ibáñez, E. 2010. "Screening for bioactive compounds from algae." *Journal of Pharmeceutical and Biomedical Analysis*. 51 : 450-455.

Prashar, P. Sachdeva, S. and Kapoor, N. 2013. "Biocontrol of plant pathogens using plant growth promoting Bacteria." 319-360. in Lichtfouse, E. Sustainable **Agriculture Reviews**. Vol 12. Dordrecht : Springer Netherlands.

Rajkumar, M. Ae, N. Prasad, M.N.V. and Freitas, H. 2010. "Potential of siderophore-producing bacteria for improving heavy metal phytoextraction." *Trends in Biotechnology*. 28 : 142–149.

Ranganayaki, S. Mohan, C. and Ally, Z. 1981. "Effect of sodium molybdate on microbial fixation of nitrogen." *Microbiology*. 21(8) : 607-10.

Rangarajan, S. Saleen, L.M. Vasudevan, P. and Nair, S. 2002. "Biological suppression of rice diseases by *Pseudomonas* spp. under saline soil conditions." *Plant and soil*. 251 : 73-82.

Radzki, W. Gutierrez Mañero, F. Alqar, E. Lucas, G.J. García-Villaraco, A. and Ramos, S.B. 2013. "Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture." *Antonie Van Leeuwenhoek*. 104(3): 321-330.

Rashid, S. Charles, T.C. and Glick, B.R. 2012. "Isolation and characterization of new plant growth-promoting bacterial endophytes." *Applied Soil Ecology*. 61: 217-224.

Reche, M.H.L.R. and Fiuza, L.M. 2005. "Bacteria diversity in rice-field water in Rio Grande Do Sul." *Brazilian Journal of Microbiology*. 36 : 253-257.

Redford, A.J. Bowers, R.M. Knight, R. Linhart, Y. and Fierer, N. 2010. "The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves." *Environmental Microbiology*. 12(11): 2885-2895.

Reetha, S. Bhuvaneswari, G. Thamizhiniyan, P. and Mycin T.R. 2014. "Isolation of indole acetic acid (IAA) producing rhizobacteria of *Pseudomonas fluorescens* and *Bacillus subtilis* and enhance growth of onion *(Allim cepa.L)*." *International Journal of Current Microbiology* and *Applied* Sciences. 3(2) : 568-574.

Ren, J.J. Shi, G.L. Wang, X.Q. Liu, J.G. and Wang, Y.N. 2013. "Identification and characterization of a novel *Bacillus subtilis* strain with potent antagonistic activity of a flagellin-like protein." *World Journal of Microbiology and Biotechnology*. 29(12) : 2343– 2352.

Rodriguez, H. and Fraga, R. 1999. "Phosphate solubilizing bacteria and their role in plant growth promotion." *Biotechnology Advances*. 17 : 319-339.

Rokhbakhsh-Zamin, F. Sachdev, D. Kazemi-Pour, N. Engineer, A. Pardesi, K.R. Zinjarde, S. Dhakephalkar, P.K. and Chopade B.A. 2011. "Characterization of plant-growth-

promoting traits of *Acinetobacter* species Isolated from Rhizosphere of *Pennisetum glaucum*." *Journal of Microbiology and Biotechnology*. 21(6): 556-566.

Saitou, N. and Nei, M. 1987. "The neighbor-joining method: a new method for reconstructing phylogenetic trees." *Molecular Biology and Evolution*. 4 : 406-425.

Saraf, M. Jha, C.K. and Patel, D. 2010. "The role of ACC deaminase producing PGPR in sustainable agriculture." 365-385. in Maheshwari, D.K. **Plant Growth and Health Promoting Bacteria**. Berlin Heidelberg : Springer.

Schwyn, B. and Neilands, J.B. 1987. "Universal chemical assay for the detection and determination of siderophores." *Analytical Biochemistry*. 160 : 47–56.

Scuderi, G. Bonaccorsi, A. Panebianco, S. Vitale, A. Polizzi, G. and Cirvilleri, G. 2009. "Some strains of *Burkholderia gladioli* are potential candidates for postharvest biocontrol of fungal rots in citrus and apple fruits." *Journal of Plant Pathology*. 91 (1), 207-213.

Sesma, A. and Osbourn, A.E. 2004. "The rice blast pathogen undergoes developmental processes typical of root-infecting fungi." *Nature*. 431 : 582–586.

Sharma, T. and Rai, N. 2015. "Isolation of plant hormone (Indole-3-acetic acid) producing rhizobacteria and study on their effects on tomato (*Lycopersicum esculentum*) seedling." *International Journal of PharmTech Research*. 7 : 99-107.

Singh, R.P. and Jha, P.N. 2015. "Plant growth promoting potential of ACC deaminase rhizospheric bacteria Isolated from *Aerva javanica*: A Plant Adapted to Saline Environments." *International Journal of Current Microciology and Applied Sciences*. 4(7) : 142-152.

Sitepu, I.R. Hashidoko, V. Santoso, V. and Tahara V. 2013. "**Potential of phosphate-solubilizing bacteria isolated from dipterocarps grown in peat swamp forest in central Kalimantan and their possible utilization for biorehabilitation of degraded peatland.**" [Online]. Available : https://www.academia.edu/28634823/Potent PhosphateSolubilizing_Bacteria_Isolated_from_Dipterocarps_Grown_in_Peat_Swa

mp_Forest_in_Central_Kalimantan_and_Their_Possible_Utilization_for_Biorehabilitation_of_Degraded_Peatland.

Soil Science Society of America (SSSA). 2017. **Agricultural bacteria: Blowing in the wind.** [Online]. Available : http://www.the-scientist.com/?articles.view/articleNo/34164/title/Bacteria-Are-Blowing-in-the-Wind/.

Souza, R. Beneduzi, A. Ambrosini, A. Costa, P.B. Meyer, J. Vargas, L.K. Schoenfeld, R. and Passaglia, L.M.P. 2013. "The effect of plant growth-promoting rhizobacteria on the growth of rice (*Oryza sativa* L.) cropped in southern Brazilian fields." 585-603. in_Lambers, H. **Plant and Soil**. 366. Boston : Martinus Nijhoff.

Souza, R. Meyer, J. Schoenfeld, R. Costa, P.B. and Passaglia, L.M.P. 2014. "Characterization of plant growth-promoting bacteria associated with rice cropped in iron-stressed soils." *Annals of Microbiology*. 65(2) : 951-964.

Souza, R. Ambrosini, A. and Passaglia L.M.P. 2015. "Plant growth-promoting bacteria as inoculants in agricultural soils." *Genetics and Molecular Biology*. 38(4) : 401-419.

Stein, T. 2005. "*Bacillus subtilis* antibiotics: structures, synthesis and specific functions." *Molecular Microbiology*. 56(4) : 845–857.

Sulistiyani, TR. and Lisdiyanti, P. 2016. "Diversity of endophytic bacteria associated with *Curcuma heyneana* and their potency for nitrogen fixation." *Widyariset*. 2(2): 106-117.

Tang, J. Zhang, Y. Meng, H. Xue, Z. and Ma J. 2013. "Complete genome sequence of *Exiguobacterium* sp. strain MH3, isolated from rhizosphere of *Lemna minor*." *Genome Announcements*. 1(6) : Doi: 10.1128/genomeA.01059-13.

Teale, W.D. Paponov, I.A. and Palme, K. 2006. "Auxin in action: signaling, transport and the control of plant growth and development." *Molecular Cell Biology*. 7(11) : 847-859.

Thompson, J.D. Higgins, D.G. and Gibson, T.J. 1994. "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." *Nucleic Acids Research*. 22 : 4673-4680.

The Scientist. 2017. **Bacteria are blowing in the wind.** [Online]. Available : http://www.the-scientist.com/?articles.view/articleNo/34164/title/Bacteria-Are-Blowing-in-the-Wind/.

Tokpah, D.P. Li, H. Wang, L. Liu, X. Mulbah, Q.S. and Liu, H. 2016. "An assessment system for screening effective bacteria as biological control agents against *Magnaporthe grisea* on rice." *Biological control*. 103: 21-29.

Toribio-Jiménez, Jeiry. Rodríguez-Barrera, M.A. Hernández-Flores, G. Ruvalcaba-Ledezma, J.C. Castellanos-Escamilla, M. and Romero-Ramírez, Y. 2017. "Isolation and screening of bacteria from *Zea mays* plants gtowth promoters." *Revista internacional de contaminación ambiental*. 33: 143-150.

Tran, D.V. 1997. "World rice production: main issues and technical possibilities." 57-69. in Chataigner, J. **Cahiers Options Méditerranéennes**. 24(2). Montpellier : CIHEAM.

Trinkley, M. and Fick, S. 2003. **Rice cultivation, processing, and marketing in the eighteenth century**. Columbia : Chicora Foundation, Inc.

Uchida, R.S. 2000. "Essential Nutrients for Plant Growth: Nutrient Functions and Deficiency Symptoms." 31-55. in Silva, A.S. and Uchida R.S. **Plant Nutrient Management in Hawaii's Soils Approaches for Tropical and Subtropical Agriculture**. Hawii : College of Tropical Agriculture and Human Resources (CTAHR).

Ueno, M. Quyet, N.T. Shinzato, N. and Matsui, T. 2016. "Antifungal activity of collected in Subtropical Region, Okinawa, against *Magnaporthe oryzae*." *Tropical Agriculture and Development*. 60(1) : 48-52.

USDA Nutrient Database. 2016. **Nutrient data laboratory**. [Online]. Available : https://ndb.nal.usda.gov/ndb/search/list.

Van de Peer, Y. Chapelle, S. and De Wachter, R. 1996. "A quantitative map of nucleotide substitution rates in bacterial rRNA." *Nucleic Acids Research*. 24(17) : 3381-3391.

Velusamy, P. Immanuel, E. and Gnanamanickan, S. 2013. "Rhizosphere bacteria for biocontrol of bacterial blight and growth promotion of rice." *Rice Science*. 20(5) : 356-362.

Walworth, J. 2013. **Nitrogen in soil and the environment.** Arizona : College of Agriculture and Life Sciences, University of Arizona.

Woese, C.R. 1987. "Bacterial evolution." *Microbiological Reviews*. 51(2) : 221–271.

Weinert, N. Piceno, Y. Ding, G. Meincke, R. Heuer, H. Berg, G. Schloter, M. Andersen, G. and Smalla, K. 2011. "PhyloChip hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato cultivars: many common and few cultivar-dependent taxa." *Microbiology ecology*. 75(3): 497-506.

Xia, Y. Dreyer, J. Scott, D. and Willams, M.A. 2015. "Characterization of culturable bacterial endophytes and their capacity to promote plant growth from plants grown using organic or conventional practices." *Frontiers in Plant Science*. 490(6). DOI: 10.3389/fpls.2015.00490.

Yadav, S. Kaushik, R. Saxena, A.K. and Arora, D.K. 2011. "Diversity and phylogeny of plant growth-promoting bacilli from moderately acidic soil." *Journal of Basic Microbiology*. 51: 98-106.

Yasmin, S. Zaka, A. Imran, A. Zahid, M.A. Yousaf, S. Rasul, G. Arif, M. and Mirza, M.S. 2016. "Plant growth promotion and suppression of bacterial leaf blight in rice by inoculated bacteria." *PLOS ONE*. 18(1) : e0160688. DOI: 10.1371/journal.pone.0160688. eCollection2016.

Zahran, H.H. 2001. "Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology." *Journal of Biotechnology*. 91 : 143–153.

Zhao, J. Xue, Q.H. Niu, G.G. Xue, L. Shen, G.H. and Du, J.Z. 2013. "Extracellular enzyme production and fungal mycelia degradation of antagonistic *Streptomyces* induced by fungal mycelia preparation of cucurbit plant pathogens." *Annals of Microbiology*. 63 : 809–812.

Appendix

Appendix A

The components of the culture media

1. Culture media preparation

1.1 Nutrient agar (NA)

Peptone	5.0	gram
Beef extract	3.0	gram
Agar	15.0	gram
Distilled water	1,000.0	milliliter

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.2 Nutrient broth (NB)

Peptone	5.0	gram
Beef extract	3.0	gram
Distilled water	1,000.0	milliliter

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.3 Trypticase soy broth (TSB)

Casein peptone	15.0	gram
Soyabean meal	5.0	gram
Sodium chloride	5.0	gram
Distilled water	1,000.0	milliliter

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.4 Potato dextrose agar (PDA)

Potato	200.0	gram
Dextrose	20.0	gram
Agar	17.0	gram
Distilled water	1,000.0	milliliter

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.5 Glucose-nitrogen-free broth

Glucose	10.000	gram
Mgso4.7H2O	0.409	gram
NaCl	0.200	gram
K2HPO4	1.000	gram
CaCO3	1.000	gram
Na2MoO4.2H2O	0.006	gram
FeSO4.7H2O	0.100	gram
Distilled water	1,000.000	milliliter

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.6 Glucose-nitrogen-free agar

Glucose	10.000	gram
Mgso4.7H2O	0.409	gram
NaCl	0.200	gram
K2HPO4	1.000	gram
CaCO3	1.000	gram
Na2MoO4.2H2O	0.006	gram
FeSO4.7H2O	0.100	gram
Agar	9.0	gram

Distilled water	1,000.000	milliliter

*Note: $(NH_4)2SO_4$ was added for glucose-nitrogen-free agar supplied with nitrogen source

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.7 Pikovskaya's (PVK) medium

Glucose	10.0	gram
Calcium phosphate	5.0	gram
Ammonium sulfate	5.0	gram
Potassium chloride	0.2	gram
Magnesium sulfate heptahydrate	0.1	gram
Manganese sulfate	0.0002	gram
Ferrous sulfate heptahydrate	0.0002	gram
Yeast extracts	0.5	gram
Agar	15.0	gram
Distilled water	1,000.000	milliliter

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.8 National botanical research institute phosphate growth (NBRIP) medium

Glucose	10.0	gram
Calcium phosphate	5.0	gram
Ammonium sulfate	5.0	gram
Potassium chloride	0.2	gram
Magnesium sulfate heptahydrate	0.1	gram
Magnesium chloride hexahydrate	5.0	gram
Agar	15.0	gram
Distilled water	1,000.000	milliliter

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.9 Dowrkin Foster's (DF) medium

KH2 PO4	4.0	gram
Na2HPO4 .2H2O	6.0	gram
MgSO4. 7H2O	0.2	gram
Glucose	2.0	gram
Citric acid	2.0	gram
FeSO4.7H2O	0.001	gram
H3BO3	0.0001	gram
MnSO4.H2O	0.0011	gram
ZnSO4 .7H2O	0.0124	gram
CuSO4 .H2O	0.0078	gram
1-aminocyclopropane carboxylic acid	0.003	gram
Distilled water	1,000.000	milliliter

*Note: 2mM of $(NH_4)2SO_4$ was added for positive control and 1-aminocyclopropane carboxylic acid was removed for negative control

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.10 Colloidal Chitin Agar

Colloidal chitin	10.0	gram
Nutrient broth	13.0	gram
Agar	15.0	gram
Distilled water	1,000.0	milliliter
pH	7.0	

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.11 Skim milk Agar

Casein Enzyme Hydrolysate	5.0	gram
Yeast extract	2.5	gram
Dextrose	1.0	gram
Skim milk powder	28.0	gram
Agar	15.0	gram
Distilled water	1,000.0	milliliter

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

1.12 Hoagland medium

1M Calcium nitrate	5.0	milliliter
1M Potassium nitrate	5.0	milliliter
1M Magnesium sulfate	2.0	milliliter
1M Potassium dihydrogen phosphate	1.0	milliliter
Fe-EDTA	1.0	milliliter
Micronutriens	1.0	milliliter
Distilled water	1,000.0	milliliter
pH	5.6	

Mix all components into distilled water. Mix well and sterilize at 121°c (15 lbs in^{-2}) for 15 minute.

2. Chemical solution preparation

2.1 Stock preparation

Calcium nitrate 23.615 gram dissolved in 100 milliliter distilled water

Potassium nitrate 10.110 gram dissolved in 100 milliliter distilled water

Magnesium sulfate 24.65 gram dissolved in 100 milliliter distilled water

Potassium dihydrogen phosphate 13.61 gram dissolved in 100 milliliter distilled water

2.2 Fe-EDTA preparation

Sodium EDTA 4.07 gram dissolved in 30 milliliter distilled water

Iron (III) chloride 2.45 gram dissolved in 60 milliliter distilled water

Mix well and bring up to the final volume of 100 milliliter

2.3 Micronutrients preparation

Boric acid	0.286	gram
Copper (II) chloride	0.005	gram
Manganese (II) chloride	0.181	gram
Zinc chloride	0.011	gram
Sodium molybdate	0.0025	gram

Mix well and bring up to the final volume of 100 milliliter

2.4 Chrome azural s; CAS

Chrome azural s	60.5	gram
Distilled water	1,000.0	milliliter

2.5 Hexadecyl trimethyl ammonium bromilde; HDTMA

HDTMA	72.9	gram
Distilled water	40.0	milliliter

2.6 Salkowski's reagent

Ferric chloride	2.0	milliliter
Perchloric acid	100.0	milliliter

2.6 0.5 McFarland standard 1%

Sulfuric acid	99.5	milliliter
Barium chloride	0.5	milliliter

Store at room temperature under dark condition

Appendix B

Appendix Table B1 Morphological characterization of epiphytic bacteria.

Isolates	Gram reaction	Cell shape	Cell morphology			
			Color	Size	Surface	Margin
1001	-	Rod	Non-pigment	Small	Smooth	Entire
1002	+	Rod	Yellow	Small	Smooth	Entire
1003	+	Rod	Creamy white	Big	Dry	Lobate
1004	-	Short rod	Non-pigment	Big	Moist	Entire
1005	-	Rod	Creamy white	Small	Smooth	Entire
1008	+	Rod	White	Big	Dry	Lobate
1009	+	Rod	Creamy white	Big	Dry	Lobate
1010	+	Rod	White	Big	Dry	Lobate
1011	+	Rod	Yellowish-orange	Big	Smooth	Entire
1012	+	Rod	White	Big	Wrinkled	Lobate
1013	+	Rod	Creamy white	Big	Dry	Lobate
1014	+	Rod	White	Big	Dry	Lobate
1015	-	Bacilli	Yellow	Small	Moist	Entire
1017	-	Rod	Yellow	Small	Moist	Entire
1018	+	Rod	Creamy white	Big	Dry	Lobate
1019	+	Rod	Creamy white	Big	Dry	Lobate
1020	-	Rod	Non-pigment	Small	Smooth	Entire
1021	+	Rod	Creamy white	Big	Mucoid	Entire
1024	+	Rod	Creamy white	Big	Dry	Lobate
2001	+	Rod	White	Big	Dry	Lobate

Isolates	Gram reaction	Cell shape	Cell morphology			
			Color	Size	Surface	Margin
2002	-	Bacilli	Yellow-cream	Big	Smooth	Entire
2003	+	Rod	White	Big	Dry	Lobate
2004	+	Rod	Yellow-cream	Big	Moist	Undulate
2006	+	Short rod	Non-pigment	Big	Moist	Entire
2007	+	Cocci	Yellow-cream	Small	Smooth	Entire
2008	+	Rod	White	Big	Dry	Lobate
2009	+	Short rod	Non-pigment	Big	Moist	Entire
2011	+	Short rod	Non-pigment	Big	Moist	Entire
3002	+	Rod	Yellowish-orange	Big	Smooth	Entire
3005	+	Cocci	Yellow with opaque	Big	Smooth	Entire
3006	+	Rod	Pink	Big	Moist	Undulate
3008	+	Bacilli	Yellowish-orange	Small	Moist	Entire
1104	-	Bacilli	Yellow	Small	Moist	Entire
1105	-	Bacilli	Yellow	Small	Moist	Entire
1109	+	Rod	Yellow-cream	Big	Smooth	Entire
1110	+	Rod	White	Big	Dry	Entire
1111	-	Rod	Ivory	Big	Mucoid	Entire
2101	-	Rod	Ivory	Big	Mucoid	Entire
2106	+	Short rod	Non-pigment	Big	Moist	Entire
3102	+	Rod	White	Big	Dry	Lobate
3103	+	Rod	White	Big	Smooth	Entire
1201	-	Rod	Red	Small	Smooth	Entire

Isolates	Gram reaction	Cell shape	Cell morphology			
			Color	Size	Surface	Margin
1202	+	Cocci	Yellow with opaque	Big	Smooth	Entire
1203	-	Rod	White	Big	Mucoid	Curled
1204	+	Rod	Creamy white	Big	Dry	Lobate
1205	+	Rod	Dark yellow	Small	Smooth	Entire
1206	-	Rod	Non-pigment	Small	Smooth	Entire
1207	-	Rod	Creamy white	Big	Moist	Entire
1208	-	Rod	White	Small	Moist	Entire
1209	-	Rod	Yellowish-brown	Small	Smooth	Entire
1210	+	Rod	Creamy white	Big	Dry	Lobate
1211	-	Rod	Non-pigment	Small	Mucoid	Entire
2201	+	Cocci	Yellow with opaque	Big	Smooth	Entire
2202	-	Rod	Purple	Small	Smooth	Entire
2206	-	Rod	Ivory	Small	Mucoid	Entire
2208	-	Rod	White	Small	Moist	Entire
2209	+	Rod	Creamy white	Big	Mucoid	Entire
2211	+	Rod	Creamy white	Big	Mucoid	Entire
2212	+	Rod	White	Big	Dry	Lobate
2213	-	Rod	Non-pigment	Small	Smooth	Entire
2214	-	Rod	Purple	Small	Smooth	Entire
3203		Rod	Brow	Big	Wrinkled	Undulate
3204	-	Rod	Brow	Big	Wrinkled	Undulate
3205	-	Rod	White	Big	Dry	Lobate
3207	+	Cocci	Yellow with	Big	Smooth	Entire

Isolates	Gram reaction	Cell shape	Cell morphology			
			Color	Size	Surface	Margin
			opaque			
3208	+	Rod	Non-pigment	Big	Moist	Entire
3209	+	Rod	White	Big	Dry	Lobate
3210	+	Rod	Creamy white	Big	Mucoid	Entire
3211	+	Rod	Creamy white	Big	Mucoid	Entire
3212	+	Rod	Creamy white	Big	Dry	Lobate
3213	+	Rod	Creamy white	Big	Mucoid	Entire
3214	+	Rod	Yellow-cream	Big	Smooth	Entire
3215	+	Rod	Creamy white	Big	Dry	Lobate
3217	-	Rod	Brown	Big	Wrinkled	Undulate
3218	-	Rod	Brown	Big	Wrinkled	Undulate
3219	+	Rod	Creamy white	Big	Dry	Lobate
3222	+	Rod	Pink	Big	Moist	Undulate
3223	+	Rod	Pink	Big	Moist	Undulate
1302	-	Rod	White	Small	Moist	Entire
1303	-	Rod	Yellow	Small	Moist	Entire
1304	-	Rod	Non-pigment	Small	Moist	Entire
1307	+	Rod	Creamy white	Big	Dry	Lobate
1308	+	Rod	White	Big	Dry	Lobate
1309	+	Rod	White	Big	Wrinkled	Lobate
1310	-	Rod	Yellow	Small	Moist	Entire
1311	+	Rod	White	Big	Dry	Lobate
1312	+	Rod	White	Big	Wrinkled	Lobate
1313	-	Rod	Yellow	Small	Moist	Entire
1314	+	Rod	White	Big	Dry	Lobate
1316	-	Rod	White	Small	Moist	Entire
2301	-	Rod	White	Big	Mucoid	Curled

Isolates	Gram reaction	Cell shape	Cell morphology			
			Color	Size	Surface	Margin
2302	-	Rod	Brown	Big	Wrinkled	Undulate
2303	-	Bacilli	Yellow	Small	Smooth	Entire
2306	+	Rod	Creamy white	Big	Mucoid	Entire
2307	-	Bacilli	White	Big	Mucoid	Curled
2308	-	Rod	White	Big	Mucoid	Curled
2309	-	Rod	Non-pigment	Small	Moist	Entire
2310	-	Rod	Greenish	Small	Moist	Entire
2311	-	Rod	Creamy white	Small	Smooth	Undulate
2312	+	Rod	Creamy white	Big	Mucoid	Entire
2313	+	Rod	Creamy white	Big	Mucoid	Entire
3301	-	Rod	White	Small	Smooth	Entire
3304	-	Rod	Brown	Big	Wrinkled	Undulate
3306	+	Cocci	Yellow with opaque	Big	Smooth	Entire
3307	+	Rod	Creamy white	Big	Mucoid	Entire
3308	+	Rod	Creamy white	Big	Mucoid	Entire
3309	-	Rod	Brown	Big	Wrinkled	Undulate
3310	+	Rod	Creamy white	Big	Mucoid	Entire
3311	-	Rod	Brown	Big	Wrinkled	Undulate
3312	+	Rod	White	Big	Dry	Lobate
3313	+	Rod	Pink	Big	Moist	Undulate
3314	+	Rod	White	Big	Smooth	Entire
3315	+	Rod	White	Big	Dry	Lobate

Appendix Table B2 The pairwise-alignment analysis of the 16S rRNA gene sequences of epiphytic bacteria using EzBioCloud database.

Isolates	Closely-related Bacteria	Similarity (%)	Completeness (%)
1001	*Pseudomonas otitidis* MCC10330^T (AY953147)	99.73	77
1002	*Fictibacillus halophilus* AS8^T (KP265300)	100	55.5
1003	*Bacillus megaterium* NBRC 15308^T (JJMH01000057)	99.91	76
1004	*Acinetobacter soli* CIP 110264^T (APPU01000012)	99.63	73.4
1005	*Aquitalea pelogenes* P1297^T (KC178611)	99.91	65.5
1008	*Bacillus altitudinis* 41KF2b^T (ASJC01000029)	100	65
1009	*Fictibacillus rigui* WPCB074^T (WPCB074)	100	34.7
1010	*Bacillus aryabhattai* B8W22^T (EF114313)	100	65
1011	*Bacillus indicus* LMG 22858^T (JGVU01000003)	99.86	47
1012	*Bacillus safensis* FO-36b^T (ASJD01000027)	100	93.8
1013	*Bacillus megaterium* NBRC 15308^T (JJMH01000057)	100	49.7
1014	*Bacillus altitudinis* 41KF2b^T (ASJC01000029)	100	69.1
1015	*Chryseobacterium bernardetii* NCTC 13530^T (JX100816)	99.2	34.9
1017	*Xanthomonas melonis* LMG 8670^T (Y10756)	99.81	36.3
1018	*Bacillus megaterium* NBRC 15308^T (JJMH01000057)	99.91	71.4
1019	*Fictibacillus rigui* WPCB074^T (EU939689)	100	68.7
1020	*Pseudomonas otitidis* MCC10330^T (AY953147)	99.73	77.0
1021	*Bacillus siamensis* KCTC 13613^T (AJVF01000043)	100	34
1024	*Bacillus megaterium* NBRC 15308^T	99.93	93.8

Isolates	Closely-related Bacteria	Similarity (%)	Completeness (%)
	(JJMH01000057)		
2001	*Bacillus aryabhattai* B8W22^{T} (EF114313)	99.82	75.2
2002	*Sphingobacterium multivorum* IAM14316^{T} (AB100738)	99.25	64.4
2003	*Bacillus altitudinis* 41KF2b^{T} (ASJC01000029)	99.93	93.8
2004	*Bacillus koreensis* DSM 16467^{T} (LILC01000014)	100	64.3
2006	*Acinetobacter soli* CIP 110264^{T} (APPU01000012)	100	33.7
2007	*Staphylococcus sciuri* DSM 20345^{T} (AJ421446)	100	49.8
2008	*Bacillus altitudinis* 41KF2b^{T} (ASJC01000029)	99.1	77.2
2009	*Acinetobacter soli* CIP 110264^{T} (APPU01000012)	100	70.8
2011	*Acinetobacter soli* CIP 110264^{T} (APPU01000012)	100	36.6
3002	*Bacillus indicus* LMG 22858^{T} (JGVU01000003)	99.9	70.2
3005	*Staphylococcus arlettae* ATCC 43957^{T} (AB009933)	99.9	66.5
3006	*Bacillus oryzaecorticis* R1^{T} (KF548480)	99.81	70.4
3008	*Brevundimonas vesicularis* NBRC 12165^{T} (KF548480)	99.25	75.9
1104	*Chryseobacterium bernardetii* NCTC 13530^{T} (JX100816)	99.7	69.1
1105	*Chryseobacterium bernardetii* NCTC 13530^{T} (JX100816)	99.13	71.7

Isolates	Closely-related Bacteria	Similarity (%)	Completeness (%)
1109	*Bacillus marisflavi* JCM 11544^{T} (LGUE01000011)	100	93.6
1110	*Bacillus amyloliquefaciens* DSM^{T} (FN597644)	99.27	47.5
1111	*Klebsiella pneumoniae* subsp. pneumoniae DSM 30104^{T} (AJJI01000018)	99.36	95.8
2101	*Klebsiella pneumoniae* subsp. Ozaenae ATCC 11296^{T} (Y17654)	99.52	70.9
2106	*Acinetobacter soli* CIP 110264^{T} (APPU01000012)	99.91	74.1
3102	*Bacillus altitudinis* 41KF2b^{T} (ASJC01000029)	100	64.5
3103	*Bacillus haikouensis* C-89^{T} (KJ868191)	98.85	83.1
1201	*Serratia marcescens* subsp. marcescens ATCC 13880^{T} (JMPQ01000005)	99.89	60.7
1202	*Staphylococcus arlettae* ATCC 43957^{T} (AB009933)	100	55.2
1203	*Enterobacter tabaci* YIM Hb-3^{T} (KP990658)	99.55	61.3
1204	*Bacillus megaterium* NBRC 15308^{T} (JJMH01000057)	99.81	71.6
1205	*Exiguobacterium indicum* HHS31^{T} (AJ846291)	99.72	72.1
1206	*Aeromonas taiwanensis* LMG 24683^{T} (CDDD01000060)	100	48.6
1207	*Pseudomonas plecoglossicida* NBRC 103162^{T} (BBIV01000080)	100	48.6
1208	*Burkholderia gladioli* NBRC 13700^{T} (BBJG01000151)	99.2	59.7
1209	*Vogesella alkaliphila* JC141^{T} (HE819389)	100	60.8
1210	*Bacillus siamensis* KCTC 13613^{T}	99.82	76.1

Isolates	Closely-related Bacteria	Similarity (%)	Completeness (%)
	(AJVF01000043)		
1211	*Aeromonas taiwanensis* LMG 24683^{T} (CDDD01000060)	99.6	67.6
2201	*Staphylococcus arlettae* ATCC 43957^{T} (AB009933)	99.88	54.7
2202	*Chromobacterium pseudoviolaceum* CCM 2076^{T} (AJ871128)	99.79	63.8
2206	*Klebsiella pneumoniae* subsp. pneumoniae DSM 30104^{T} (AJJI01000018)	99.71	94.5
2208	*Burkholderia gladioli* NBRC 13700^{T} (BBJG01000151)	99.9	68
2209	*Bacillus siamensis* KCTC 13613^{T} (AJVF01000043)	99.9	60
2211	Bacillus *siamensis* KCTC 13613^{T} (AJVF01000043)	99.9	65.4
2212	*Bacillus aryabhattai* B8W22^{T} (EF114313)	99.9	65.4
2213	*Citrobacter youngae* GTC 1314^{T} (AB273741)	99.06	94
2214	*Chromobacterium pseudoviolaceum* CCM 2076^{T} (AJ871128)	100	70.6
3203	*Pseudomonas psychrotolerans* DSM 15758^{T} (FMWB01000061)	99.9	67.3
3204	*Pseudomonas psychrotolerans* DSM 15758^{T} (FMWB01000061)	99.51	70.4
3205	*Bacillus aryabhattai* B8W22^{T} (EF114313)	100	67

Isolates	Closely-related Bacteria	Similarity (%)	Completeness (%)
3207	*Staphylococcus arlettae* ATCC 43957^{T} (AB009933)	99.93	96.5
3208	*Acinetobacter soli* CIP 110264^{T} (APPU01000012)	99.63	73.7
3209	*Bacillus altitudinis* 41KF2b^{T} (ASJC01000029)	99.42	47.4
3210	*Bacillus siamensis* KCTC13613^{T} (AJVF01000043)	100	73.6
3211	*Bacillus siamensis* KCTC13613^{T} (AJVF01000043)	99.91	73.6
3212	*Bacillus velezensis* CR-502^{T} (AY603658)	99.86	98.5
3213	*Bacillus siamensis* KCTC13613^{T} (AJVF01000043)	99.64	95.1
3214	*Bacillus marisflavi* JCM 11544^{T} (LGUE01000011)	99.79	64
3215	*Bacillus megaterium* NBRC15308^{T} (JJMH01000057)	100	40.22
3217	*Pseudomonas psychrotolerans* DSM 15758^{T} (FMWB01000061)	100	40.2
3218	*Pseudomonas psychrotolerans* DSM 15758^{T} (FMWB01000061)	99.01	63.7
3219	*Fictibacillus rigui* WPCB074^{T} (EU939689)	100	39.3
3222	*Bacillus oryzaecorticis* R1^{T} (KF548480)	99.9	70.7
3223	*Bacillus oryzaecorticis* R1^{T} (KF548480)	98.96	85.2

Isolates	Closely-related Bacteria	Similarity (%)	Completeness (%)
1302	*Rahnella aquatilis* CIP 78.65^{T} (CP003244)	99.63	70
1303	*Chitinophaga eiseniae* DSM 22224^{T} (jgi.1055333)	99.32	40.3
1304	*Pandoraea pnomenusa* DSM 16536^{T} (CP009553)	100	72.9
1307	*Bacillus velezensis* CR-502^{T} (AY603658)	99.93	96.9
1308	*Bacillus cereus* ATCC 14579^{T} (AE016877)	99.9	71.1
1309	*Bacillus safensis* FO-36b^{T} (ASJD01000027)	100	93.9
1310	*Chitinophaga eiseniae* DSM 22224^{T} (jgi.1055333)	100	74.4
1311	*Bacillus altitudinis* 41KF2b^{T} (ASJC01000029)	100	68.2
1312	*Bacillus safensis* FO-36b^{T} (ASJD01000027)	100	68.2
1313	*Chitinophaga eiseniae* DSM 22224^{T} (jgi.1055333)	99.38	89.2
1314	*Bacillus altitudinis* 41KF2b^{T} (ASJC01000029)	98.85	87.7
1316	*Burkholderia vietnamiensis* LMG 10929^{T} (CP009631)	99.77	59.6
2301	*Enterobacter tabaci* YIM Hb-3^{T} (KP990658)	100	39
2302	*Pseudomonas psychrotolerans* DSM 15758^{T} (FMWB01000061)	99.39	67.4
2303	*Chryseobacterium endophyticum* CC-YTH209^{T} (KU358716)	99.63	93
2306	*Bacillus siamensis* KCTC13613^{T} (AJVF01000043)	99.93	93.9
2307	*Enterobacter tabaci* YIM Hb-3^{T} (KP990658)	99.78	62.7

Isolates	Closely-related Bacteria	Similarity (%)	Completeness (%)
2308	*Enterobacter tabaci* YIM Hb-3^{T} (KP990658)	100	55
2309	*Pseudomonas indoloxydans* IPL-1^{T} (DQ916277)	98.5	63.8
2310	*Burkholderia cenocepacia* LMG 16656^{T} (JTDP01000003)	99.89	60.6
2311	*Kinneretia asaccharophila* KIN192^{T} (AY136099)	99.6	50.8
2312	*Bacillus siamensis* KCTC 13613^{T} (AJVF01000043)	100	61.7
2313	*Bacillus siamensis* KCTC 13613^{T} (AJVF01000043)	100	94.2
3301	*Roseateles depolymerans* KCTC 42856^{T} (CP013729)	99.69	66.2
3304	*Pseudomonas psychrotolerans* DSM 15758^{T} (FMWB01000061)	99.71	47.1
3306	*Staphylococcus arlettae* ATCC 43957^{T} (AB009933)	100	75.6
3307	*Bacillus siamensis* KCTC 13613^{T} (AJVF01000043)	99.08	66.3
3308	*Bacillus siamensis* KCTC 13613^{T} (AJVF01000043)	99.74	52.2
3309	*Pseudomonas psychrotolerans* DSM 15758^{T} (FMWB01000061)	99.49	67.5
3310	*Bacillus siamensis* KCTC 13613^{T} (AJVF01000043)	99.57	95.4
3311	*Pseudomonas psychrotolerans* DSM 15758^{T} (FMWB01000061)	99.3	88.4
3312	*Bacillus siamensis* KCTC 13613^{T}	99.71	94.2

Isolates	Closely-related Bacteria	Similarity (%)	Completeness (%)
	(AJVF01000043)		
3313	*Bacillus oryzaecorticis* R1^{T} (KF548480)	99.89	62.3
3314	*Bacillus haikouensis* C-89^{T} (KJ868191)	99.5	79.6
3315	*Bacillus altitudinis* 41KF2b^{T} (ASJC01000029)	99.93	99.93

Appendix Table B3 Classification of epiphytic bacteria.

Phylum	Genus	Isolate
Firmicutes	*Bacillus* sp.	1003, 1008, 1010, 1011, 1012, 1013, 1014, 1018, 1021, 1024, 2001, 2003, 2004, 2008, 3002, 3006, 3008, 1109, 1110, 3102, 3103, 1204, 1210, 2209, 2211, 2212, 3205, 3209, 3210, 3211, 3212, 3213, 3214, 3215, 3222, 3223, 1307, 1308, 1309, 1311, 1312, 1314, 2306, 2312, 2313, 3307, 3308, 3310, 3312, 3313, 3314, 3315
	Exiguobacterium sp.	1205
	Fictibacillus sp.	1002, 1009, 1019, 3219
	Staphylococcus sp.	2007, 3005, 1202, 2201, 3207, 3306
Proteobacteria	*Acinetobacter* sp.	1004, 2006, 2009, 2011, 2106, 3208
	Aeromonas sp.	1206, 1211
	Aquitalea sp.	1005
	Brevundimonas sp.	3008
	Burkholderia sp.	1208, 2208, 1316, 2310
	Citrobacter sp.	2213
	Chromobacterium sp.	2202, 2214
	Enterobacter sp.	1203, 2301, 2307, 2308,
	Kinneretia sp.	2311
	Klebsiella sp.	1111, 2101, 2206
	Pandoraea sp.	1304
	Pseudomonas sp.	1001, 1020, 1207, 3203, 3204, 3217, 3218, 2302, 2309, 3304, 3309, 3311
	Rahnella sp.	1302
	Roseateles sp.	3301
	Serratia sp.	1201
	Vogesella sp.	1209

Phylum	Genus	Isolate
	Xanthomonas sp.	1017
Bacteroidetes	*Chitinophaga* sp.	1303, 1310, 1313
	Chryseobacterium sp.	1015, 1104, 1105, 2303
	Sphingobacterium sp.	2002

Appendix Table B4 Plant-growth-promoting activities of epiphytic bacteria isolates.

Isolate	IAA production	phosphate solubilization	siderophore production	ACC deaminase production	Nitrogen fixation
1001	+	-	+	-	-
1002	-	-	-	-	+
1003	-	-	-	-	-
1004	+	-	-	-	-
1005	-	-	-	-	-
1008	-	-	+	-	+
1009	-	-	+	-	-
1010	-	-	-	-	+
1011	-	-	-	-	+
1012	-	-	-	+	-
1013	-	-	-	-	-
1014	-	-	-	+	-
1015	-	-	+	-	-
1017	-	-	-	-	-
1018	-	-	-	-	-
1019	-	-	-	-	-
1020	-	-	-	-	-
1021	-	-	-	-	-
1024	-	-	-	-	+
2001	-	-	+	-	-
2002	-	-	+	+	-
2003	-	-	+	-	-
2004	-	-	+	-	-
2006	+	-	+	+	-
.2007	-	-	-	-	+
2008	-	-	+	+	-

Isolate	IAA production	phosphate solubilization	siderophore production	ACC deaminase production	Nitrogen fixation
2009	-	-	-	+	+
2011	-	-	-	-	+
3002	-	-	-	-	+
3005	-	-	-	-	+
3006	-	-	-	-	+
3008	-	-	-	-	+
1104	-	-	-	-	+
1105	-	-	-	-	-
1109	-	-	-	+	+
1110	-	-	-	-	-
1111	+	-	-	-	-
2101	+	-	+	-	-
2106	-	-	-	+	+
3102	-	-	-	-	+
3103	-	-	-	-	+
1201	-	-	+	+	-
1202	-	-	+	-	-
1203	+	-	-	+	-
1204	-	-	+	-	-
1205	-	-	+	+	+
1206	-	-	+	-	-
1207	+	-	+	-	-
1208	-	-	-	-	-
1209	-	-	-	-	-
1210	-	-	+	-	-
1211	-	-	+	-	+
2201	-	-	+	-	-

Isolate	IAA production	phosphate solubilization	siderophore production	ACC deaminase production	Nitrogen fixation
2202	-	-	-	-	-
2206	-	-	+	-	+
2208	-	+	-	-	-
2209	-	-	+	-	-
2211	+	-	+	+	-
2212	-	-	+	-	-
2213	-	-	-	-	-
2214	-	-	-	-	-
3203	-	+	-	-	-
3204	-	+	-	-	+
3205	-	-	-	-	-
3207	-	-	-	-	-
3208	+	-	+	-	-
3209	-	-	+	+	+
3210	-	-	-	-	-
3211	-	-	-	-	-
3212	-	-	+	-	-
3213	-	-	+	-	-
3214	-	-	+	-	+
3215	-	-	-	-	+
3217	-	-	-	-	+
3218	-	+	-	+	+
3219	-	-	-	-	+
3222	+	-	+	-	-
3223	-	-	+	-	+
1302	+	-	-	-	-
1303	-	-	+	-	-

Isolate	IAA production	phosphate solubilization	siderophore production	ACC deaminase production	Nitrogen fixation
1304	+	-	+	-	+
1307	-	-	+	-	-
1308	-	-	+	+	+
1309	-	-	+	+	-
1310	-	-	-	-	+
1311	-	-	+	-	+
1312	-	-	+	-	-
1313	-	-	+	-	-
1314	-	-	-	-	-
1316	-	-	+	+	-
2301	+	-	-	+	-
2302	-	-	+	-	-
2303	-	-	-	-	-
2306	-	-	+	-	-
2307	+	-	-	-	-
2308	-	-	-	-	-
2309	+	-	-	+	-
2310	+	+	+	+	-
2311	-	+	+	-	-
2312	-	+	+	+	-
2313	-	-	+	+	-
3301	-	+	-	+	-
3304	+	-	-	+	-
3306	+	-	-	-	+
3307	-	-	+	+	-
3308	-	-	+	+	-
3309	-	-	-	+	-

Isolate	IAA production	phosphate solubilization	siderophore production	ACC deaminase production	Nitrogen fixation
3310	-	-	+	-	-
3311	-	-	-	+	+
3312	-	-	-	+	+
3313	-	+	-	-	+
3314	-	+	-	+	-
3315	-	-	-	+	-

*Note

+ : Positive result

- : Negative result.

Appendix Table B5 Epiphytic bacterial isolates with antagonistic effects against *X. oryzae* and *P. grisea* by the dual culture method.

Isolate	Antagonistic effects	
	X. oryzae	*P. grisea*
1001	-	-
1002	-	-
1003	-	-
1004	-	-
1005	-	-
1008	-	-
1009	-	-
1010	-	-
1011	-	-
1012	+	+
1013	-	-
1014	-	-
1015	-	-
1017	-	-
1018	-	-
1019	-	-
1020	-	-
1021	-	+
1024	-	-
2001	-	-
2002	-	-
2003	-	-
2004	-	-
2006	-	-
2007	-	-
2008	-	+

Isolate	Antagonistic effects	
	X. oryzae	*P. grisea*
2009	-	-
2011	-	-
3002	-	-
3005	-	-
3006	-	-
3008	-	-
1104	-	-
1105	-	-
1109	-	-
1110	-	+
1111	-	-
2101	-	-
2106	-	-
3102	-	+
3103	-	-
1201	-	-
1202	-	-
1203	-	-
1204	-	-
1205	-	-
1206	-	-
1207	-	-
1208	-	+
1209	-	-
1210	-	-
1211	-	-
2201	-	-
2202	-	-

Isolate	Antagonistic effects	
	X. oryzae	*P. grisea*
2206	-	-
2208	-	+
2209	-	-
2211	-	+
2212	-	-
2213	-	-
2214	-	-
3203	-	-
3204	-	-
3205	-	-
3207	-	-
3208	-	-
3209	-	+
3210	-	+
3211	-	-
3212	-	-
3213	-	-
3214	-	-
3215	-	-
3217	-	-
3218	-	-
3219	-	-
3220	-	-
3222	-	+
3223	-	-
1302	-	-
1303	+	+
1304	-	-

Isolate	Antagonistic effects	
	X. oryzae	*P. grisea*
1307	-	-
1308	-	-
1309	-	-
1310	+	-
1311	-	-
1312	-	-
1313	+	+
1314	-	+
1316	-	+
2301	-	-
2302	-	+
2303	-	-
2306	-	+
2307	-	-
2308	-	-
2309	-	-
2310	+	+
2311	-	-
2312	-	+
2313	-	-
3301	-	-
3304	-	-
3306	-	-
3307	-	-
3308	-	+
3309	-	-
3310	-	+
3311	-	-

Isolate	Antagonistic effects	
	X. oryzae	*P. grisea*
3312	-	+
3313	-	-
3314	-	-
3315	-	+

*Note

+ : Positive result

- : Negative result.

Appendix Table B6 Percentage of *X. oryzae* growth inhibited by epiphytic bacteria obtained from dual culture method.

Isolate	*X. oryzae* growth inhibition (%)
Control	0.00±0.00
Bacillus sp. 1012	15.50±2.60
Burkholderia sp. 2310	10.00±0.00
Chitinophaga sp. 1303	36.50±3.84
Chitinophaga sp. 1310	20.50±3.57
Chitinophaga sp. 1313	12.50±4.33

*Note : Control was *X. oryzae* streaked on NA plates without epiphytic bacteria

Appendix Table B7 Percentage of *P. grisea* growth inhibited by epiphytic bacteria obtained from dual culture method.

Isolate	*P. grisea* growth inhibition (%)
Control	0.00±0.00
Bacillus sp. 1012	87.50±1.80
Bacillus sp. 1021	100.00±0.00
Bacillus sp. 1110	100.00±2.10
Bacillus sp. 1314	87.50±1.10
Bacillus sp. 2008	79.40±2.10
Bacillus sp. 2211	100.00±0.00
Bacillus sp. 2306	100.00±0.00
Bacillus sp. 2312	98.12±1.10
Bacillus sp. 3102	90.62±2.10
Bacillus sp. 3209	84.40±2.10
Bacillus sp. 3210	100.00±0.00
Bacillus sp. 3222	83.12±1.10
Bacillus sp. 3308	100.00±0.00
Bacillus sp. 3310	100.00±0.00
Bacillus sp. 3312	82.50±0.00

Isolate	*P. grisea* growth inhibition (%)
Bacillus sp. 3315	73.75±1.25
Burkholderia sp. 1208	90.00±1.80
Burkholderia sp. 1316	86.25±1.25
Burkholderia sp. 2208	88.12±1.10
Burkholderia sp. 2310	74.37±1.10
Chitinophaga sp. 1303	73.10±3.24
Chitinophaga sp. 1313	73.75±2.17
Pseudomonas sp. 2302	100.00±0.00

*Note : Control was *P. grisea* streaked on NA plates without epiphytic bacteria

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