SOIL BACTERIA CAPABLE OF DESTROYING STRUCTURE OF CASSAVA MEALY BUGS

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แบคทีเรียในดินที่สามารถทำลายโครงสร้างของเพลี้ยแป้งมันสำปะหลัง

นางสาวพัชริดา คำภูเมือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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SOIL BACTERIA CAPABLE OF DESTROYING STRUCTURE OF CASSAVA MEALY BUGS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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พัชริดา คำภูเมือง : แบคทีเรียในดินที่สามารถทำลายโครงสร้างของเพลี้ยแป้งมันสำปะหลัง (BACTERIA IN SOIL CAPABLE OF DESTROYING STRUCTURE OF CASSAVA MEALY BUGS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุรีลักษณ์ รอดทอง, 155 หน้า.

เพลี้ยแป้งทำลายพืชโดยดูดกินน้ำเลี้ยงจากทุกส่วนของพืชทำให้พืชเหี่ยวตาย เป็นแมลงศัตรูพืชที่สำคัญของมันสำปะหลัง การควบคุมเพลี้ยแป้งมันสำปะหลังโดยอาศัยแบคทีเรียในดินมีแนวโน้มเป็นวิธีที่ประหยัดและยั่งยืน จากการศึกษาโครงสร้างและส่วนประกอบทางเคมีของเพลี้ยแป้งที่เป็นสารอาหารของแบคทีเรีย และทดสอบความเป็นไปได้ของแบคทีเรียสายพันธุ์ที่คัดเลือกในการทำลายโครงสร้างของเพลี้ยแป้งมันสำปะหลังที่พบในพื้นที่ที่มีการทำลายพืชในภาคตะวันออกเฉียงเหนือของประเทศไทย ชนิดเพลี้ยแป้งสีชมพู (*Phenacoccus manihoti* Matile-Ferrero) ซึ่งพบมากกว่าอีก 3 ชนิด ที่พบคือ เพลี้ยแป้งสีเขียว (*Phenacoccus maderiensis* Green) เพลี้ยแป้งแจ๊คเบียดเลย์ (*Pseudococcus jackbeardsleyi* Gimpel & Miller) และเพลี้ยแป้งลาย (*Ferrisia virgata* Cockerel) ลำตัวของเพลี้ยแป้งสีชมพูที่ศึกษา มีชั้นคิวติเคิลหนาประมาณ 3-4 ไมโครเมตร มีช่องเปิดที่แบคทีเรียสามารถบุกรุก ได้แก่ ช่องบริเวณด้านบนของส่วนหัวและส่วนท้ายลำตัว (Ostioles) ช่องของต่อมสร้างไขที่พบกระจายอยู่ทั่วไปตามผนังลำตัว ช่องเปิดบริเวณส่วนท้อง (Circulus) และรูหายใจ (Spiracles) มีเส้นผ่าศูนย์กลาง 2-59 2-7 17-20 และ 19-30 ไมโครเมตร ตามลำดับ ตัวเพลี้ยแป้งมีปริมาณคาร์บอนทั้งหมด ไนโตรเจนทั้งหมด และความชื้นโดยเฉลี่ยร้อยละ 46.35 6.74 และ 77.30 โดยน้ำหนักแห้ง ตามลำดับ จากการคัดแยกแบคทีเรียในดินได้จำนวน 416 ไอโซเลต และคัดเลือกจากลักษณะทางสัณฐานวิทยาของเซลล์ ความสามารถในการสร้างเอนไซม์โปรติเอส ไลเปส และไคติเนส และการเจริญในอาหารเหลวที่เตรียมจากเพลี้ยแป้งได้จำนวน 8 ไอโซเลต ที่ให้รหัสคือ PCSMA53 CCSMA59 SSTBA15 SSTBA16 SSSMA5 SSTBA26 KBSMA65 และ KSSMA80 เมื่อเตรียมเชื้อที่คัดเลือกในสารละลายน้ำเกลือปลอดเชื้อให้ได้ปริมาณ 10^8 เซลล์ต่อมิลลิลิตร ฉีดพ่นด้วยปริมาตร 2 มิลลิลิตร บนลำตัวเพลี้ยแป้ง 40 ตัว ที่เกาะบนยอดมันสำปะหลังคลอบคลุมพื้นที่ 16 ตารางเซนติเมตร และฉีดพ่นสารละลายแขวนลอยของแบคทีเรียปริมาตรเท่ากันบนเพลี้ยแป้ง 40 ตัว ที่กระจายอยู่ในดิน (ชุดดินโคราช) และได้รับอาหารจากใบมันสำปะหลัง คลอบคลุมพื้นที่ 50 ตารางเซนติเมตร ทำการทดลอง 3 ซ้ำ และใช้สารละลายน้ำเกลือปลอดเชื้ออย่างเดียวฉีดพ่นเพลี้ยแป้งสำหรับชุดควบคุม ควบคุมสภาวะทดลองที่อุณหภูมิ 25-28 องศาเซลเซียส ความชื้นสัมพัทธ์ร้อยละ 70 เป็นเวลา 5 วัน พบว่ามีการตายของเพลี้ยแป้งจากการฉีดพ่นด้วยแบคทีเรียไอโซเลต PCSMA53 CCSMA59 SSTBA15 SSTBA16 SSSMA5 SSTBA26 KBSMA65 และ KSSMA80 จากที่อาศัยบนยอดมันสำปะหลังร้อยละ 81.67 70.0 38.33

35.84 40.0 23.35 28.33 และ 18.38 ตามลำดับ และที่อยู่ในดินร้อยละ 92.50 90.83 80.0 64.17 57.50 50.85 44.18 และ 27.50 ตามลำดับ ที่มีความแตกต่างอย่างมีนัยสำคัญ ($P < 0.05$) จากกล้องจุลทรรศน์แบบส่องกราดพบเซลล์แบคทีเรียจำนวนมากบริเวณช่องเปิดบริเวณด้านบนของส่วนหัวและส่วนท้ายลำตัว และช่องเปิดของต่อมสร้างเส้นไขตามผิวลำตัว เมื่อศึกษาสมบัติทางสรีรวิทยาของแบคทีเรียจำนวน 8 ไอโซเลต PCSMA53 CCSMA59 SSTBA15 SSTBA26 SSTBA16 KSSMA80 KBSMA65 และ SSSMA5 พบความหลากหลายของสกุลของแบคทีเรีย จำนวน 4 สกุล คือ Pseudomonas Serratia Chromobacterium และ Chryseobacterium จากนั้นนำมาวิเคราะห์เพื่อหาลำดับนิวคลีโอไทด์ของ 16S ribosomal RNA gene และเมื่อเปรียบเทียบความเหมือนกับข้อมูลจากฐานข้อมูล GenBank มีความเหมือนกับ *Pseudomonas aeruginosa* KVD14-MG *Serratia marcescens* 7/18r *Chromobacterium pseudoviolacium* LMG3953 *Chryseobacterium gleum* ALR-8 *Chromobacterium piscinae* LMG3947 *Pseudomonas fluorescens* DKP1 *Chryseobacterium indologenes* MUT2 และ *Chromobacterium piscinae* LMG3947 ร้อยละ 98.0 96.8 99.3 99.7 98.3 99.8 99.2 และ 96.6 ตามลำดับ

สาขาวิชาชีววิทยา
ปีการศึกษา 2558

ลายมือชื่อนักศึกษา พัชริดา คำเมือง
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

PATCHARIDA KHUMPUMUANG : BACTERIA IN SOIL CAPABLE OF DESTROYING STRUCTURE OF CASSAVA MEALY BUGS. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 155 PP.

CASSAVA MEALYBUGS/SOIL BACTERIA/ STRUCTURE OF MEALY BUGS

Mealy bug is important pest insect of cassava, could destroy plant by suck to eat the nutrient from every the part of the plant were depressed plant dies. The control cassava mealy bugs by soil bacteria have tendency economical and sustainable methods. From the study of structures and chemical components of cassava mealy bugs that could serve as nutrients of bacteria and the possibility of using selected isolates of soil bacteria for destroying structures of cassava mealy bug, that destroy the plant in Northeastern Thailand. Found species, pink mealy bug (*Phenacoccus manihoti* Matile-Ferrero) higher than three species, green mealy bug (*Phenacoccus maderiensis* Green), jack beard mealy bug (*Pseudococcus jackbeardsleyi* Gimpel & Miller), and striped mealy bug (*Ferrisia virgata* Cockerel). Pink mealy bugs were studied, have cuticle layer size, 3-4 μm. Found the structure of open pores can destroy by using soil bacteria such as anterior and posterior pores (ostioles), several pores for producing wax filament, abdomen pores (circulus) and respiration pores (spiracles) have diameter 2-59, 2-7, 17-20, and 9-30 μm, respectively. Chemical compositions of the body of mealy bug found total carbon, total nitrogen, and moisture content 46.35, 6.74, and 77.30% by dry basis, respectively. A total of 416 bacterial isolates from soil samples were collected and screened by morphological characteristic, producing protease lipase and chitinase enzymes, and can grow in cassava mealy bug broth. Eight isolates of PCSMA53, CCSMA59, SSTBA15, SSTBA16, SSSMA5, SSTBA26,

KBSMA65, and KSSMA80, after sprayed 2 ml of bacterial suspension (10^8 cells/ml) with 0.85% normal saline on third instar stages of forty cassava mealy bug feed on peak of cassava stem, the area 16 square centimeter and compared with in soil, the area 50 square centimeter, 70 humidity at 25-28 ºC for 5 days (triplicate test). Found structure that bacteria can be destroyed mealy bug such as anterior and posterior pores (ostioles), and several pores for producing wax filament on the body. Found the motility of mealy bug on soil 92.5, 90.83, 80.0, 64.17, 57.5, 50.85, 44.18, and 27.50%, respectively and compared with on cassava, 81.67, 70.0, 38.33, 35.84, 40.0, 23.35, 28.33, and 18.38%, respectively, these were significantly ($p>0.05$). Eight bacterial isolates, PCSMA53, CCSMA59, SSTBA15, SSTBA26, SSTBA16, KSSMA80, KBSMA65, and SSSMA5, from physiological characterization, four genera: Pseudomonas, Serratia, Chromobacterium and Chryseobacterium were classified. Then representative isolates of these bacteria were selected for 16S ribosomal RNA gene. When compared to sequences from GenBank database, had 98.0, 96.8, 99.3, 99.7, 98.3, 99.8, 99.2 and 96.6% similarity to *Pseudomonas aeruginosa* KVD14-MG, *Serratia marcescens* 7/18r, *Chromobacterium pseudoviolacium* LMG3953, *Chryseobacterium gleum* ALR-8, *Chromobacterium piscinae* LMG3947, *Chryseobacterium indologenes* MUT2, *Pseudomonas fluorescens* DKP1, and *Chromobacterium piscinae* LMG3947, respectively.

School of Biology

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LIST OF ABBREVIATIONS

ANOVA	Analysis of varience
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
α	Alpha
BLAST	Basic local alignment search tool
bp	Base pair
ºC	Degree Celsius
CCA	Colloidal chitin agar
CFU	Colony forming unit
CO_2	Carbon dioxide gas
Co., Ltd.	Limited company
cm	Centrimeter
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example

LIST OF ABBREVIATIONS (Continued)

et al.	et alia (and others)
g	Gram
(m, μ) g	(milli, micro) Gram
h	Hour
ha	Hectare
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
M	Molar
MMB	Mealy bug broth
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
NCBI	National Center for Biotechnological Information
ng	Nanogram
nm	Nanometer
OD	Optical density
%	Percentage
pM /pmol	Picomolar
PCR	Polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

RNA	Ribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecylsulphate
sec	Second
SEM	Scanning electron microscopy
SMA	Skim milk agar
sp.	Species
T_m	Melting temperature
TBA	Tributyrin agar
U	Unit
U.K.	United Kingdom
µl	Microliter
µm	Micrometer
U.S.A.	United States of America
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

Bacteria are normally the most abundant microbes in soil. Populations of bacteria can boom or bust in soil in a few days according to response to changes in soil moisture, soil temperature or nutrients particularly carbon substrate. In this way, some bacterial species can suppress other disease-causing microorganisms. Some can play an important role in decomposition of organic materials, especially in the early stages of decomposition when moisture levels are high (Christian *et al*., 2004). For destroying insects, the cuticle is considered to function as a protective barrier against desiccation as well as microbial attack, and is the major barrier against absorption of foliar applied agrichemicals. Bacterial chitinase has been reported. The principal bacteria found are *Bacillus* spp., (*Bacillus cereus, Bacillus amyloliquefaciens*), *Penibacillus* sp., *Pseudomonas* spp., (e.g. *Pseudomonas aeruginosa*), and *Serratia* sp. Secretion of chitinase and antibiotics by *Bacillus* sp. facilitates the utilization of chitin as a carbon source by the bacterium (Guelder *et al*., 1988; Dahiya *et al*., 2005; Chae *et al*., 2006). Bacterial chtinases of *Streptomyces scabies* and *Pseudomonas putida* are well-characterized in molecular weight, amino acid composition, and carbohydrate content (Dutta *et al*., 2009; Sebastian *et al*., 1987; Sebastian and Kolattukudy, 1988). Proteins are another main component of animals. Although a wide range of microorganisms are known to date to produce proteases, a large proportion of the commercially available alkaline proteases are derived from *Bacillus* strains because

of its ability to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures (Jenal and Fuchs, 1998).

Cassava, a root crop from which tapioca is made, is one of the major crops for the Thai economy. Not only is cassava a food crop, it is also used as animal feeds and raw materials for a number of industries. The plant is one of the important crops in Northeastern Thailand. Mealy bugs cause severe damage to the cassava in Northeastern (Chiamchamnanja, 1999). The cassava mealy bugs, *Phenacoccus manihoti* Matile-Ferrero, is a native of Central South America and it devastated cassava when inadvertently introduced to Congo in early 1970s, and from there, it spread to the rest of cassava growing areas in the continent of Africa (Neuenschwander, 2003). The bug is oligophagous and feeds on plants in several families but it prefers cassava. It colonizes tender shoots and causes distortion and cessation of leaf production. In 2009, *P. manihoti* was reported in Thailand, Cambodia and Laos, when was the first introduction to Asia (Winotai *et al*., 2010) and in Indonesia in 2010 (Muniappan *et al*., 2011). There are several species of cassava mealy bugs being found, including stripe mealy bugs (Ferrisia virgata), Jack-beardsley mealy bugs (*Pseudococcus jackbeardsleyi*), Green's mealy bugs (*Phenacoccus madeirensis*), and Pinkish cassava mealy bugs *(Phenacoccus manihoti).* *Phenacoccus manihoti* and *Ferrisia virgata* have now spread to cassava fields in more than 25 provinces of Thailand, especially in Nakhon Ratchasima, Lopburi and Saraburi. The severe damages to the shoots can be easily seen in the field (Chiamchamnanja, 1999). The spread of cassava mealy bugs to about 200,000 hectares has been confirmed in eastern and northeastern Thailand, where the pest is

causing yield losses as high as 50 percent. Since the country's cassava industry generates more than 1.5 billion of income each year and the overall Thai cassava industry is worth 3 billon reductions of that magnitude could translate into hundreds of millions of dollars in economic losses, especially if the pest is allowed to spread further (Limsakda and Limsila, 1994). The control measures are generally by chemical control as well as others. Malathion is an organophosphate insecticide frequently used in the home garden because it is relatively safe to apply, and its residues disappear quickly (Thapinta and Hudak, 2000). Chemical control is economically not viable, due to the crop's low yield, long cycle, and to the limited resources of producers (Tanticharoen, 2004). These chemical may contaminate the environment and pose short-term and long-term effect on health of organisms in the surrounding area, including humans (Sematong, 2008). The use of biological control is another alternative method and being done as it was successfully applied in other countries (Roy *et al*., 1996). After a lengthy foreign exploration, the *Anagyrus lopezi* De Santis (Hymenoptera: Encyrtidae) was found in Paraguay, Brazil and Bolivia. This parasitoid was imported to International Institute of Tropical Agriculture (IITA) at Benin and starting 1981 releases were made throughout equatorial Africa. It took about two years to reduce the population of the mealy bugs since the release of the parasitoid in each site. A stock rearing colony of the parasitoid *A. lopezi* was imported from IITA Benin into Thailand in 2009. It was multiplied and field released in the East, Northeast and Central plain region of the country (Neuenschwander, 2003). Several tactics are used in the control of cassava mealy bugs, such as: resistant cultivars (Nukenine *et al*., 2000), cultural practices, and entomopathogens, such as the fungus, *Beauveria bassiana* and *Metarhizium flavoviride* have the potential use as

biological control agents against mealy bugs because they were relatively safe on non target insects, such as natural enemies and beneficial soil insects, but fungus have specific condition, they slowly destroy insect pest and require knowledge as using (Milner, 1997; Thungrabeab and Tongma, 2007). Environmental safe methods for controlling cassava mealy bugs are still needed. This research aims to investigate soil bacteria that can destroy structure of cassava mealy bugs. These bacteria will be useful for further application in agriculture.

1.1 Research objectives

1.2.1 To investigate soil bacteria that can destroy structure of cassava mealy bugs.

1.2.2 To study structure and some chemical components of cassava mealy bugs that could serve as nutrients.

1.2.3 To determine the possibility of using selected isolates of soil bacteria for killing cassava mealy bugs.

1.2 Scope and limitation of the study

Cassava mealy bugs and soil samples will be collected from at least 10 cassava plantation areas in Northeastern Thailand, where the cassava devastation has been recently reported. Structures and chemical component of the cassava mealy bugs that could be served as bacterial nutrients, will be analyzed. Bacteria producing enzymes to digest the crucial structure of cassava mealy bugs will be isolated, selected, and identified by their morphological and biochemical characteristics as well as their 16S

ribosomal RNA gene sequences. The possibility of using selected isolates of soil bacteria for killing cassava mealy bugs will be preliminary investigated.

1.3 Expected results

The following results are expected: bacteria isolated from soil that can destroy structure of cassava mealy bugs, chemical components of cassava mealy bugs that could serve as bacterial nutrients, data of physiological and biochemical characteristics of selected bacterial isolates that could destroy cassava mealy bugs structures, and the possibility of using the selected isolates of soil bacteria for killing cassava mealy bugs. These data will be useful for further application of these soil bacteria in agriculture.

CHAPTER II

LITERATURE REVIEW

2.1 Cassava mealy bugs

Cassava mealy bugs are pest insects covered with white waxy secretions occurs on the undersurfaces of cassava leaves, shoot tips, petioles, and stems (Meyer *et al*., 2008). Cassava bug damage symptoms include shortened internode lengths, compression of terminal leaves together into "bunchy tops", distortion of stem portions and defoliation. It is major pests of a wide range of agricultural, horticultural and ornamental plants worldwide (Miller *et al*., 2002).

2.1.1 Classification of cassava mealy bugs

Cassava mealy bugs were classified in the Pseudococcidae family and member of the Sternorrhyncha (formerly Homoptera) order. They are so called due to the thin to-thick mealy or cottony wax secretion covering the insects (Gavrilov, 2004). The entomologic classification of mealy bugs:

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Hemoptera

Suborder: Sternorrhyncha

Family: Pseudococcidae

Most basic information on plant mealy bug interactions during the last decade has come from research on the cassava, *Manihot esculenta* Crantz (Euphorbiaceae) system with two mealy bug species, namely *Phenacoccus manihoti* and *Phenacoccus herreni*. Both these Pseudococcidae species cause severe damage to cassava. Several Pseudococcidae species have been found on cassava and on other species (Calatayud and Ru, 2006).

2.1.2 Life cycle of cassava mealy bugs

Cassava mealy bugs (Hemiptera, Coccoidea, Pseudococcidae) are plant sap-sucking insects that constitute a family with about 2,000 species, some of which are major pests of agricultural plants. They feed by inserting their thin, lonmouthparts through the plant tissue to suck up phloem sap. Female mealy bugs are soft, often elongate or oval, and usually attached to plant surfaces. Frequently they are covered with a mealy or cottony wax secretion. Males are short lived and rarely seen or, in some species, are absent. Most mealy bug species lay eggs, and most have sexual reproduction, life cycle of mealy bugs show in (Figure 1) (Kono *et al*., 2008; James *et al*., 2000). Mealy bugs are generally located on the underside of the cassava canopy leaves (Figures 2.2) and at low density inside growing tips. With increasing density, they spread over the entire plant. *Phenacoccus manihoti* has four developmental instars. *Phenacoccus manihoti* has four developmental instars *Phenacoccus manihoti* has four developmental instars during its life cycle, producing only females (thelytokous parthenogenesis) (Figure 2.2a). The adult female lays up to 500 eggs in an ovisac. The first instar is the most mobile stage and is responsible for plant colonization within the same cultivated plot. The entire life cycle from egg to adult takes about 21 days. *Phenacoccus herreni* is bisexual, with a strong sexual

dimorphism after the second instar (Figure 2.2b). During the third and fourth instars the males complete development to winged adults inside a cocoon. Similar to *P. manihoti*, adult females laid their eggs inside an ovisac. In this species, the first instar is also the most mobile stage. The life cycle between egg and adult is 21 days (Calatayud and Ru, 2006).

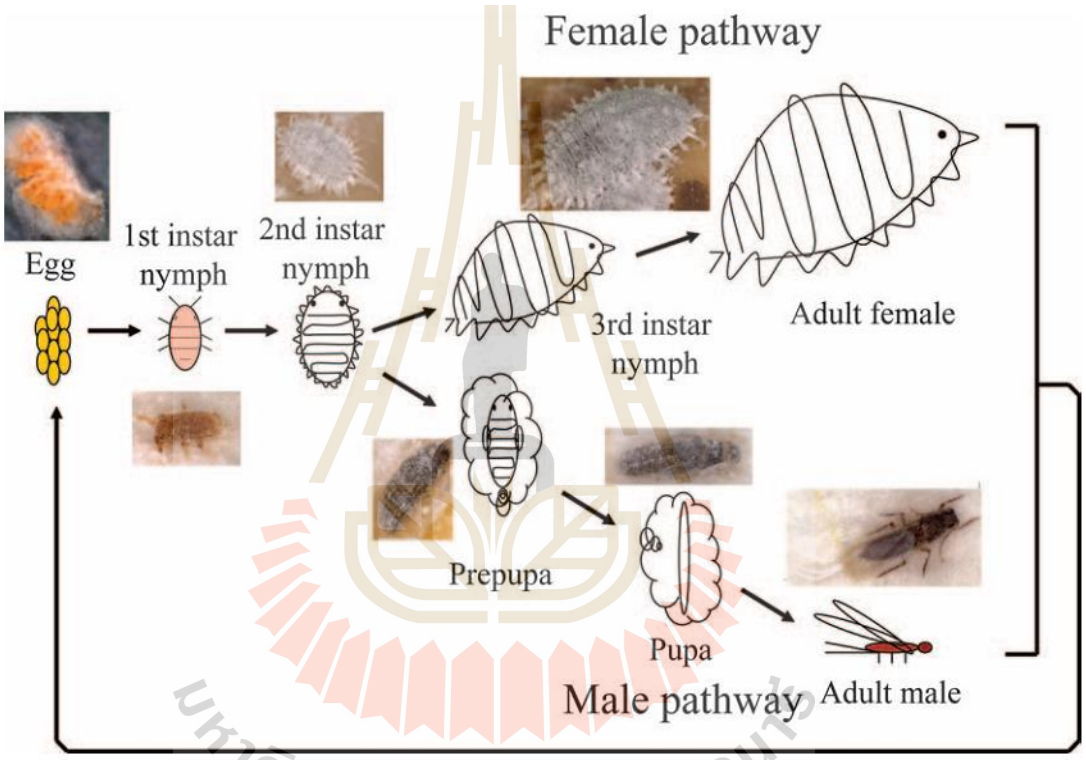


Figure 2.1 Life cycle of mealy bugs, mealy bugs has four developmental instars *Phenacoccus manihoti* has four developmental instars during its life cycle, producing only females.

Source: Kono *et al*. (2008).

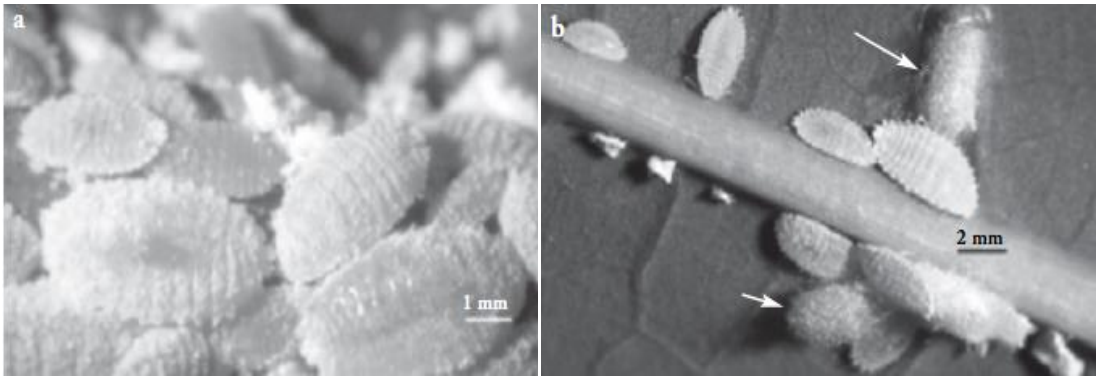


Figure 2.2 Mealy bugs reproduction. In case of *Phenacoccus mannihoti*, only females are produced (a). In *Phenacoccus herreni*, the males develop inside a cocoon (b).

Source: Calatayud and Ru (2006).

The female and immature young tend to hang out under the leaves of an assortment of plants, while the males have wings and are more active (Nassar *et al*., 2008). Mealy bugs begin life as an egg. The eggs are contained in an egg sac that the female deposits on the underside of a plant leaf where they will be left to mature. The eggs hatch and the first stage of the mealy bugs, known as a crawler or first instar, travels in search of food (Vincent, 2009). They will disperse along the host tree or can be transferred to other trees by hitching a ride on an unsuspecting animal. Second-instar mealy bugs also have the capability to move around, though most prefer to remain sheltered under leaves. It is at this point that the male and female mealy bugs differ in their development. Male mealy bugs will create cocoons and undergo traditional metamorphosis to emerge as winged adults at strategic intervals to ensure successful mating. Females will transition from second instar to third instar before ultimately molting into an adult, skipping metamorphosis. The mealy bug larvae will

mature into adults in about 21 days, when reproduction commences almost immediately (Winotai *et al.*, 2010). The males will generally only live for two to three days, and the females will remain alive for up to three weeks, allowing them to mature their eggs and lay them before they die. Most of the eggs are laid within 10 days to two weeks of fertilization. Mealy bugs will produce at least two generations of offspring per summer and spend the winter. While, they do not undergo a state of dormancy, up to 90 percent of eggs are lost during the winter and spring. There is a high rate of mortality for mealy bugs that emerge during the winter and spring months, with only a few first-instar mealy bugs surviving to summer. Maturation tends to occur more slowly with these bugs because they only feed during warm days (Jonathan *et al*., 2010).

2.1.3 Distribution of mealy bugs and destruction of cassava mealy bugs

Approximately 15 species of mealy bugs have been reported feeding on cassava but only *Phenacoccus mannihoti* and *Phenacoccus herreni* are economically important. The nymphs and adults feed on the leaves, causing yellowing and malformation. *P. mannihoti* is found in Northern South Amarica and high population cause serious yield losses. Those populations peak during the dry season; however, the rains reduce them and permit the recovery of the crop. The pest is the cassava mealy bugs, known to scientists as *P. manihoti*. The plants and causing them originally from South America, it feeds only on cassava, sucking sap from to shrivel. Also a South American native, cassava was carried by Portuguese traders to Africa and Asia, where it thrived in the absence of the insect pests that inhabit its home territory. But eventually, the mealy bug and others caught up with cassava, devastating crops first in sub-Saharan Africa and now in Southeast Asia. The cassava

mealybug, *P. manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae), is one of the most severe pests of cassava (*Manihot esculenta*) (Figure 2.4), in the world It is native to South America, but it has become naturalized throughout sub-Saharan Africa since its inadvertent introduction into the continent in the early 1970s. *P. manihoti* was not known to occur in Asia until 2008 (Figure 2.3), when it was first detected in Thailand. Since that year, it has spread aggressively throughout Thailand's cassava-growing region, also invading its neighboring countries and Indonesia and raising significant concern over its potential arrival to more countries (Parsa *et al*., 2012).

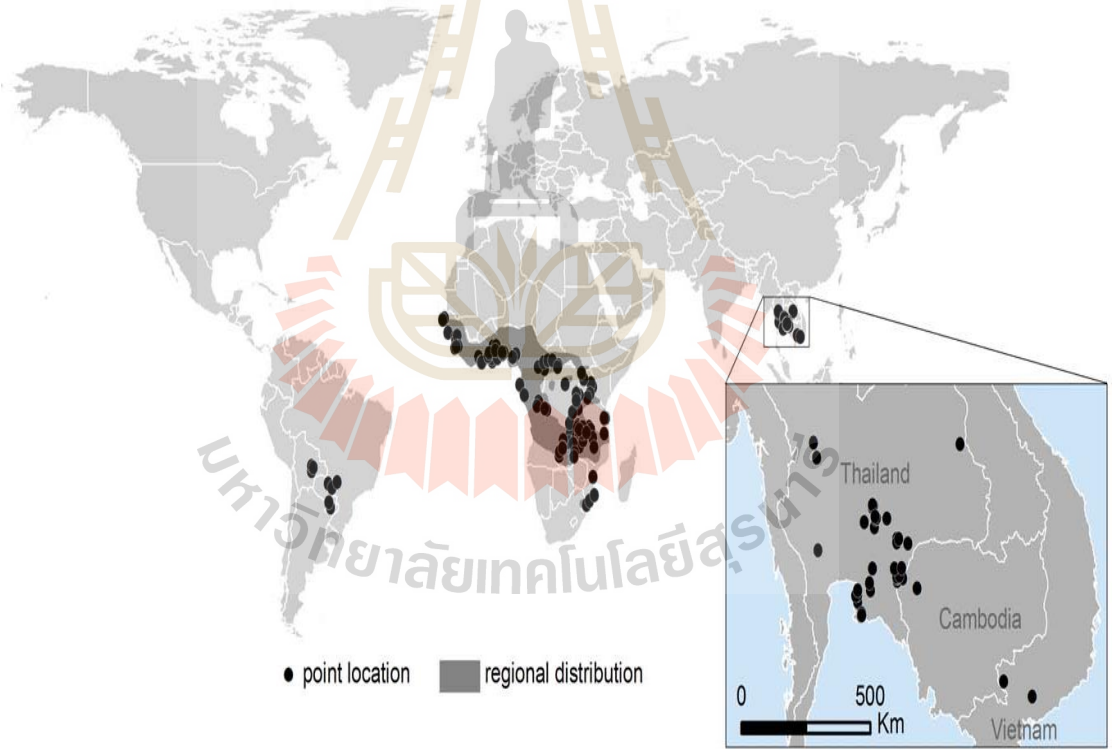


Figure 2.3 Known distribution of *Phenacoccus manihoti*, Point locations in South America correspond to its native distribution.

Source: Parsa *et al*. (2012).

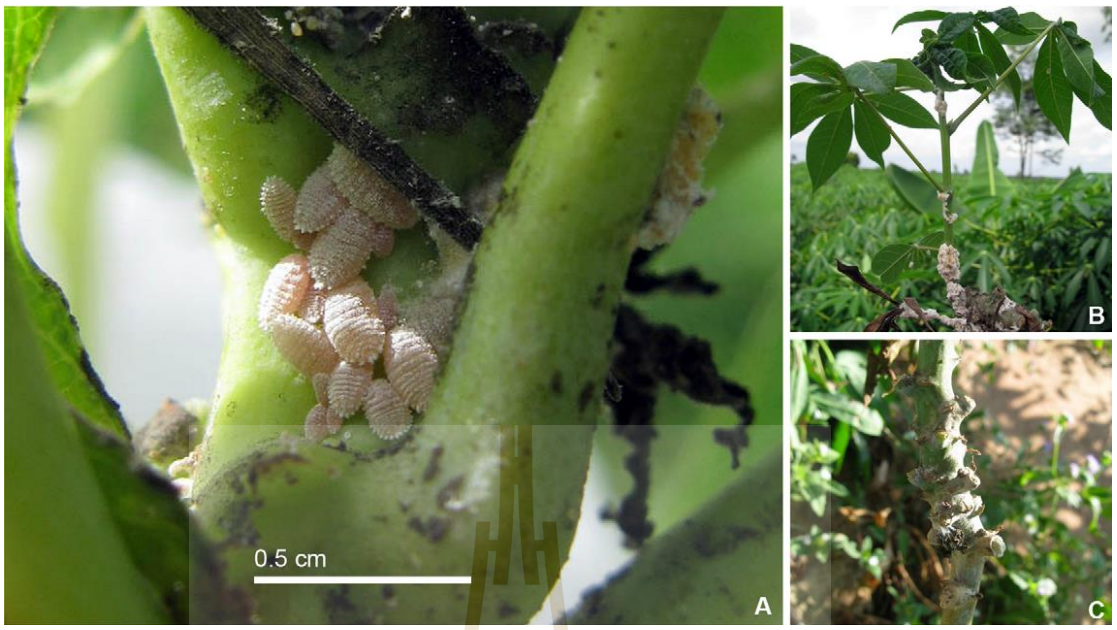


Figure 2.4 *P. manihoti* infestations and associated symptoms on cassava in Asia (A), *P. manihoti* populations at upper nodes (B), Heavy infestations and associated terminal shoot distortion (C), Stem deformation associated with *P. manihoti* infestations.

Source: Parsa *et al*. (2012).

2.1.4 Mealy bugs of cassava in Northeastern Thailand

Cassava (Manihot Esculenta, Crantz, Euphorbiaceae, Dicotyledons) is the sixth most important crop after wheat, rice, maize, potato and barley and is the primary step for more than 800 million people in the world, mostly in the poorest tropical countries (Hillocks *et al*., 2002). *Phenacoccus manihoti* was reported from Thailand, Cambodia and Laos, a first introduction to Asia in 2009 (Winotai *et al*., 2010) and in Indonesia in 2010 (Muniappan *et al*., 2011). There are some cassava mealy bugs, including stripe mealy bugs (Ferrisia virgata), Jack-beardsley mealy bugs (*Pseudococcus jackbeardsleyi*), Green mealy bugs (*Phenacoccus madeirensis*), and

Pinkish cassava mealy bugs *(Phenacoccus manihoti). P. manihoti* and *F. virgata* have now spread to cassava fields in more than 25 provinces of Thailand especially in Nakhon Ratchasima, Lopburi, and Saraburi. The severe damages to the shoots can be easily seen in the field (Chiamchamnanja, 1999). The spread of cassava mealy bugs to about 200,000 hectares has been confirmed in eastern and northeastern Thailand, where the pest is causing yield losses as high as 50 percent. Since the country's cassava industry generates more than 1.5 billion of income each year and the overall Thai cassava industry is worth 3 billon reductions of that magnitude could translate into hundreds of millions of dollars in economic losses, especially if the pest is allowed to spread further (Limsakda and Limsila, 1994).

2.2 Structure and some chemical composition of mealy bugs

Mealy bugs get its name from the white, sticky, powdery coating that covers its body. This coating resembles white cornmeal. The various types of wax glands may be positioned throughout the body or confined to a specific region (Figure 2.5 and Figure 2.6). The total number of wax glands, the number of dominant gland types, their size, and patterns, exhibit consistent differences among families (Tewari *et al*., 1994). The cuticle exhibits great diversity of thickness, structure and composition. The cuticulin layer is colorless and transparent. The cuticles of first and second nymphal instars of either sex do not differ significantly from those of the corresponding instars of other Pseudococcidae. The Pseudococcidae or mealy bugs are one of two largest scale insect families and comprises more than 2000 species in world fauna. The main taxonomic characters used in the analysis of the genera: wax glands and cylindrical wax glands (Table 1).

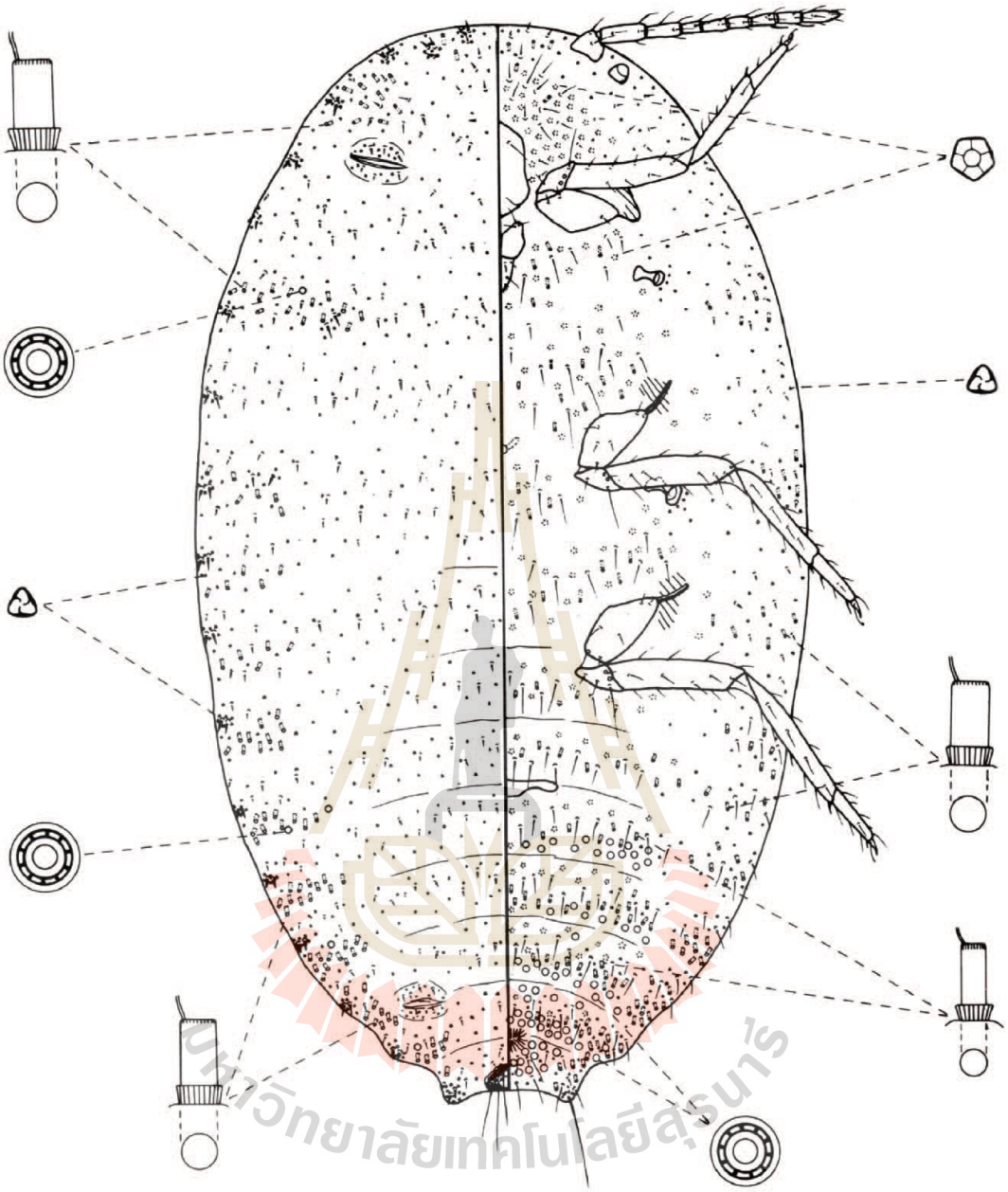


Figure 2.5 *Phenacoccus manihoti* Matile-Ferrero, adult female. Illustration after Cox & Williams, with modification.

Source: Parsa *et al*. (2012).

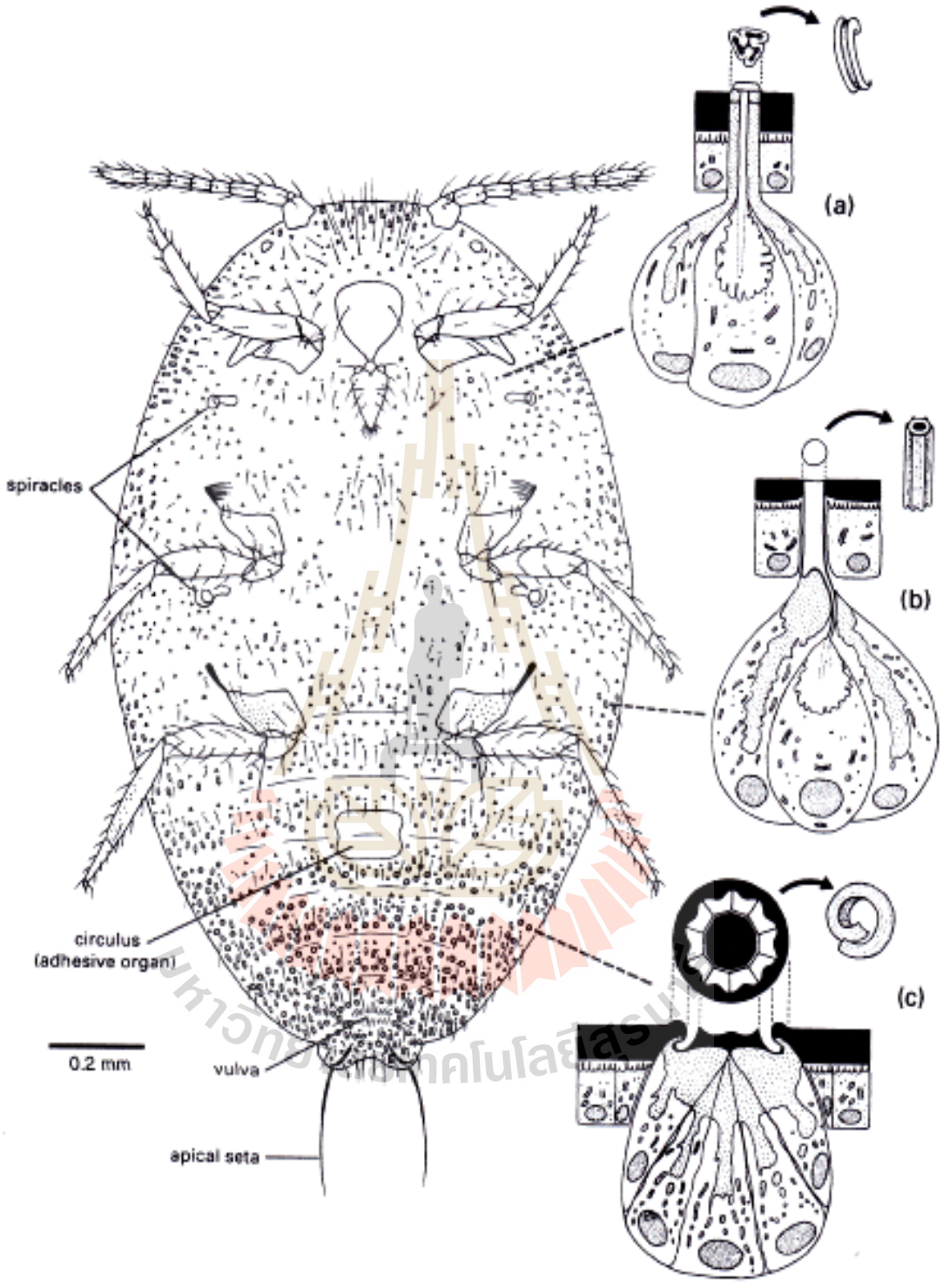
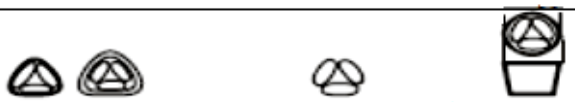
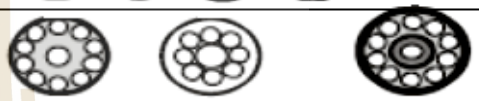
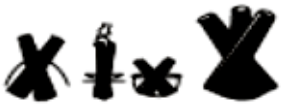


Figure 2.6 The various types of wax glands of adult female mealy bugs.

Source: Gulla and Cranston (2010).

Table 2.1 Wax glands in Pseudococcidae.

Wax glands	
Simple discoidal pores	
Trilocular pore	
Quinquelocular pores	
Multilocular pores	
Cylindrical wax glands	
Simple tubular ducts	
Collar tubular ducts — Fungiform ducts	
Collar tubular ducts — Oral collar ducts	
Collar tubular ducts — Middle collar ducts	
Collar tubular ducts — Double collar ducts	
Collar tubular ducts — Crateriform ducts	
Collar tubular ducts — Mitral ducts	
Bitubular/ Tritubular ducts	
Peculiar types	

Source: Gavrilov-Zimin (2015).

The structure and distribution of wax glands are the main diagnostic characters in the systematics of mealy bugs. Cuticular sclerotized parts of these glands are clearly visible because they absorb stain during preparation of scale insects and can be subdivided into 3 main groups: discoidal glands, cylindrical glands and glands connected with conical setae ("spines" in non-English literature). In the English coccidological tradition the term "wax glands" is usually not used, probably because the soft parts of the glands are lost during preparation and only "ducts" and "pores" are visible on specimens mounted in Canada-balsam. In Russian and other old European coccidological schools the term "glands" is widely used, because there are no special terms to indicate the soft parts of the wax glands (Gavrilov-Zimin, 2015; Xie *et al*., 2014; Zhang *et al*., 2012).

Simple discoidal pores; have a single opening (loculus). Such pores are often scattered in more or less quantity on both body surfaces of mealybugs and/or present in groups with other wax glands or associated with different types of setae. Monotypic genus *Hopefoldia* is characterized by very peculiar compact groups of discoidal pores, so-called "poraria" located on sclerotized plates on both body sides.

Trilocular (3-locular) pores have 3 excretory openings and approximately triangular in form. These pores are one of the synapomorphic characters of mealybugs and the monotypic family Phenacoleachiidae and can be found in majority of mealybugs on all body surfaces. Only some mealybug genera, such as from *Heterococcus*, *Boreococcus*, *Neotrionymus*, *Metad enopus*, and also some genera from other groups demonstrate reduction in the number of trilocular pores or their secondary total absence. Sometimes trilocular pores are scattered on body very irregularly; thus, they can be absent or very few in medial zone of the thoracic and

anterior abdominal sternites (as, for example, in some species of the species-rich genus *Phenacoccus*) or form dense marginal or medial bands (as in *Trimerococcus* and *Kiritshenkella*, respectively). The size of the trilocular pores is usually equal throughout the body. However, in the species of *Puto*, dorsal trilocular pores (and especially cerarian ones) are larger than ventral pores. Trilocular pores of peculiar structure are known in some species and genera, for example, in *Cucullococcus arrabidensis* (pores without fringe) and in the species of *Iberococcus* (pores are strongly convex and located in a deep cup).

Quinquelocular (5-locular) pores have one central loculus and 5 peripheral loculi; sometimes central loculus absent. These pores are present mainly in the species of the subfamily Phenacoccinae (in Pseudococcinae they are known mainly in *Metadenopus*) and usually scattered on the ventral surface of body, especially around mouthparts and near spiracles. In some cases (for example, in *Heterococcus* and *Metadenopus*) 5-locular pores are numerous on both body sides and replace partly or totally reduced trilocular pores.

Multilocular pores usually have one central loculus and 8-10 peripheral loculi and form transverse rows on abdominal sternites. More rarely multilocular pores present on other surface of body and/or show a peculiar distribution, structure and size. For example, in *Mirococcus sphaeroides* some of multilocular pores have several central loculi; species of the genera *Pelionella* and *Rosebankia* show 2 different types of multilocular pores in the same female (Gavrilov-Zimin, 2015; Xie *et al*., 2014; Zhang *et al*., 2012).

Cylindrical glands produce wax filaments. There is a great diversity of these glands, probably, exceeding the diversity of such glands in other scale insects all together (Gavrilov-Zimin, 2015; Xie *et al*., 2014; Zhang *et al*., 2012).

Simple tubular ducts: They have one simple excretory duct. The opening of this duct usually lies on the surface of the cuticle or more rarely it is slightly raised or slightly invaginated from the surrounded cuticle area. Most genera of subfamily Phenacoccinae have only this type of cylindrical glands. The deviation of this type is socalled "bottle-shaped" ducts in the species of *Coccidohystrix*: distal (distant from the excretory opening) part of such duct is enlarged in the form of a bottle.

Collar tubular ducts: In these glands the duct is surrounded by collars of different sizes and forms. The greatest diversity of collar ducts takes place in the subfamily Pseudococcinae, whereas among more primitive Phenacoccinae these ducts are known in several genera only, for example in *Heliococcus*, *Seyneria*, *Ehrhornia*. There are at least the following main variants of collar ducts. a) Fungiform ducts. The collar of these ducts is very wide and similar to a cap of fungus. Most of species with fungiform ducts are members of *Pseudococcus* and only some species with such ducts are known in other genera, for example, in the genera *Mirococcopsis* and *Humococcus*. b) Oral collar ducts, with narrow collar which surrounds the excretory opening and part or all tube of the duct. Such ducts are most common and known in most genera of the subfamily Pseudococcinae. The length of the collar can be rather different, from very small to longer than the length of the duct itself. c) Middle collar ducts, with collar located in the middle part of the duct (for example, in *Volvicoccus volvifer*, *Metadenopus caudatus.* d) Double collar ducts. Proximal part of such collar is always more sclerotized than the distal part (for example, in some *Trionymus*,

Paraputo, etc.). f) Crateriform ducts. The collar of such ducts bears one or several conical setae. These are known in the genus *Heliococcus* only. g) Mitral ducts; resemble a headwear ("mitre") of orthodox religious superiors. These ducts are known, for example, in the genera *Ehrhornia*, *Discococcus*, *Paradiscococcus*.

Bitubular and tritubular ducts: are structures, consisted of 2 or 3 crossed ducts, with or without general collar; these are present in many (but not in all) species of *Rhizoecus*. Peculiar types of tubular ducts are known in different genera. For example, in the species of the genus *Nudicauda*, and in the monotypic genus *Malekoccus*, the duct opening is encircled by a flat sclerotized ring; in the species of pantropical genus *Ferrisia*, the opening is encircled by a sclerotized zone of the cuticle with several associated flagellate setae. In addition to these rather large ducts which are well visible under usual translucent microscope, many mealybugs have numerous poorly visible minute microtubular ducts, which can be located on different parts of the body and sometimes on the hind coxae. Fine structure and function of these microducts are unknown till now (Gavrilov-Zimin, 2015; Xie *et al*., 2014).

Important morphological structure (i.e. the trilocular, multiocular pores, tubular ducts, ostioles, and the female genitalia in the different stages) were studied under the scanning electron microscope. Further, it was also observed that the wax secreted by numerous pores and ducts around the ovipositional opening played an important role in forming a waxy sac around the laid eggs (Xie *et al*., 2014; Nassar *et al*., 2008). The wax gland (Figure 2.6) contained two large central cells and several small lateral cells. The two center cells were close ranged together and surrounded by the lateral cells, which possessed very large nuclei (Xie *et al*., 2014). The main chemical compositions of the honey dew extracted vine mealy bugs *planococcus vitis*. Sucros

were present in both the honey dew and the sap of potatoes indicating a lack of invertase activity in the insect gut. Other sugars demonstrated were glucose, fructose, and raffinose. Sixteen amino acid were detected in the honey dew; aspartic acid, glutamic acid, proline, valine, histidine, serine, tyrosine, arginine, alanine, isoleucine, leucine, methionine, threonine, and glycine. Critic and tartaric acid were also detected in the honey dew Calatayud and Ru (2006). Five carbohydrate components and thirteen amino acids were identified in the honey dew of sugar cane mealy bug *Saccharicoccus sacchari* by chromatographic analysis. The surgars are fructose, sucrose, melizitose and raffinose Salama and Rizk (1969). Insect cuticle is a very complex structure composed of protein, chitin, and fatty acid. Protein constitutes nearly 50-80 % of the total mass (Gupta *et al*., 1992). The exact nature of the linkage between protein and carbohydrates is not known (Brunet, 1980). However, the structure varies considerably depending upon the insect source.

Previous investigations on honeydew indicated that its major components were: sucrose, D-fructose, mannose, trehalose, myo-inositol, ribitol, galactose, quinic acid, and malic acid. The chemical compositions of waxes produced by Hemiptera have been investigated mainly in whiteflies and scale insects. The surface lipids on nymphs and exuvia of several whitefly species (Aleyrodidae) contained largely wax esters, long-chain aldehydes, hydrocarbons and long-chain alcohols (Ammar *et al*., 2013).

2.3 Diversity of bacteria in soil

The microbial population in soil is very diverse. Bacteria, fungi and yeasts, microalgae, protozoa, virus, nematodes, and other microscopic invertebrate animals

are used soil as a microhabitat (Eldor, 2007; Metting, 1993). Soil is the naturally occurring, unconsolidated mineral and organic material at the earth's surface that provides an environment for living organisms. The presence of about 6,000 different bacterial genomes per gram of soil by taking the genome size of *Escherichia coli* as a unit was calculated. Relative numbers of living organisms and approximate biomass of the soil show in Table 2.2.

Table 2.2 Numbers of living organisms and approximate biomass of the soil.

Organism	Number		Biomass
	Per m^2	Per g	(wet kg/ha)
Bacteria	10^{13}-10^{14}	10^{8}-10^{9}	300-3,000
Actinomycetes	10^{12}-10^{13}	10^{7}-10^{8}	300-3,000
Fungi	10^{10}-10^{11}	10^{5}-10^{6}	500-5,000
Microalgae	10^{9}-10^{10}	10^{3}-10^{6}	10-1,500
Protozao	10^{9}-10^{10}	10^{3}-10^{5}	5-200
Nematodes	10^{6}-10^{17}	10-10^{2}	1-100
Earthworms	30-300		10-1,000
Other invertebrate microfauna	10^{3}-10^{5}		1-200

Source: Metting (1993).

Bacteria are the most dominant group of microorganisms in soil and probably equal one half of the microbial biomass in soil. They are present in all type of soil but their population decreases as the depth of soil increase. Bacteria live in soil as cocci (Spheres, 0.5 µ), bacilli (rod, 0.5 to 3.0 µ) or spirilli (spirals). The bacilli are common in soil whereas spirilli are very rare in natural environment (Levin *et al*., 1992). The

classification soil microorganisms in general and bacteria in particular into two broad categories, the autochthonous and the zymogenous organisms. The autochthonous or indigenous population is always uniform and constant in soil since their nutrition is derived from native soil organic matter (examples, *Arthobacter* and *Nocardia*). On the other hand, zymogenous or fermentative organism require an external source of energy and their normal population in soil is low (examples, *Pseudomonas* and *Bacillus*) (Barker and Dickman, 1993). Growth in the presence or absence of oxygen is taken as the criterion to distinguish bacteria into anaerobic, aerobic and facultative anaerobic, that is, those capable of developing under oxygenated as well as non-oxygenated conditions. The most common soil bacteria come under the genera *Pseudomonas*, *Arthobacter*, *Clostridium*, *Achromobacte*r, *Bacillus*, *Micrococcus*, *Flavobacterium*, *Corynibacterium*, *Sarcina* and *Mycobacterium*. *Escherichia* is encountered rarely in soils except as a contaminant from sewage whereas *Aerobacter* is frequently encountered and is probably a normal inhabitant of certain soils. Another group of bacteria common in soils is the myxobacteria belonging to the genera *Myxoxoccus*, *Chondrococcus*, *Archangium*, *Polyangium*, *Cytophaga* and *Sporocytophaga*. Bacteria are also classified on the basis of their nutritional requirements (Subba Rao, 1995). Autotrophic as well as heterotrophic bacteria are present in soil. Autrotrophs synthesize their own food whereas heterotrophs depend on pre-formed food for nutrition. Photoautotrophs are those whose food energy is derived through mediation of sunlight, as in the instance of photosynthetic bacteria as opposed to chemoatotrophs which oxidize inorganic materials to derive energy and at the same time utilize to carbon from CO_2 for growth. In the latter category, a group of bacteria known as obligate chemoautotrophs are included which prefer specific

substrates (Nannipieri *et al*., 2003). Microbial and biochemical characteristics are used as potential indicators of soil quality, even if soil quality depends on a complex of physical, chemical and biological properties. The rationale for the use of microbial and biochemical characteristics as soil quality indicators is their central role in cycling of carbon dioxide and nitrogen and their sensitivity to change. The relationships between bacterial community diversity and stability in soil were investigated. *Chloroflexus*, *Stenotrophomonas*, and *Azospirillum* were restricted to the *Leucadendron xanthoconus* rhizosphere soil. Barker and Dickman (1993) isolated nitrogen-fixing and denitrifying bacteria in an acid forest soil which were *Stella humosa*, *Azospirillum* sp., *Frateuria* sp., *Acidobacterium capsulatum*, *Acidimicrobium* sp., and *Conexibacter* sp. (Bohme *et al*., 2005).

2.4 Bacteria as insect pathogens and symbionts

Bacterial pathogens used for insect control are spore forming, rod-shaped bacteria in the genus *Bacillus*. They occur commonly in soils, and most insecticidal strains have been isolated from soil samples. Bacterial insecticides must be eaten to be effective; they are not contact poisons. Insecticidal products comprised of a single *Bacillus* species may be active against an entire order of insects, or they may be effective against only one or a few species. For example, products containing *Bacillus thuringiensis* kill the caterpillar stage of a wide array of butterflies and moths. In contrast, *Bacillus popillae* (milky spore disease) kills Japanese beetle larvae but is not effective against the closely related annual white grubs (masked chafers in the genus Cyclocephala) that commonly infest lawns (Sanahuja *et al*., 2011). The microbial insecticides most widely used in the United States since the 1960s are preparations of

the bacterium *Bacillus thuringiensis* (abbreviated as Bt). Bt products are produced commercially in large industrial fermentation tanks. As the bacteria live and multiply in the right conditions, each cell produces (internally) a spore and a crystalline protein toxin called an endotoxin. Most commercial Bt products contain the protein toxin and spores, but some are cultured in a manner that yields only the toxin component (Weinzierl *et al*., 2000). *B. thuringiensis* is regarded as an insect pathogen commonly used as a crop protection agent against insects from the orders of Lepidoptera, Diptera, and Coleoptera (Sanchez-Contreras, 2008).

Positive symbiotic interactions have been reviewed elsewhere and will not be considered here. The strategies discussed here include the interactions between: The ability to multiply in the host. Innate immunity effector mechanisms that control infection and that possess a certain degree of specificity to different classes of microorganisms. *Yersinia pestis* with *Xenopsylla cheopis* (Siphonaptera; fleas); *Photorhabdus* species and *Xenorhabdus* species with *Manduca sexta* (Lepidoptera; moths and butterflies); *Bacillus thuringiensis* or *Bacillus cereus* with various insect orders; *Serratia entomophila* with the grass grub *Costelytra zealandica* (Coleoptera); and *Erwinia carotovora*, *Pseudomonas entomophila* and *Serratia marcescens* with *Drosophila* spp. (Diptera). Other interactions (Table 2.3) have been reviewed elsewhere but these infections will not be discussed here because of a lack of detailed molecular or genetic information (Vallet-Gely *et al*., 2008).

Table 2.3 Examples of interactions between bacteria and insects.

Bacteria	Type of interaction	Host (mode of interaction)
Erwinia aphidicola	Pathogen	Pea aphid (ingestion)
Dickeya dadantii	Pathogen	Pea aphid (ingestion)
Pseudomonas entomophila	Pathogen	*Drosophila*, *Bombyx*, (ingestion)
Yersinia pestis	Pathogen	Rat flea (ingestion)
Serratia entomophila	Pathogen	Grass grub (ingestion)
Serratia marcescens	Pathogen	*Drosophila* (ingestion)
Photorhabdus sp.	Pathogen	Lepidopteran (assisted entry)
Xenorhabdus sp.	Pathogen	Lepidopteran (assisted entry)
Vibrio cholerae	Pathogen	Drosophila (ingestion)
Melissococcus pluton	Pathogen	Honey bee (ingestion)
Bacillus thuringiensis	Pathogen	Different orders (ingestion)
Bacillus papillae	Pathogen	Scarab larvae (ingestion)
Paenibacillus lentimorbus	Pathogen	Scarab larvae (ingestion)
Paenibacillus larvae	Pathogen	Honey bee larvae (ingestion)
Bacillus sphaericus	Pathogen	Mosquito (ingestion)
Bacillus laterosporus	Pathogen	Bee larvae, dipteran (ingestion)
Pseudomonas aeruginosa	Opportunistic	Caterpillar (ingestion)
Pseudomonas aeruginosa	Opportunistic	*Drosophila* (direct injection)
Bacillus cereus	Opportunistic	*Galleria mellonella* (ingestion)
Erwinia carotovora	Infectious	*Drosophila* larvae (ingestion)
Shigella spp.	Passive vector	House fly (ingestion)
Rickettsia spp.	Vector	Cat flea (ingestion)
Bartonella spp.	Vector	Cat flea (ingestion)

Source: Vallet-Gely *et al*. (2008).

Hexapoda, the largest class of invertebrates, is involved in several types of symbiosis, primarily with bacteria. All insects live in close association with bacteria;

however, bacteria are present on the integument, inhabit the digestive tract, and, in some highly evolved cases, inhabit unique structures within the insect body (Carol *et al*., 2001). Many types of bacteria have been identified from different insects, such as the gypsy moth, migratory grasshopper, cabbage moth, and cotton bollworm. The bacterial association in insects plays a significant role in host insect morphogenesis, food digestion, nutrition, antifungal toxin production, pheromone production, pH regulation, vitamin synthesis, temperature tolerance, resistance against parasitoid development, and detoxification of noxious compounds (Harriet *et al*., 2010). Arthropods, particularly insects, form successful long-term symbioses with endosymbiotic bacteria. The associations between insects and endosymbionts are remarkably stable; many stretch back several hundred million years in evolutionary time. With the exception, perhaps, of the filarial nematodes no other group of metazoans shows such a proclivity for their intracellular symbionts (Hoy and Jeyaprakash, 2008). The identification and classification of bacterial symbionts and hosts has grown rapidly over the last two decades and these relationships form a continuum from classical mutualism to parasitism. Complete genomes have been sequenced for many of these bacteria and some of their hosts. Now more intractable questions regarding endosymbiosis are being addressed. Bacterial symbionts are important promoters of insect diversity and speciation. The display of potent virulence mechanisms, including a variety of toxins, and the development of strategies to evade the host immune system are required for the success of the infection (Shahina *et al*., 2011).

Multiple endosymbiotic bacteria are often housed in the same host organisms. Many insects harbor an obligate endosymbiotic bacterium, referred to as the “primary

symbiont" in specialized cells called bacteriocytes or mycetocytes. The primary symbiont is essential for the survival and reproduction of the host and is therefore fixed in the host population. Frequently, these insects also possess a facultative endosymbiotic bacterium, referred to as the "secondary symbiont" often in different types of cells and tissues. The secondary symbiont is, in most cases, nonessential for the host and usually shows partial infection in the host population (Ammar and Hogenhout, 2006). The bacteriocytes harboring the primary symbiont and the bacteriocytes housing the secondary symbiont often form a huge symbiotic organ, called the bacteriome or mycetome (Riegler and Neill, 2007). Endosymbiotic associations comprising an obligate primary symbiont and one or several optional secondary symbionts are commonly found in diverse. The body cavity of a female mealybug is a large structure called a bacteriome, composed of large cells called bacteriocytes. In the older literature, there are descriptions of a variety of different bacterial types embedded within "mucous spherules," and it has been stressed that this is a morphologically unique type of insect endosymbiosis. Initially, it was established that mealybugs contain primary endosymbionts (P-endosymbionts) that are members of the β-subdivision of the Proteobacteria, in markedcontrast to most other described insect endosymbionts (Thao *et al*., 2002). Interesting the primary endosymbionts are *Buchnera*, *Blochmannia*, and *Wigglesworthia*. The secondary symbiont of the tsetse fly is the *Sodalis glossinidius* (Wille and Hartman, 2009; Sanchez-Contreras and Vlisidou, 2008).

Table 2.4 Commercially available bacterial pesticides.

Bacteria	Target pest/pathogen	Countries of registration
Fungicides		
Bacillus subtilis	*Rhizoctonia, Fusarium Alternaria* and *Aspergillus* root rots and seedling Diseases	Canada, Korea, India, Japan, New Zealand, Switzerland, Mexico, USA
Pseudomonas fluorescens	*Erwinia amylovora* on apple, cherry, almond peach, pear, potato straw-berry, tomato	
Burkholderia cepacia (formerly *Pseudomonas cepacia*)	*Fusarium, Rhizoctonia*	India, USA
Streptomyces griseoviridis	Soil pathogens *Fusarium, Alternaria Rhizoctonia, Phomopsis Botrytis* that cause wilt, seed, root and stem rots	Canada, Denmark, Finland, Hungary, Iceland, Italy, Netherlands, Norway Sweden, Switzerland, USA Spain
Insecticides		
Bacillus popilliae	Larvae of Japanese beetle	USA
Bacillus thuringiensis	Several insect species *Helicoverpa armigera* European corn borer *Plutella xylostella* spruce budworm gypsy moth caterpillars	
Serratia entomophila	Grass grub (White)	New Zealand
Bacillus sphaericus	Mosquito control	

Source: Arora and Saikia, 2005; Chavan *et al*., 2008; Hynes and Boyetchko, 2006; Rabindra, 2005.

The most widely used microbial control agent is *Bacillus thuringiensis* (Bt). Today a number of isolates of the bacterium are commercially produced with activity against insect as well as fungal pests (Table 2.4). Most of the insecticidal activity of *B. thuringiensis* is associated with the proteinacious toxins. Their mode of action is thought to involve a cascade of events leading to insect death within several hours following ingestion (Hofte and Whiteley, 1989; Gill *et al*., 1992; Knowles, 1994). The largest share of the biopesticide market currently goes to *B. thuringiensis*. Other species of bacteria are used on a much smaller scale for insect control. These include *Serratia entomophila*, the only non-bacillus bacterial microbial insecticide registered for pastureland grass grub control in New Zealand (Klein and Kaya, 1995). *Bacillus sphaericus* is now commercially produced for the control of mosquito and has some advantages over *B. thuringiensis* in that it is more persistent in polluted habitats and may recycle under certain conditions, but has a narrow host range (Lacey and Undeen, 1986; Charles *et al*., 1996; Nicolas *et al*., 1994).

After a few decades of research on microbial pest management dominated by *Bacillus thuringiensis* (*Bt*), novel bacterial species with innovative modes of action are being discovered and developed into new products. Significant cases include the entomopathogenic nematode symbionts *Photorhabdus* spp. and *Xenorhabdus* spp., *Serratia* species, *Yersinia entomophaga, Pseudomonas entomophila*, and the recently discovered Betaproteobacteria species *Burkholderia* spp. and *Chromobacterium* spp. Lastly, Actinobacteria species like *Streptomyces* spp. and *Saccharopolyspora* spp. have gained high commercial interest for the production of a variety of metabolites acting as potent insecticides. Bacteria are widespread in the environment and they have evolved a variety of interactions with insects including essential symbiosis.

While many bacterial species inhabit bodies of insects establishing different levels of mutualistic relationships, only a limited number of them behave as insect pathogens. The latter have evolved a multiplicity of strategies to invade the host, to overcome its immune responses, to infect and to kill it. The mechanisms leading to these kinds of interactions are presumed to have ancient origin and to have developed throughout a long co-evolution process. In line with this concept, a variety of insecticidal toxins produced by certain spore forming entomopathogenic bacteria, have a similar structure and mode of action. Important information to understand the molecular mechanisms involved in diverse pathogen-host interactions are being produced as a result of modern studies. However, many aspects are still unrevealed and after few decades of microbial pest management dominated by *B. thuringiensis*, novel bacterial species with innovative modes of action have been discovered and formulated as new biopesticidal products. As a result of continuous industrial and academic screening activities, the discovery of new bacterial species and insecticidal metabolites is expected in the near future. This trend is also the result of modern legislative frameworks fostering the use of bioinsecticides in Integrated Pest Management (IPM) programs (Ruiu *et al*., 2015).

2.5 Detection and identification of bacteria in soil

2.5.1 Detection of bacteria in soil

There are several methods for the detection of bacteria in soil. Conventional culture methods and genetic methods are wildly used for the detection of these bacteria. Conventional culture methods for the detection of dominant bacterial strains are based on directly using cultural medium.

2.5.2 Identification of heterotrophic bacteria

2.5.2.1 Morphological characterization

One of the properties of major importance in classifying bacteria is morphology of the individual bacterial cell particularly cell shape (rods or bacilli, cocci and spiral shape), cell arrangement (singly, or in chains, clusters, or regular packets), flagella arrangement, and the reaction after straining by the method of Gram. Gram-strained bacterium reveals another significant morphological character. It tells whether a bacterium does or does not possess an outer membrane and whether it has a thick or thin peptidoglycan wall. This simple test distinguishes two major divisions of bacteria, Gram-negative and Gram-positive (Pattison *et al*., 1995).

2.5.2.2 Physiological characterization

Physiological characterization used to classify bacteria are based on conditions that support their growth including a supply of suitable nutrient, a source of energy, an appropriate temperature, an appropriate pH, and appropriate level oxygen (Singleton, 2004). Carbon sources that support growth are a particularly useful set of characters because most bacteria can use so many different carbon sources. Bacteria are divided in to thermophilic, mesophilic and psychrophilic bacteria depending on their appropriate temperature for growth. Most bacteria grow best at or near pH 7 (neutral), and the majority cannot grow under strongly acidic or strongly alkaline. However, some of bacterial species are tolerated to those conditions. They are acidophiles or alkalophiles. An appropriate level of oxygen has effected to bacterial growth. Bacteria are sensitive to oxygen classified as obligate anaerobes, tolerance of it is aerobes or requirement for it is strict aerobes.

Biochemical characterizations of bacteria are also useful characters for bacterial metabolisms.

2.5.2.3 Genetic characterization

Genetic characterization of bacteria has been acquired through the application of techniques based on the complementary nature of the double-helix structure of deoxyribonucleic acid (DNA) (Ogunseitan, 2005). The techniques related with percentage of guanine+cytosine (% G+C) and DNA hybridization which are highly indirect methods of evaluating bacterial genomes. The mole % G+C expresses the percentage of total base pairs in DNA that are G-C, as opposed to adenine-thymine (A-T). Higher % G+C means a higher DNA melting point and density. Closely related bacteria have similar % G+C values. DNA hybridization involves heating DNA melts it, allowing the two strands of the double helix to separate, DNA is cooled, and the two single strands anneal. When single strands of DNA from different sources are mixed, they anneal in regions where the sequences are the same or similar, creating a hybrid molecule. Therefore, the extent of annealing is a quantitative index of the similarity of base sequences in the DNA from the two sources. Polymerase chain reaction (PCR) is a method for copying specific sequences of nucleotides in DNA or in a modified form of the process in RNA and repeated replication of a given sequence of nucleotides (the PCR product usually less than 2 kb long) forms millions of copies within hours (Singleton, 2004). This method is a powerful technique which has been widely used for the identification of bacteria.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, reagents, and media

3.1.1 Determination of structure and some chemical compositions of cassava mealy bugs

The chemicals and reagents used for electron microscopy techniques were composing of 5% glutaraldehyde, 1% osmiumtetoxide, 0.2 M phosphate buffer, alcohol series (20, 40, 60, 80, and 100%), carbon fuchsin staining, and methylene blue staining. Preparation of these chemical were described on section 5 in Appendix A.

3.1.2 Isolation, screening and selection of soil bacteria

The microbiological media preparations for cultivation, screening, and selection were colloidal chitin agar (CCA), tributyrin agar (TBA), skim milk agar (SMA) and trypticase soy agar (TSA) (Appendix A.1.1-1.5, respectively). The media were described, according to Atlas (2010), using for screening and culturing of bacteria isolated from soil sample. Media for physiological identification of bacteria were composed of triple sugar iron (TSI) medium, lysine indole motile (LIM) medium, methyl red Voges-Proskauer (MR-VP) medium, Simmons citrate agar, nitrate reduction broth, oxidation-fermentation medium, lauryl sulfate tryptose (LST) broth, motility test medium, nutrient gelatin, and phenol red broth (Appendix A.1.6-1.15, respectively).

Chemicals used for medium preparation including D-glucose, yeast extract, tryptone, soy peptone, and calcium carbonate were purchased from Himedia (Himedia Laboratories, Mumbai, India). Sodium acetate, magnesium sulfate, manganese sulfate, and sodium chloride were obtained from Carlo Erba (Montedison group, Milan, Italy). Iron (II) sulfate was a product of BDH (BDH Labolatory supplies, Radnor, PA., U.S.A.). L-Histidine, L-leucine, and L-lysine were ordered from Sigma (Sigma-Aldrich Chemical Company, St. Louis, MO., U.S.A.). L-Ornithine and L-tyrosine were obtained from Fluka (Sigma-Aldrich Chemical Company, U.S.A.). Diaminoheptane, dansyl chloride, and acetonitrile were ordered from Sigma (Sigma-Aldrich Chemical Company).

3.1.3 Identification of the selected bacteria

3.1.3.1 Morphological and physiological characterization

Chemicals used for morphological and physiological characterization were crystal violet (POCH, POCH SA, Poland); iodine, potassium iodide, ethanol, safranin O, hydrogen peroxide, paraffin oil, and sodium chloride (Carlo Erba, Montedison group); Tween 80 (ACRŌS organics, Acros Organics, Morristown, NJ., USA); skim milk (Himedia, Himedia Laboratories); and copper(II) sulfate (Sigma, Sigma-Aldrich Chemical Company) and tetramethyl-p-phenylenediamine dihydrochloride (Fluka, Sigma-Aldrich Chemical Company). Sugars used for sugar fermentation were D-arabinose and D-fructose (Merck, Merck Chemicals, Darmstadt, Germany); D-mannose and D-sucrose (Carlo Erba, Montedison group); D-mannitol (Univar, Ajax Finechem Pty. Ltd., New South Wales, Australia); and D-trehalose and D-maltose (Fluka, Sigma-Aldrich Chemical Company). API 20E and strips (bioMérieux, bioMérieux Industry, Marcy l'Etoile,

France) were also used for biochemical test. Chemicals and reagents used for identification of the bacterial isolates based on conventional method were 1% solution of dimethyl-p-phenylenediamine dihydrochloride, Gram staining reagents, 3% hydrogen peroxide, Kovács' reagent, methyl red test reagent, nitrate test reagents, and Voges-Proskauer reagents (Appendix A.2.1-2.10, respectively). The preparations of these reagents were according to Beishier, 1991, and Smibert and Krieg, 1994.

3.1.3.2 16S rRNA gene characterization

Chemicals and reagents used for genomic DNA extraction and detection were absolute ethanol and sodium chloride (Merck, Merck Chemicals); ethidium bromide and tris-HCl (Promega, Promega Corporation, Madison, WI., U.S.A.); phenol (BDH, BDH Labolatory supplies); sodium citrate and chloroform (Carlo Erba); sodium dodecylsulfate (SDS) (Fluka, Sigma-Aldrich Chemical Company); lysozyme and ethylenediaminetetraacetic acid (EDTA), RNase and proteinase K (Sigma, Sigma-Aldrich Chemical Company); and LE Agarose (Seakem, Cambrex Bio Science Rockland Inc., Rockland, ME., USA).

Chemicals and reagents used for polymerase chain reaction (PCR) amplification were 10X reaction buffer, $MgCl_2$ solution, and *Taq* DNA polymerase (Invitrogen^{TM}, Invitrogen life technologies, Foster, CA., U.S.A.); and dNTPs (dATP, dCTP, dGTP, and dTTP) (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, CA., U.S.A.). The oligonucleotide primers were ordered from the Science Pacific Company, Ltd. (Bangkok, Thailand). The molecular weight marker (1 kb plus DNA ladder) was purchased from Invitrogen^{TM} (Invitrogen life technologies).

The reagent preparations for nucleic acids analysis are given in Appendix A.4. Reagents used for DNA extraction were STE buffer, TE buffer, and

Trissaturated phenol (pH 8.0). Reagents used for amplification of 16S rDNA were PCR buffer without $MgCl_2$ (100 mM tris-HCl, pH 9.1; 500 mM KCl and Triton™X-100), $MgCl_2$ solution (25 mM $MgCl_2$ in sterile water), dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), primers, and *Taq* DNA polymerase (Vivantis, 30 VIVANTIS TECHNOLOGIES SDN BHD**,** Malaysia). The reagents used for sequencing were prepared as described in the manual of ABI377 Autometed DNA sequence (Perkin Elmer, Applied Biosystems, Inc., Foster, CA, U.S.A.). The Big Dye Terminator Ready Reaction kit (Perkin Elmer, Applied Biosystems Inc., U.S.A.) was used for the nucleotide sequencing reaction

3.2 Instrumentation

Instruments required for cultivation, screening, selection, and identification of bacteria were located at the Instrument Buildings of the Center for Scientific and Technological Equipment and Research Center for cassava and products, Suranaree University of Technology, Nakhon Ratchasima Thailand.

3.3 Collection of mealy bugs from cassava plantation areas in Northeastern Thailand

Cassava mealy bugs were collected at different locations from cassava in plantation areas, Northeastern Thailand, where the cassava devastation has been reported during March, 2011 and April, 2013. Data cassava mealy bug and growth of mealy bugs, collection locality, date was record and take photographs. After returning to the laboratory, some chemical composition and structure of specimen were

performed. Mealy bugs will be reared and studied in the Research Center for cassava and products at Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.4 Determination of structure and some chemical compositions of mealy bugs collected from cassava plantation areas in Northeastern Thailand

Live pink cassava mealy bugs, *Phenacoccus manihoti* were used for studied under stereo microscope, light micro scope from serial section and scanning electron microscope (SEM) (Parsa *et al*., 2012; Sirisena *et al*., 2015; Zhang *et al*., 2009 and 2011; Xie *et al*., 2010).

3.4.1 Determination of structure of cassava mealy bugs

3.4.1.1 Stereo microscope

In laboratory, cassava mealy bugs specimens were collected from different locations were observed and photographed under on Olympus stereo-microscope. Shape, size, color, a part of head thorax, and abdomen were observed and recorded (Appendix B). Other samples were fixed in 2.5% glutaraldehyde (v/v) in 0.2 M phosphate buffer (PBS) at pH 7.2 and at 4 °C for paraffin sections was examined under light microscope (Light microscope, Olypus BX51, Olympus optical Co., Ltd., Japan). Finally, the samples were observed under a JEOL JSM-610 Scanning electron microscope (JEOL Ltd., Japan) at an operating voltage of 15Kv, and micrographs were taken using a canon EOS350D digital camera (Parsa *et al*., 2012).

3.4.1.2 Scanning electron microscope (SEM)

For SEM study, three adult females were fixed in 4% glutaraldehyde for 24-48 h, rinsed three times in 0.2M phosphate buffer and then dehydrated through successive 5 min changes in 70, 80, 90, 100% ethanol [v/v] and three further changes in 100%. The ethanol was then displaced by liquid carbon dioxide and samples were dried using EMS 850 critical point dryer, mounted on copper stubs, then coated with gold in the sputter coater and finally scanned at different angles using the SEM (JSM-6010, JEOL, Japan) at 15 kv. (Zhang *et al*., 2012; Xie *et al*., 2010).

3.4.1.3 Light microscope (LM)

Three samples, pink mealy bugs were embedded in paraffin by first dehydrating the tissues in a series of increasing concentrations of ethyl alcohol (10 min each in 35, 55, 75, 85, 95, and 100% [v/v]) and xylene (10 min each in 35, 55, 75, 85, 95, and 100% [v/v]). The tissues were then immersed in an equal volume mixture of xylene:paraffin for 48 h at 56 °C and then in paraffin for 48 h at 56 °C before embedding in paraffin. Embedded specimens were serially sectioned to a thickness of 0.6 μm and immersed in 100% xylene and then decreasing concentrations of ethyl alcohol (10 min each in 100, 95, 85, 75, 55, and 35% [v/v]) followed by a rinse with distilled water (10 min). These were then stained with carbol fuchsin and methylene blue and intermediately rinsed in distilled water (10 min). Xylene was used as a mounting medium. Integument sections were observed and photographed with optic microscopy (Olympus BX51, Olympus Optical Co., Japan (Zhang *et al*., 2012; Xie *et al*., 2010).

3.4.2 Determination of some chemical compositions of mealy bugs

The samples were treated with 0.25M HCl solution at ambient temperature with a solution-to-solid ratio of 40 mL/g. The resulting solid washed with distilled water until neutral. Then, the demineralized samples were dried and weighed. Deproteinizaton of chitin was carried out using 1.0M NaOH (0.2 ml/g) at 70 °C. The treatment was repeated several times. The absence of proteins was indicated by the absence of color of the medium at the last treatment, which was left over night. The resulting solution then washed to neutrality. Finally, it was washed with hot ethanol (10 Ml/g) and later boiled in acetone to remove any impurities. The purified chitin was then dried. The chitin content was determined from the weight difference of the raw materials and that of the chitin obtained after acid and alkaline treatment (Al Sagheer *et al*., 2009). Waxy particles collected from cassava mealy bugs with adults and larvae were dissolved in chloroform, then the chloroform was evaporated and the waxy particles extracted with n-haxane to remove short-chain compounds which were more soluble in hexane than were the long-chain compound (Soroker *et al*., 2003).

Determination of crude protein was done by Kjeldahl method at the Food technology, Suranaree University of Technology, Nakhonratchasima, Thailand. In the presence of digestion mixture, the digestion was made by heating the samples with concentrated Sulphuric acid (H_2SO_4) to find protein content. The mixture was then made alkaline. Thus Ammonium sulphate ($(NH_4)_2SO_4$) was formed and the released ammonia was collected in 2% boric acid (H_3BO_3) and against standard hydrogen chloride (HCl) it was titrated (AOAC, 2005; Official Methods of Analysis of AOAC International, Method 968.06 and 992.15). For total protein calculation, the amount of nitrogen was multiplied with constant factor (6.25) (Shah *et al*., 2016).

Soxhlet apparatus was used to perform crude fat extraction. In a clean and previously dried extraction thimble, about 2-4 gm moisture free sample was taken. Then the thimble was placed in an extraction tube. Weight of previously cleaned and dried 200 ml round bottom flask was taken. Then with the solvent (petroleum ether 40-60), it was filled up to one third and was connected with the extraction tube. Top water and burner apparatus remained on. The extraction process took 3-4 hours. After every 5-10 minutes, siphoning occurred at the condensation rate of 3 to 4 drops per second. When the process was finished, thimble was then removed from the extractor and flask was heated to collect all the solvent for future use. The apparatus was allowed to cool down. Then at 105 °C, the flask was dried for 1 hour. Finally it was cooled and weighted again (AOAC, 2005 Official Methods of Analysis of AOAC International Methods 960.39 and 948.22). The oil content of the sample was calculated in percentage as: % fat = (weight of flask+fat) – (weight of empty flask) x100 Weight of sample (Shah *et al*., 2016).

Total carbon and nitrogen were analyzed with Leco CHN-628 analyzer. Soil samples were dried at 105 °C for one hour prior to analysis. The 0.2 g of the soil was weighted into a 502-186 Tin Foil Cup and seal. The samples were then transferred to the analyzer (Leco CHN-628, LECO Corporation, MI., U.S.A.).

3.5 Collection of soil samples in cassava plantation areas in Northeastern Thailand where have been reported to have problems with mealy bugs

Soil samples were collected from different areas of cassava plantation area. Soil sample were collected below 10-15 cm depth. Date and time were noted on sample collecting of 500 g were collected in a plastic bag. Surface of soil sample locations was collected and triplicates samples were obtained at each location in a cassava flied. In each cassava plantation area, soil samples. Soil samples were carried back to the Research cassava center and products, Suranaree University of Technology (Janssen *et al*., 2002).

3.6 Isolation of bacteria that have their ability to digest crucial compositions of mealy bugs

3.6.1 Isolation of soil bacteria

The techniques used for isolation of bacteria were serial dilution and pour plate method (Beishier, 1991; Steubing, 1993). Ten-grams of soil sample were mixed with 90 ml of sterile normal saline. The suspension was thorough mixed, and serial dilution (10^{-1} to 10^{-6}) of each soil sample were carried out 1 ml of each aliquot was pour in skim milk agar (1%) (Appendix A.1.3) tributyrin agar (1%) (Appendix A.1.2) and (1%) colloidal chitin agar (Appendix A.1.1) incubated at 35 °C for 18-24 hr. The zone of hydrolysis was noted for each sample. Colonies with different morphological characteristics and show highest zone were selected. The pure colony was grown on trypticase soy agar (TSA) plate repeatedly and preserved on trypicase

soy agar slant at 4 °C and stock at -80 °C for further study. The pure bacterial isolates were characterized and identified using the method of Holt *et al*. (1994).

3.6.2 Screening and selection of soil bacteria

3.6.2.1 Morphology of colony and cell

Characteristics of morphology of selected isolates were observed by light microscopy with gram staining cells and grown of colony on TSA at 35 °C for 18-24 hr. Cells of selected isolates have to small size and no endospore

3.6.2.2 Extracellular enzyme, protease, lipase and chitinase

Proteinase, lipase and chitinase producing ability of selected isolates was tested again by point inoculation in skim milk agar (1%) (Appendix A.1.3) tributyrin agar (1%) (Appendix A.1.2) and (1%) colloidal chitin agar (Appendix A.1.1) incubated at 35 °C for 18-24 hr. The presence of a clear zone surrounding the colonies in skim milk agar, tributyrin agar and colloidal chitin agar indicated extracellular enzyme producing bacteria (Chae *et al*., 2006).

3.6.2.3 Growth in mealy bug broth (MBB)

Bacterial isolates were tested for growth in mealy bug broth (MBB) containing 0.5% NaCl and K_2HPO_4 0.25% and mealy bugs 2.25% (g/l). Mealy bug broth was prepared by boiling 1 part of cassava mealy bugs (*Phenacoccus manihoti*) with 2 parts of distilled water for 20 min modify methods of Yongsawatdigul, Rodtong, and Raksakulthai (2007). The mealy bug slurry was filtered through cheesecloth and supernatant was collected. The pH was adjusted to 7.0 and the media was autoclaved at 115 °C for 10 min. The purified isolates were cultured in 5 ml of TSB incubated at 35 °C for 18-24 h. The inoculum size of 1% (approximate 10^6 CFU/ml) was added to 10 ml MBB and incubated at 35 °C for 24 h. Growth was enumerated using TSA and incubated

at 35 ℃ for 48 h. The pH was measured using a pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland). Eight out of 44 isolates exhibited growth on mealy bug broth.

3.7 The possibility of selected bacteria to destroy mealy bugs

3.7.1 Cassava mealy bug

Cassava mealy bug, larvae and adult were collected from cassava plantation area of Nakhon Ratchasima province and obtained from Pest management center, Nakhon Ratchasima province, Thailand. Mealy bugs will be studied and reared on pumpkin at room temperature under a 12:12 photoperiod for 1-2 week in the Research Center for cassava and products at Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.7.2 Preparation of bacteria and bioassay

Bacterial isolates were streaked onto trypticase soy agar (TSA) plates (Appendix A.1.5) to obtain single colonies for each isolate. The obtained single colonies were inoculated into trypticase soy broth (TSB) (Appendix A.1.4) and incubated at 35 °C overnight. After incubation, the bacterial density was measured at optical density (OD600) and approximately 10^8 cfu/ml. Five milliliters of this culture was centrifuged at 8,000-10,000 rpm for 10 min. After that, the pellet was resuspended in 5 mL of normal saline and used in bioassays (Ozkan-Cakici *et al*., 2014).

The healthy larvae and adult were selected at random and used in bioassays. Fresh small pieces of cassava stem (approximately 8-9 cm) were used as the diet. Experiments were performed with 40 larvae and adult per replicate, and 3

replicates of each treatment group were used. A 2 mL bacterial suspension of each isolate prepared as described above was saturated spray on fresh small pieces of cassava stem with cassava mealy bugs and placed into sterile Petri dish (10 cm in length and 10 cm in width) containing a sterile tissue paper compared with sterile soil sample and sterile 0.85% normal saline served as controls.

3.7.3 Investigations of isolated bacteria effects on cassava mealy bugs

Cassava mealy bugs mortality was recorded for 5 days (no movement or response to stimulus and changed color). Three replicates for each treatment were established and investigate under light microscopy (LM), stereo microscope, scanning electron microscope (SEM) and culture on plates (Parsa *et al*., 2012; Sirisena *et al*., 2015; Zhang, 2009 and 2012; Xie, 2010).

3.8 Identification of dominant bacterial strains

3.8.1 Morphological characterization

Bacterial isolates were identified by morphological and physiological characterization, and 16S rRNA gene sequence analysis as follow: morphological characterization including Gram stain reaction and cell morphology of bacterial isolates were determined and compared to data of known organisms described in the Bergey's Manual of Systematic Bacteriology (Krieg *et al*., 2001), Microbiology: A Laboratory Manual (Cappuccino and Sherman, 1999), and Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

3.8.2 Physiological characterization

Some physiological characteristics were determined according to Krieg *et al*. (2001), Cappuccino and Sherman (1999), and Holt *et al*. (1994) as follows:

3.8.2.1 Catalase test

The overnight cultures of bacterial isolates cells were transferred to the surface of a glass slide. One or 2 drops of 3% hydrogen peroxide (Appendix A.2.6) was added over the cells. Rapid appearance of gas bubbles indicated a positive result.

3.8.2.2 Oxidase test

The overnight cultures of bacterial isolates were streaked onto filter paper (Whatman no. 4) which was placed into a petri dish and wet with 0.5 mL of 1% dimethyl-p-phenylenediamine dihydrochloride (Appendix A.2.1). The development of a deep blue color at the inoculation site within 10 sec indicated a positive result.

3.8.2.3 Motility test

Pure cultures of bacterial islolate were streaked on TSA (Appendix A.1.5) and incubated at 30 °C for 16-18 h. Colonies were stabbed into motility medium (Appendix A.1.13) and incubated at 35 °C for 24-48 h. The growth of bacteria out of needle stabbing indicated a positive test while no spreading growth indicates a negative test.

3.8.2.4 Gelatin hydrolysis

The overnight cultures of isolated bacteria was stabbed into gelatin medium (Appendix A.1.14) and incubated at 35 °C for 48 h. Gelatin hydrolysis was indicated by the liquification of the medium after the tube was kept at 4 °C for 30 min.

3.8.2.5 Nitrate reduction test

Pure cultures of isolated bacteria were streaked on TSA and incubated at 35 °C for 16-18 h. The colonies were inoculated to the nitrate reduction broth (Appendix A.1.10) and incubated at 35 °C for 48 h. Then, 1 mL of solution A (Appendix A.2.9) was added to each culture tube, followed by 1 mL of solution B (Appendix A.2.9) and mixed thoroughly. The development of a red color indicated a positive test while no red color developed indicates a negative test. The result of a negative test was confirmed by adding small amounts of zinc powder. The true negative test was indicated by the development of the red color.

3.8.2.6 Methyl red test

The overnight cultures of isolated bacteria on TSA were inoculated to MR-VP medium (Appendix A.1.8) and incubated at 35 °C for 48 h. Five drops of the methyl red solution (Appendix A.2.8) were added directly to the broth. The development of a stable red color in the medium indicated a positive result.

3.8.2.7 Voges-Proskauer test

The overnight cultures of isolated bacteria on TSA were inoculated to MR-VP medium (Appendix A.1.8) and incubated at 35 °C for 48 h. Five milliliters of solution A (Appendix A.5.4) were added to the medium, followed by 3 mL of solution B (Appendix A.5.4), then mixed gently to allow the medium to expose atmospheric oxygen, and left to be undisturbed for 30 min. A positive result was indicated by the development of a red color within 15 min or more but was not more than 1 h after reagents were added.

3.8.2.8 Indole formation

The overnight cultures of isolated bacteria on TSA were inoculated to LIM medium (Appendix A.1.7) and incubated at 35 °C for 48 h. A few drops of Kovács' reagent solution (Appendix A.2.7) were then added. The positive result indicated by a bright fuchsia red color at the interface of the reagent and the semisolid medium within seconds after adding the reagent.

3.8.2.9 Carbohydrate fermentation

Acid production from carbohydrate tested using the method according to Chookietwattana (2003). Colonies of isolated bacteria grown on TSA at 35 °C for 16-18 h were transferred to 5 mL of sterile phosphate buffer (Appendix A. 2.10) to obtain a 4-6 McFarland unit suspension compared to McFarland turbidity standards (Appendix A.3.1). Then, 200 µL of McFarland unit suspension were transferred to 6 mL of phenol red broth (Appendix A.1.15) and mixed thoroughly. The 6.8% concentration of eight sugars (L-arabinose, D-fructose, Dgalactose, D-glucose, lactose, D-mannitol, D-mannose, and D-xylose) were prepared by filtration through a 0.45 µm pore size membrane filter (Whatman, PuradiscTM, England) onto the sterile container. Fifty microlitters of each sugar (for obtaining the final concentration of sugar at 2%) was distributed in each well of a sterile micro titer plate (recorded of which well contains which sugar). Then, 120 µL of the phenol red broth containing bacterial suspension was added to each carbohydrate in the microtiter plate. The plate was incubated at 35 °C for 24-48 h. Acid production was indicated by the color of the broth changing from red to yellow.

3.8.2.10 Hydrogen sulfide production

The overnight cultures of isolated bacteria was stabbed deep into the TSI agar (Appendix A.1.6) slant and also streaked on the surface of the slant, then incubated at 35 °C for 48 h. Black color formed on the surface and along the stabbed line indicated the positive result.

3.8.2.11 Biochemical characterization using API identification system

Biochemical characteristics of bacterial isolates were also determined using API identification system (bioMérieur® Inc., France). Gram-positive cocci were determined by API 20E was used for Gram-negative rod bacteria according to the manufacturer follows as: API 20 E is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods bacteria. The overnight cultures of bacteria on TSA were used to prepared suspension with turbidity equal to 0.5 McFarland (Appendix A.3.1). API 20 NE strip was opened with an aseptic technique. Sterile distilled water was added to the tray of test kit. Then, the strips were put on the tray with water. The inoculum was filled in the strip tubes. Sterile emulsion oil (Appendix A.3.3) was dropped on the cupules of the 3 underlined tests, GLU, ADH, and URE until a convex meniscus is formed. The kit was covered with lid and incubated at 29 °C (± 2 °C) for 24 h (± 2 h). After the incubation period, one drop of NIT 1 and NIT 2 reagents were added into the NO3 capsule for nitrate reduction test. After 5 min, a red color indicates a positive reaction to be recorded on the result sheet. One drop of JAMES reagent was added into TRP tube for indole formation test. The reaction take place immediately, a pink color which develops in the whole cupule indicates a positive reaction. A yellow color and opaque of various

sugars were indicated as positive. All of results were recorded compared to known organisms for API database (bioMérieux, Inc.).

3.8.3 16S rRNA gene characterization

Sequencing of 16S rRNA was used as a tool for species identification. There are 5 major steps for 16S rRNA gene characterization of bacterial isolates in this study: genomic DNA extraction, 16S rRNA amplification, PCR product purification, 16S rRNA sequencing, and 16S rRNA sequence analysis and phylogenetic tree construction.

3.8.3.1 Genomic DNA extraction

Genomic DNA extraction from bacteria isolates was performed as described by Sambrook and Russell (2001). Genomic DNA of the selected isolate bacteria that is cultivates on agar medium, and incubated at 37 °C for 18 h. Bacterial cells are washed by scraping into microcentrifuge tube containing 1 ml of 50 Mm saline-EDTA (pH 8.0) (Appendix A.4.2) and centrifuged at 12,000 rpm for 5 min at 4 °C. The cell pellet is resuspended with 240 µl of 50 mM EDTA (pH 8.0), then 30 µl of 10 mg/ml lysozyme (Appendix A.2.2.6) is added. The mixture is incubated at 37 °C for 90 min for cell lysis, followed by the addition of 30 µl of 10% SDS (Appendix A.4.9). Samples are then incubated at 80 °C for 5 min and left to cool down to room temperature. Subsequently, the mixture is added with 30 µl of 20 mg/ml proteinase K and 2 µl of RNase solution (10 mg/ml), mixed by inversion, and incubated at 37 °C for 30 min. Add an equal volume of phenol:chloroform (1:1) (Appendix A.4.10) for protein removal and mix well but very gently to avoid shearing the DNA by inverting the tube until the phases are completely mixed. Centrifuge at 12,000 rpm for 5 min at 4 °C. The supernatant is transferred into a new sterilized microcentrifuge tube. Add

(1:10) volume of sodium acetate (Appendix A.4.13) mix and add 0.6 volumes of isopropanol and mix gently until the DNA precipitates and centrifuged at 12,500 rpm for 10 min at 4 °C. The supernatant is discarded. The DNA pellet was washed with 600 µl of 70% ethanol then dried at 37 °C for 1 h. Fifty microliters of TE buffer (Appendix A.4.3) are added, and kept overnight at 4 °C to allow DNA to dissolve. The extracted DNA is detected using 1% agarose (Low EEO Agrarose, BIO 101, Inc., U.S.A.) gels electrophoresis (Bio-Rad, Bio-Rad Laboratories Inc.) in TBE buffer (pH 8.3), stained with ethidium bromide (1 mg/ml), and examined under UV transilluminator (Bio-Rad, Bio-Rad Laboratories Inc.). The concentration of DNA was measured by SmartSpecTm 3000 spectrophotometer at 260 nm (Bio-Rad, Bio-Rad Laboratories Inc.) and DNA purity with respect to contaminats, such as protein, was calculated from the the ratio of optical density at A260/280. Then, purified DNA solution was maintained at -20 °C until use.

3.8.3.2 16S rRNA amplification

The 16S rRNA of bacteria was amplified by polymerase chain reaction (PCR) using primers fD1 and rP2 (Weisburg *et al*., 1991) as forward and reverse primers to obtain approximately 1,500 bp of 16S rRNA gene (Table 3.1). Each 50 µL reaction mixture contained 2.5 µL of genomic DNA (approximately 100 ng/µL), 14.25 µL of MilliQ water, 5 µL of 10× buffer (200mM Tris-HCl, pH 9.1; 500 mM KCl), 1.5 Ml of 25 mM $MgCl_2$, 1.0 µL of dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), 1.0 µL of 20 µM (20.0 pmoles/ µL) of each primer (fD1 and rP2) (Table 3.1), 0.5 µL of *Taq* DNA polymerase (5 units/µL; New England Biolabs Inc**,** U.S.A.), and adjusted volume to 50 µL with deionized water. The PCR was performed using a thermal cycle (Bio-Rad, Bio-Rad Laboratory Inc). The

program of amplification consisted an initial denaturation at 95 °C for 2 min was followed by 35 cycles with denaturation at of 95 °C (45 sec), annealing at 55 °C (45 sec) and extension at 72 °C (2 min), and a final extension at 72 °C for 7 min and holding at 4 °C . The PCR amplified products were examined by gel electrophoresis. Five microliters of PCR-amplified product was thoroughly mixed with 6X loading buffer (Appendix A.4.5). The mixture was loaded into the gel, and electrophoresis was carried out at constant 100 volts for 5 min and 50 volts for 30 min. DNA ladder (1 kbp; Fermentas International Inc., Canada) was used size reference of the PCR products. After electrophoresis, the agarose gel was stained with ethidium bromide (Appendix A.4.6) by soaking the gel in a solution containing 10 μg/mL of ethidium bromide, and visualized under UV transilluminator (BioRad UV-Transilluminator Universal Hood, BioRad, U.K.). The agarose gel was photographed for being reference.

Table 3.1 Oligonucleotide primers used for PCR amplification and direct sequencing of 16S rRNA gene.

Primer	Primer sequence (5′ to 3′)	Target region[a]	Reference
fD1 (PCR amplification)	5′-AGAGTTTGATCCTGGCTCAG -3′	8-27	Weisburg *et al.* (1991)
rP2 (PCR amplification)	5′-ACGGCTACCTTGTTACGACTT-3′	1490-1511	Weisburg *et al.* (1991)
Forward primer (Sequencing)	5′-TAACTACGTGCCAGCAGCC-3′	515-533	Udomsil (2008)

[a]: *Escherichia coli* numbering.

3.8.3.3 16S rRNA sequencing

The cloned ribosomal DNA was sequenced using the BigDye® Terminator Ready Reaction kit version 3.1 (BigDye®, Applied Biosystems, Inc., U.S.A.) according to the manufacturer's protocol. The 10 µL cycle sequencing reaction mixture contained 80-200 ng DNA, 4 µL BigDye, and 5 pM primer. Primers used for sequencing 16S rRNA were provided in the kit. The thermal profile consisted of 25 cycles of 10 sec at 96 °C, 5 sec at 50 °C, and 4 min at 60 °C. The cycle sequencing was performed in the thermal cycle GeneAmp® 9700 (ABI, Applied Biosystems, Inc., U.S.A.). The PCR mixtures were spun down briefly before DNA precipitation. The sequencing DNA fragments were precipitated by adding 16 µL of deionized water and 64 µL of 95% ethanol. The tube was vortexed briefly, incubated at 4 °C for 15 min, and then spun at 12,000 rpm for 20 min at 4 °C. The DNA pellet was washed with 300 µL of 70% ethanol, centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatant was discarded. The DNA pellet was dried at room temperature in the dark. The sequencing gel used for an ABI 377 automated DNA sequencer (Perkin Elmer) was prepared as described in the manufacturer's protocol. The 6% polyacrylamide gel was casted in slab gel glass plates. The DNA pellet was dissolved in 3 µL of loading buffer, and loaded onto the gel. Electrophoresis was carried out at constant 750 volts for 8 h. Fluorescent signals were detected with ABI collection software. Base calling was performed using sequencing analysis software, and nucleotide sequence determination was performed using sequence navigator software. The resulting sequences were assembled and manually corrected by using Chromas 1.56 program (Technelysium Pty. Ltd). The sequences obtained were deposited to the GenBank (NCBI, U.S.A.).

3.8.3.4 16S rRNA sequence analysis and phylogenetic tree construction

Nucleotide sequence data obtained from DNA sequencing software of ABI377 Automated DNA (Perkin Elmer, Applied Biosystems, Thermo Fisher Scientific Inc). Sequence was interpreted and converted to single letter code in text file format by the Chromas 1.56 program. The sequence was also corrected by manual inspection of the chromatogram, and compared to local alignment search of the GenBank database using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnological Information (NCBI). (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic tree was inferred using the neighbour-joining method with software MEGA version 4 (Kumar, Tamura, and Nei, 2004). The stability relationships were evaluated by a boot strap analysis of 1,000 replications.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Collection of mealy bugs from cassava plantation areas in Northeastern Thailand

This study was collected samples of cassava mealy bugs from infested different cassava field in Northeastern Thailand. Cassava plantation area, in Thailand has reported 45 provinces, Nakhon Ratchasima province was highest plantation areas. In 2011-2013, found 20 provinces have spread of cassava mealy bugs and high cause severe in three provinces such as Nakhon Ratchasima, Chaiyaphum and Buriram province (Figure 4.1) reported by Office of Agricultural Economics (2012). This studied were collected cassava mealy bugs from these three provinces, where the cassava devastation has been reported during March 2011 and April, 2013 (Figure 4.2). Fourteen plantation of cassava was collected in different districts, such as Muang, Pakthongchai, Chockchai, Konburi, Serngsarng, Nongbunmark, Seakeaw, Khamthalasore, Sungnern, and Dankhunthod of Nakhon Ratchasima province, Pakhum and Lahan Sai districts of Buriram provinces, and Chatturat and Muang districts of Chaiyaphum provinces in Northeastern Thailand (Table 4.1). Collected larvae were removed and put into plastic boxes (25 cm in length and 18 cm in width) with perforated covers to permit airflow, and small pieces of cassava leaves were provided as food and the boxes were immediately transported to the laboratory. After the transportation, larvae were carefully taken by a soft paintbrush in to on pumpkin

for fed increased number of cassava mealy bugs at the room temperature under a 12:12 photoperoid for 1 week. The pumpkin was washed twice in sterile distilled water and dried before being given to the larvae. Mealy bugs will be reared in the Research Center for cassava and products at Suranaree University of Technology, Nakhon Ratchasima provinces, Thailand. Healthy and diseased larvae were then separated and used for bacterial isolation.

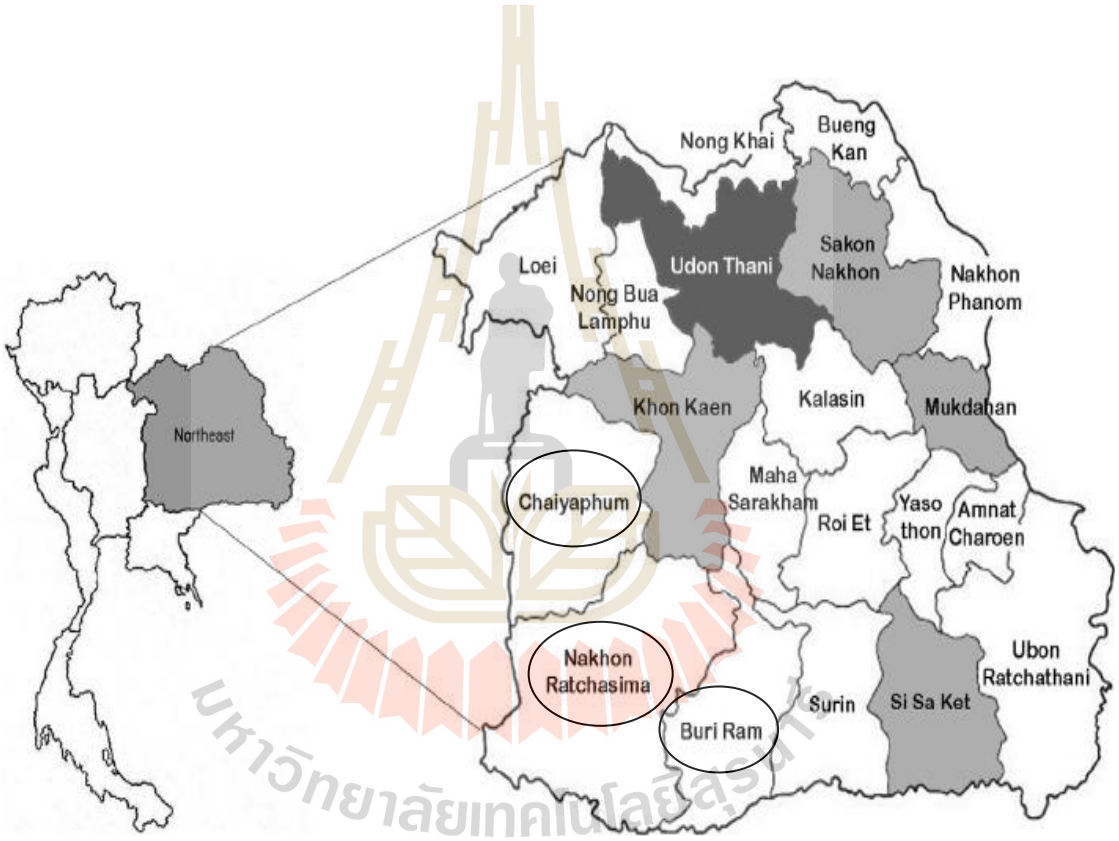


Figure 4.1 Different plantation area was collected of cassava mealy bug, with high severe cause, Nakon Ratchasima, Chaiyaphum and Buriram provinces in Northeastern Thailand.

Mealy bug both adults and nymphs are found to completely cover shoots on branches, at which point, the shoots turn brown and abscise, resulting in 70–80% of loss Feeding directly affects the growth of plant by the extraction of nutrients from shoots and twigs (Chen *et al*., 2009). Infestation can cause growth reduction or malformation, loss of plant vigor, stunting, defoliation, reduced seed production, and timber yield, and even death of the plant Sooty mold induced by honeydew secreted from mealy bugs also hampers photosynthesis in plant and indirectly affects plant growth (Chen *et al*., 2009). The infestation-induced effects reported in the physical environment of plant habitats include changes in humidity, temperature, water sources, local air conditions, soil fertility, and canopy openings. These changes can further alter ecosystem functions and services (Xie *et al*., 2001; Jin *et al*., 2011). The cassava mealy bug uses its stylets to feed in specific tissues of plant. The route of mealybug mealy bugs stylets in plants was monitored by electro-penetrography. After penetrating the epidermis, the stylets mainly follow an extra-cellular route in the mesophyll before reaching the xylem and phloem cells in general. Mealy bugs has been reported as being a phloem feeder (like aphids and whiteflies) but has a more sedentary way of life (except for whitefly nymphs), and this is reflected in mealy bugs feeding behavior. Mealy bugs produce a much lower number of probes of longer duration than those produced by aphids. The sequence of feeding behavior events reported for mealybugs is quite similar to that reported for aphids: insertion of the stylet in the plant, intercellular stylet pathway with a series of intracellular stylet punctures into the mesophyll and, immersed on the intercellular pathway, periods of xylem ingestion and phloem-related activities (presumably salivation followed by long periods of phloem sap ingestion). This is not surprising as aphids (Aphidoidea)

and mealybugs (Coccoidea) are the most closely related superfamilies within the Sternorrhyncha suborder according to molecular phylogenetic studies (Cid and Fereres, 2010).

Figure 4.2 Different cassava field were collected, stage 8-12 mouths (A, B and C), 1- 4 mouths (D, E and F) and cassava mealy bug were found on the underside of leaves of the plant, especially around the main veins (G, K), the top of leaves (H), stem (I, J and L), root (M) and in soil (N).

Table 4.1 Collection of cassava mealy bugs in cassava plantation areas where have been reported high problem with mealy bugs in Northeastern Thailand.

Year	Location (provinces)	Cassava variety	Growth stage of cassava	Part of cassava to found mealy bugs	Growth stage of mealy bug
2011-2013	Nakhon Ratchasima	Kasetsart 50, Huay Bong 60 and Rayong 72	stage 1-4 months, 4-8 months, 8-12 months	The underside of leaves, on stems, leafstalk, the top of leaves and roots	Adults , Nymphs and eggs
	Buriram	Kasetsart 50, Huay Bong 60 and Rayong 72	stage 1-4 months, 4-8 months	The underside of leaves, on stems, leafstalk and high to found on the top of leaves	Adults , Nymphs and eggs
	Chaiyaphum	Kasetsart 50, Huay Bong 60 and Rayong 72	stage 1-4 months, 4-8 months	The underside of leaves, on stems, leafstalk and high to found on the top of leaves	Adults , Nymphs and eggs

4.2 Determination of structure and some chemical compositions of mealy bugs collected from cassava plantation areas in Northeastern Thailand

4.2.1 Determination of structure of cassava mealy bugs under stereo microscopy

A total of cassava mealy bugs specimens were confirmed for their identifications relied on morphological characteristics which belong to genera; *Phenococcus*, *Pseudococcus*, and *Ferrisia*. (Appendix B). In the laboratory, specimens insect samples included the 1st instar nymph, 2nd instar nymph, 3rd instar nymph and female adult were observed and photographed under an Olympus stereo-microscope (Figure 4.4). This studied found four different species (Figure 4.3). Early workers used the number of antennal setae as primary characters to separate species (Maskell, 1894; Essig, 1909; Smith, 1911; and Hollinger, 1917). In recent years, several species of economically important mealybugs (Hemiptera: Pseudococcidae) have been introduced into different countries in the Mediterranean and other areas of the Palaearctic Region. These include, *P. madeirensis* Green (Pellizzari and Germain, 2010; Kaydan *et al*., 2012), According to CABI invasive species mealy bugs species, *Phenacoccus manihoti* Matile-Ferrero, are regarded as important invasive species worldwide. Among these species, the pink cassava mealy bug *P. manihoti* Matile-Ferrero, is an important pest of cassava, *Manihot esculenta* Crantz, accidentally introduced into Africa from the New World around 1973. *P. manihoti* is mainly phloemophagous and reproduces by thelytokous parthenogenesis with three nymphal instars. It usually develops the largest populations during the dry season and decreases

during the rainy season (Souissi *et al*., 1998). Pink mealy bug is regarded an important plant pest worldwide (Hodgson *et al*., 2008). This mealy bug may reach high populations and may cause reduced plant growth or plant death. Mealy bug has the potential to inflict significant damage to field crops in all growing regions (Kaydan *et al*., 2013). The overall length of the antenna, as well as the lengths of the individual segments, were recorded in detail by numerous workers until Ferris (1918) decided that variation made is unreliable as a taxonomic character. However, the setae associated with the antennal segments, clearly visible under the SEM, might be useful for separating closely related species (Sirisena *et al*., 2015).

Figure 4.3 Samples of mealy bugs found on cassava (A) and B), the study structure under stereo microscope (50x), *Pseudococcus jackbeardsleyi* Gimpel & Miller (C), *Ferrisia virgata* Cockerell (D), *Phenacoccus manihoti* Matile-Ferrero (E) and *Phenacoccus maderiensis* Green (F).

Figure 4.4 Cassava mealy bugs under stereo microscopy the stage of 1: nymph (10x), 2: adults (50x), 3: adult with eggs (100x) 4: eggs (100x) respectively of species, *Phenacoccus manihoti* Matile-Ferrero (A1, A2, A3 and A4), *Phenacoccus maderiensis* Green (B1, B2, B3 and B4), *Pseudococcus jackbeardsleyi* Gimpel & Miller (C1, C2, C3 and C4), and *Ferrisia virgata* Cockerell (D1, D2, D3 and D4).

4.2.2 Determination of structure of cassava mealy bugs under scanning electron microscopy (SEM)

The pink cassava mealy bug *Phenacoccus manihoti* Matile-Ferrero, is an important pest of cassava, *Manihot esculenta* Crantz, the external morphological characteristics of cassava mealy bugs, species *P. manihoti* Matile-Ferrero were studied under scanning electron microcopy (SEM). Found various wax glands, associated wax secretions, spiracles, ostiole, and different open holes on body wall of mealy bugs (Figure 4.5). Foldi (2004) described and illustrated the morphological characters of all the development stages of mealy bug and noted that the wax filaments around the spiracles of both the first instar and second-instar nymphs were secreted by multilocular pores located at the base of atrium of the spiracles. In the present study, a complex wax gland structure with a central wax reservoir In addition, the wax secreting cells possessed large nuclei and abundant rough endoplasmic reticulum. In biochemistry, the rough endoplasmic reticulum is often associated with lipid synthesis in the cell, and lipids are the primary constituent of the wax secretions of scale insects (Tamaki, 1997; Xie *et al*., 2006). Multilocular disc pores are usually present on the venter, at least around the vulva; sometimes they may be present also on the dorsum. Each pore contains a circle of more than five openings (loculi); the outer ring contained 8-10 loculi. The wax curls produced by the multilocular pores surrounding the vulva become attached to each egg as it leaves the genital opening (Kumar *et al*., 1997). The outer ring of multilocular disc pores has 8-10 loculi in *M. hirsutus,* whereas 6-10 loculi, situated around the circumference, is more common in three other pesudococcids (Cox and Pearce, 1983). The short curly type of wax filaments has been observed on the egg surface of most families of mealybugs and

scale insects, and appears to be universally produced by some forms of multilocular or quinquelocular pores (Hashimoto and Kitaoka, 1971; Hamon *et al.*, 1975; Gerson, 1980). Hashimoto and Ueda (1985) reported the coil-form wax on the egg surface of mealy bug and further compared it with eight other species of the family further, the 'multilocular disc pores located on the ventral surface are :more numerous in oviparous than in viviparous species, whereas in the second instar male they are more scattered over the dorsum. Tamaki *et al.* (1969) reported multilocular disc pores around the genital opening which produced a white powdery wax adhering to the eggs in the coccid *Ceroplastes pseudoceriferus* Green (Kumar *et al*., 1997). Trilocular pores are characteristic of the family Pseudococcidae and the pores are more abundant on dorsum and venter than on the sides (Cox and Pearce, 1983) the trilocular pores situated on the lateral sides of the body are supported by a pair of small setae (cerarian setae), whereas the trilocular pores of dorsum and venter are devoid of setae. Cox and Pearce (1983) studied three species from three genera of Pseudococcidae and found similar trilocular pores in all the instars. The biolocular pores were observed using SEM and represented 70% of all the wax glands. The adult female of mealy bug secreted wax filaments through multilocular disc pores to form an ovisac (Young and Yao, 1986). The wax filaments of these latter species likely exhibit a simple structure and ancestral characteristics because Matsucoccidae and Monophlebidae are relatively ancient groups in the Coccoidea. In contrast, the Coccidae, Eriococcidae and Pseudococcidae are relatively evolutionary groups in the Coccoidea, and most of their wax filaments are hollow. This hollow structure increased the strength of the wax filaments but decreased the amount of material consumption, which may be a derived trait (Foldi, 1991 and 1997; Xie *et al*., 2006 and 2010; Zhang *et al*., 2011). The

circulus is a large, membranous structure found on the midline of the abdominal venter in many mealy bug species; it helps the mealy bug adhere to the substrate. Cerarii, these paired, dorsal structures on the body margins are present only in mealy bugs; they secrete and support the lateral wax filaments in life (Cox and Pearce, 1983). Each cerarius consists of an aggregation of setae of one or two types, trilocular pores, and sometimes discoidal pores (Sirisena *et al*., 2015).

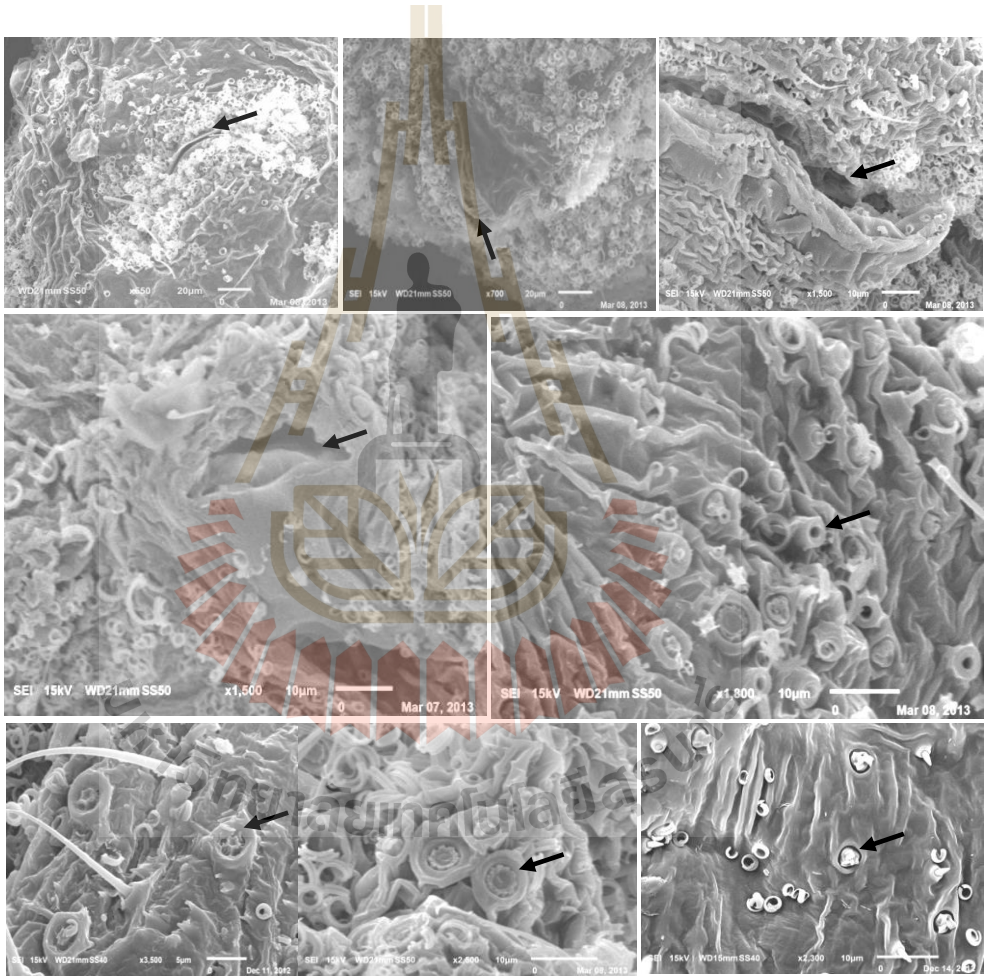


Figure 4.5 Scanning electron microcopy (SEM) micrographs of *Phenacoccus manihoti* Matile-Ferrero, ostiole (A and B), circulus (C), spiracle (D), discoidal pore (E) quinquelocular pore (F), tilocular pore (G), and multilocular pore (H).

4.2.3 Determination of structure of cassava mealy bugs under light microscope (LM) with paraffin section technique by rotary microtome

The internal structure and cytological characters of the wax glands were observed using under light microscope (LM) with paraffin section technique by rotary microtome. In the cross section of the adult female, specie *Phenacoccus manihoti*, a series of wax glands were observed close together in the epidermis (Figure 4.6). There were more wax glands on the dorsal than ventral surface. However, the wax glands were dense and arranged in multiple layers in the V-shaped vulva region. The multilocular pore had a complex structure. The transverse sections of the multilocular pores containing eight loculi clearly revealed a central cell and eight surrounding lateral cells. The longitudinal sections showed a central, bottle-shaped cell at the center of the gland, several lateral cells encompassing the central cell, a wax reservoir located at the top of the central cell, and a tube duct with its bottom end connected to the center cell. The tube duct, through which the wax substances excreted led to the outer cuticle surface (Xie *et al*., 2010). Differences in wax pore ultrastructure may be taxonomically useful. Such differences have been used to revise the generic placement of some scale insect species into families, such as Coccidae, Diaspididae and Monophlebidae (Takagi, 1990; Ulgenturk and Wilhem, 2001; Unruh, 2008; Unruh and Gullan, 2008). Waku (1978), studied wax glands in nymphs of another Asian citrus psyllid by transmission electron microscopy, and indicated that these glands consisted of two kinds of cells, derived from epidermal cells: wax cells, which produce and secrete the wax, and flat interstitial cells found among these cells. Each wax cell has a long, wide duct which opens at the cuticle.

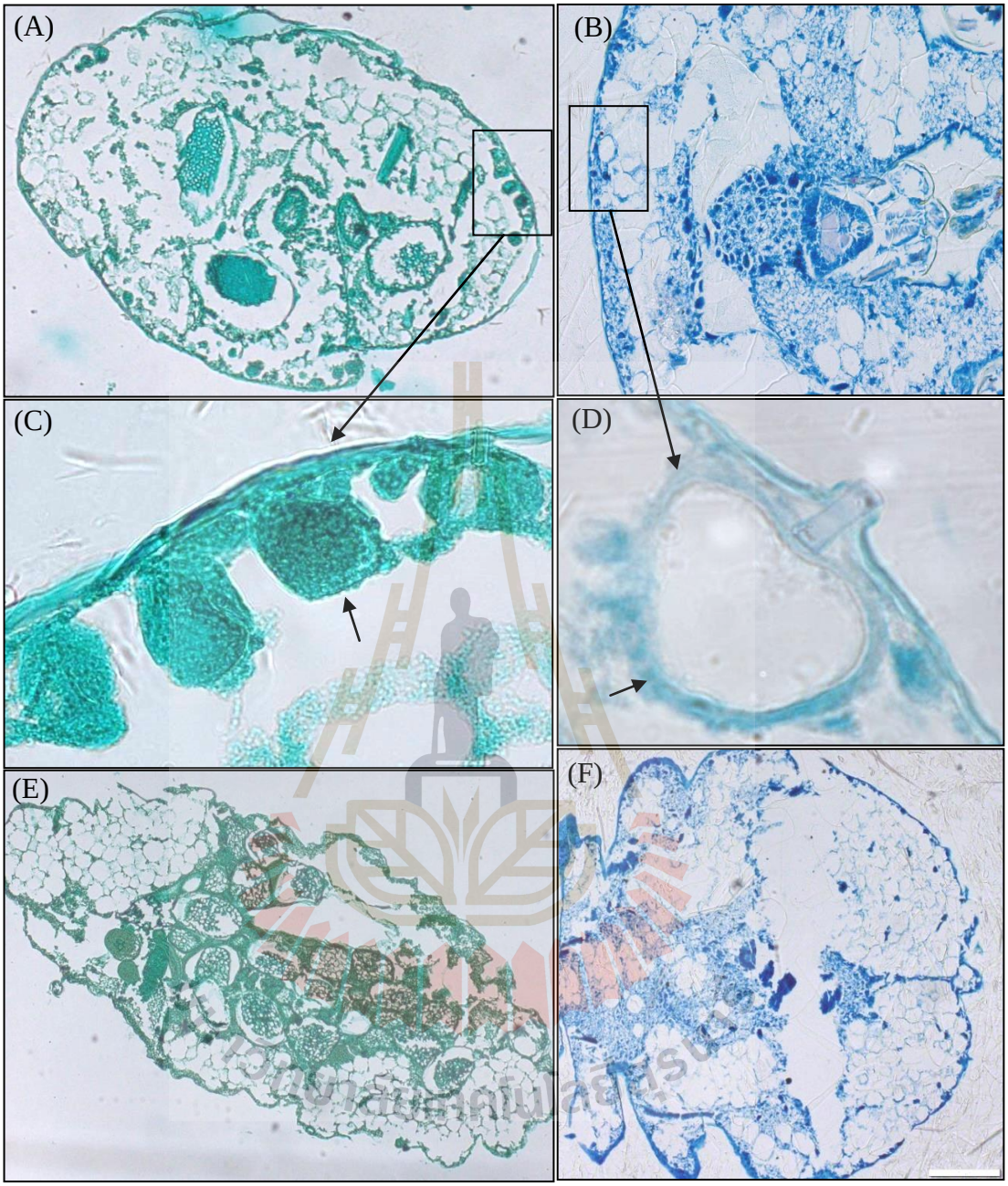


Figure 4.6 Light Micrographs of the adult female of *Phenacoccus manihoti* Matile-Ferrero, (100x) Transection of the abdomen showing the wax glands (arrow) (A, B, C and D), which were more abundant in the dorsal surface than in the ventral surface (E and F).

4.2.2 Determination of some chemical compositions

Chemical compositions of pink cassava mealy bugs consists of crude chitin 45.27%, crude wax 33.03%, crude protein 3.09%, crude fat 45.77%, total nitrogen content 6.74% and total carbon content 46.35% and found high glucose of liquid inside body of cassava mealy bug by HPLC analysis respectively (Table 4.2 and Figure 4.7). Previous investigations on honeydew indicated that its major components were: sucrose, D-fructose, mannose, trehalose, myo-inositol, ribitol, galactose, quinic acid, and malic acid. The chemical compositions of waxes produced by Hemiptera have been investigated mainly in whiteflies and scale insects. The surface lipids on nymphs and exuvia of several whitefly species (Aleyrodidae) contained largely wax esters, long-chain aldehydes, hydrocarbons and long-chain alcohols (Ammar *et al*., 2013). The main chemical compositions of the honey dew extracted vine mealy bugs planococcus vitis. Sucros were present in both the honey dew and the sap of potatoes indicating a lack of invertase activity in the insect gut. Other sugars demonstrated were glucose, fructose, and raffinose. Sixteen amino acid were detected in the honey dew; aspartic acid, glutamic acid, proline, valine, histidine, serine, tyrosine, arginine, alanine, isoleucine, leucine, methionine, threonine, and glycine. Critic and tartaric acid were also detected in the honey dew Calatayud and Ru (2006). Five carbohydrate components and thirteen amino acids were identified in the honey dew of sugar cane mealy bug *Saccharicoccus sacchari* by chromatographic analysis. The surgars are fructose, sucrose, melizitose and raffinose (Salama and Rizk, 1969). Insect cuticle is a very complex structure composed of protein, chitin, and fatty acid. Protein constitutes nearly 50-80% of the total mass (Gupta *et al*., 1992). The exact nature of the linkage between protein and carbohydrates is not known (Brunet,

1980). However, the structure varies considerably depending upon the insect source. Cameron and Drake (1976) indicated that the major components of external waxes of the woolly apple aphid, Eriosoma lanigerum, were diketo esters. More recently, Nelson *et al*. (2000) investigated the chemical composition of wax produced by nymphs of the giant whitefly, Aleurodicus dugesii Cockerell. He showed that the major lipid class associated with wax filaments consisted of saturated wax esters (89%), mainly C44, C46 and C60, and that the major lipid class of the wax 'curls' consisted of wax esters (50%), mainly C44 and C46. Also, longchain aldehydes, alcohols, hydrocarbons and wax esters were major components of the external lipids of adult Aleyrodes singularis Danzig (Ammar *et al*., 2013)

Table 4.2 Chemical compositions of percent average of pink cassava mealy bugs.

Compositions	Wet weight (g)	(%) Average
Crude chitin	2.067±0.019	45.27±1.440[a]
Crude wax	2.092±0.007	33.03±0.530[b]
Crude protein	2.070±0.004	3.09±0.040[d]
Crude fat	2.018±0.028	45.77±0.770[a]
Total nitrogen	0.256±0.003	6.74±0.760[c]
Total carbon	0.256±0.003	46.35±0.270[a]

Data are mean± standard deviation (SD) for triplicate data, letters indicate significant difference within the same column (P<0.05).

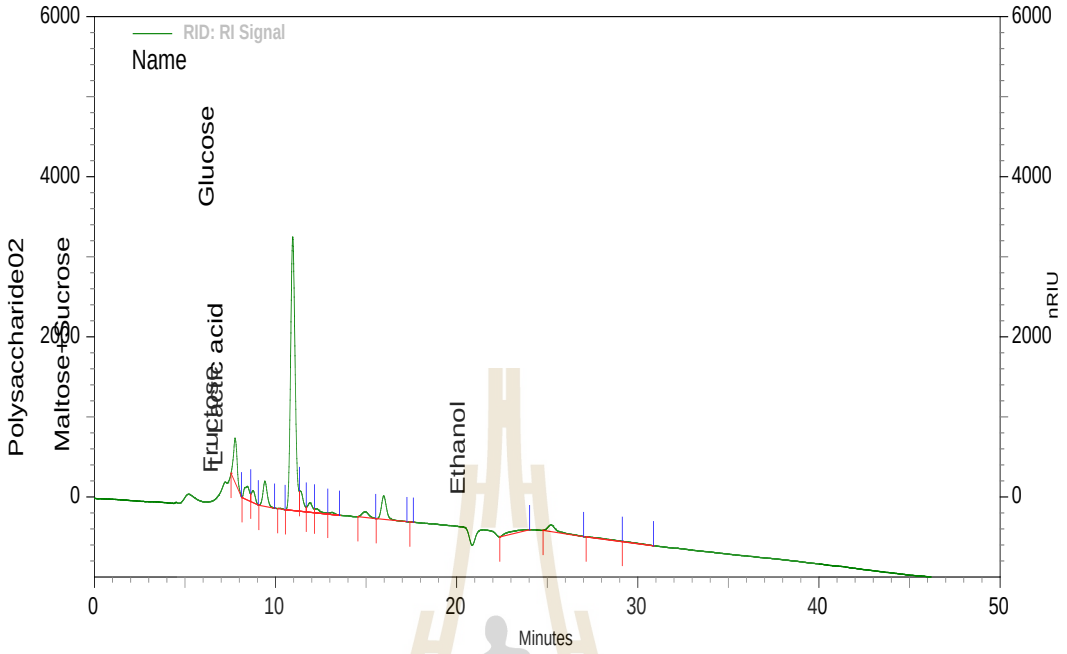


Figure 4.7 HPLC Chromatogram of liquid inside of pink cassava mealy bug with high glucose and some sugar of fructose, maltose and sucrose.

4.3 Collection of soil samples in cassava plantation areas in Northeastern Thailand where have been reported to have problems with mealy bugs

Sources of bacteria isolated were collected at different locations from cassava in plantation areas, Northeastern Thailand, where the cassava devastation has been reported. Soil samples were collected from different areas of cassava plantation area. Soil sample were collected below 10-15 cm depth. Fourteen plantation of cassava was collected in different districts, such as Muang, Pakthongchai, Chockchai, Konburi, Serngsarng, Nongbunmark, Seakeaw, Khamthalasore, Sungnern, and Dankhunthod of Nakhon Ratchasima province, Pakhum and Lahan Sai districts of Buriram provinces,

and Chatturat and Muang districts of Chaiyaphum provinces in Northeastern Thailand. Four hundred and sixteen bacteria isolated from cassava samples were examined for the (Table 4.3).

Table 4.3 Sources of bacteria isolated from soil samples at different locations from cassava in plantation areas, Northeastern Thailand.

No.	Sources of bacteria (province)	Number of areas for collection (district)	Number of isolates
1	Nakhon Ratchasima (Munag, Pakthongchai, Chockchai, Konburi, Serngsarng, Nongbunmark, Seakeaw, Khamthalasore, Sungnern and Dankhunthod districts)	10	302
2	Buri Rum (Pakhum and Lahan Sai districts)	2	49
3	Chaiyaphum (Muang and Chatturat districts)	2	65
	Total	14	416

4.4 Isolation of bacteria that have their ability to digest crucial composition of mealy bugs

4.4.1 Isolation of soil bacteria

Bacteria were isolated from soil samples from differences cassava plantation areas, using skim milk agar (SMA) containing pancreatic digestion of casein 15, yeast extract 5, glucose 2.5, skim milk 10 (g/l) (Atlas, 2010), tributyrin agar (TBA) containing tributyrin 10 ml, peptone 5, yeast extract 3, (g/l) (Atlas, 2010) and colloidal chitin agar (CCA) containing colloidal chitin 10, Na_2HPO_4 6, KH_2PO_4 3,

NH_4Cl 1, NaCl 0.5, yeast extract 0.05, $MgSO_4.7H_2O$ 0.2 (g/l) and all medium added agar 15 (g/l) and adjusted with distilled water (Nurderbyandaru *et al*., 2010) (example of bacterial colonies a shown in Figure 4.8). Streak plate technique was used for the isolation of bacterial isolates on trypticase soy agar (TSB). The plates were incubated at 35 °C for 18-24 h. After incubation, colonies with different morphological characteristics were randomly selected for further identification (Table 4.4).

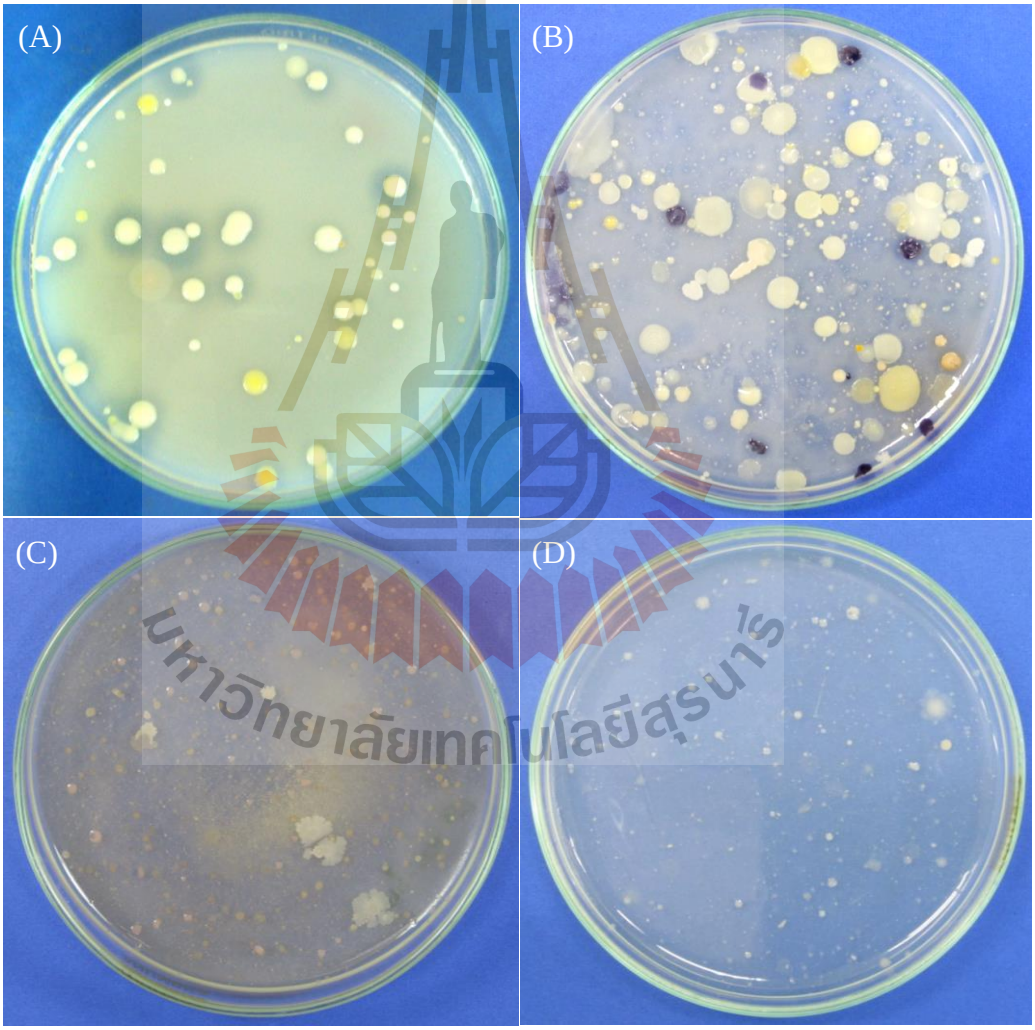


Figure 4.8 Examples of bacterial colonies isolated from soil sample of cassava plantation areas on skim milk agar (SMA) (A), tributyrin (TBA) (B) and colloidal chitin agar (CCA) (C and D) media.

Table 4.4 Codes of selected bacteria isolated from soil samples.

Isolates codes[a]	Source of soil samples	Culture selective medium[b]	Number of bacteria
MG	Nakhon Ratchasima province, Muang district	SMA, CCA,TBA	54
PC	Nakhon Ratchasima province, Pakthongchai district	SMA, CCA,TBA	35
CC	Nakhon Ratchasima province, Chockchai district	SMA, CCA,TBA	48
KB	Nakhon Ratchasima province, Konburi district	SMA, CCA,TBA	27
SS	Nakhon Ratchasima province, Serngsarng district	SMA, CCA,TBA	22
NM	Nakhon Ratchasima province, Nongbunmark district	SMA, CCA,TBA	19
SK	Nakhon Ratchasima province, Seakeaw district	SMA, CCA,TBA	35
KS	Nakhon Ratchasima province, Khamthalasore district	SMA, CCA,TBA	24
SN	Nakhon Ratchasima province, Sungnern district	SMA, CCA,TBA	18
DK	Nakhon Ratchasima province, Dankhunthod district	SMA, CCA,TBA	20
PK	Buri Rum province, Pakhum district	SMA, CCA,TBA	17
LS	Buri Rum province, Lahan Sai district	SMA, CCA,TBA	32
MC	Chaiyaphum province, Muang district	SMA, CCA,TBA	37
CR	Chaiyaphum province, Chatturat district	SMA, CCA,TBA	28
	Total		416

aMG: Muang (Nakhon Ratchasima); PC: Pakthongchai; CC: Chockchai; KB: Konburi SS: Serngsarng; NM: Nongbunmark; SK: Seakeaw; KS: Khamthalasore; SN: Sungnern; DK: Dankhunthod; PK: Pakhum; LS: Lahan Sai; MC: Muang (Chaiyaphum); and CR: Chatturat. bSMA: skim milk agar; TBA: tributytin agar; CCA: colloidal chitin agar.

4.4.2 Screening and selection of soil bacteria

4.4.2.1 Morphology of colonies and cells

From a total of 416 isolates studied, small cell size and no spore-forming for destroy small structure of cassava mealy bugs. 192 isolates were Gram-positive rods have spore-forming and cell sizes between 0.5-0.65×1.0-1.5 μm and 0.70-1.55×3.5-6.5 μm, 98 isolates were Gram-positive rods no have spore-forming and cell sizes between 00.3-0.5×2.0-2.5 μm and 0.5-1.0×2.5-5.0, 75 isolates were Gram-positive cocci having cell sizes of 0.1-0.2 to 0.4-1.2 μm, and 42 isolates were Gram-negative rods and cell sizes between 0.5-0.6×1.0-1.5 and 0.8-1.0×1.5-3.5 μm, which occurred singly, in pairs, or in short chains (Table 4.5) Their colony morphology was observed after incubating on TSA agar medium after 18-24 h incubation under aerobic condition. Circular and irregular colonies with entire and undulate margins; and flat, low convex, convex and umbonate were found. These included colonies punctiform, small, moderate and large colonies with 0.1-4.0 mm in diameters. These isolates were re-streaked on the TSA medium for bacterial purification. Pure cultures were kept in 5% skim milk (final concentration) at -80 °C for further selection.

Table 4.5 Group of bacteria isolated from soil samples according to their cell morphology.

Bacterial group	Cell size (μm)	Endospore	Amount of isolate
Gram-positive rods	0.5-0.65×1.0-1.5 to 0.70-1.55×3.5-6.5	+	192
Gram-positive rods	0.3-0.5×2.0-2.5 to 0.5-1.0×2.5-5.0	-	98
Gram-positive cocci	0.1-0.2 to 0.4-1.2	-	75
Gram-negative rods	0.5-0.6×1.0-1.5 to 0.8-1.0×1.5-3.5	-	42
ND	ND	ND	9
Total			416

ND: not detect

4.4.2.2 Extracellular enzymes, protease, lipase and chitinase

Four hundred and sixteen were tested able of extracellular enzyme for proteases, lipases and chitinases enzyme, point inoculating on skim milk agar (Atlas, 2010), tributyrin agar (Atlas, 2010) and colloidal chitin agar (Nurderbyandaru *et al*., 2010) respectively (Figure 4.9). Forty-four out of 416 isolates could utilize skim milk, tributyrin and chitin, which were observed from clear zone surrounding bacterial colonies (0.1-1.7 cm in diameters). However, skim milk, tributyrin and chitin are substrate specific. Therefore, screening for the purpose of destroying structure of cassava mealy bugs should be carried out using mealy bugs.

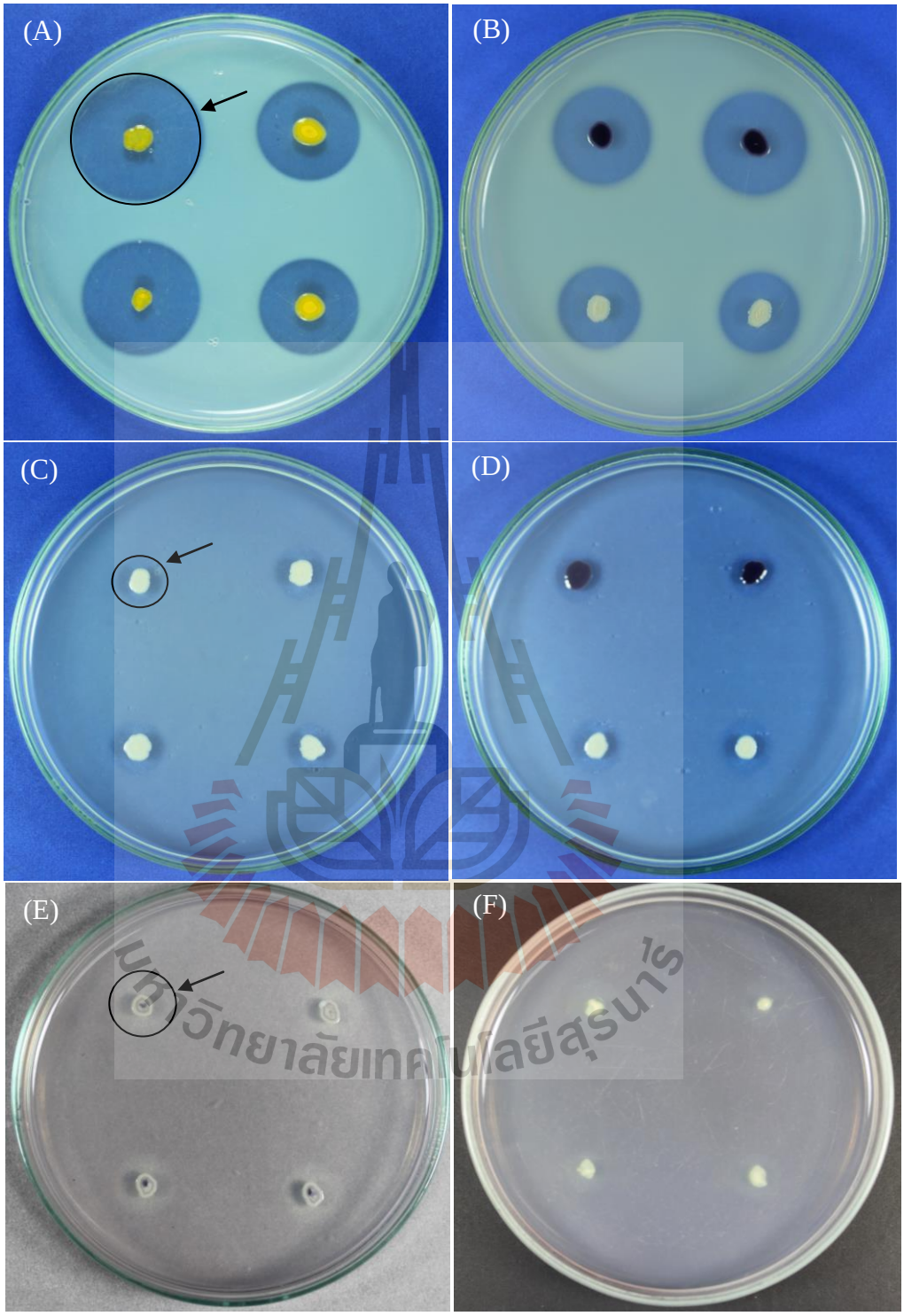


Figure 4.9 Examples of bacterial colonies isolated from soil sample of cassava plantation areas on Skim milk agar (SMA) (A and B), tributyrin (TBA) (C and D) and Colloidal chitin agar (CCA) (E and F) media.

4.4.2.3 Growth in mealy bug broth (MBB)

Eight isolates were tested for growth in mealy bug broth (MBB) containing 0.5% NaCl and K_2HPO_4 0.25% and mealy bugs 2.25% (g/l). Mealy bug broth was prepared by boiling 1 part of cassava mealy bugs (*Phenacoccus manihoti*) with 2 parts of distilled water for 20 min modify methods of Yongsawatdigul, Rodtong, and Raksakulthai, (2007). The mealy bug slurry was filtered through cheesecloth and supernatant was collected. The pH was adjusted to 7.0 and the media was autoclaved at 115 °C for 10 min. The purified isolates were cultured in 5 ml of TSB incubated at 35 °C for 18-24 h. The inoculum size of 1% (approximate 10^6 CFU/ml) was added to 10 ml MBB and incubated at 35 °C for 24 h. Growth was enumerated using TSA and incubated at 35 °C for 48 h. The pH was measured using a pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland). Eight out of 44 isolates exhibited growth on mealy bug broth (Table 4.6 and Figure 4.10).

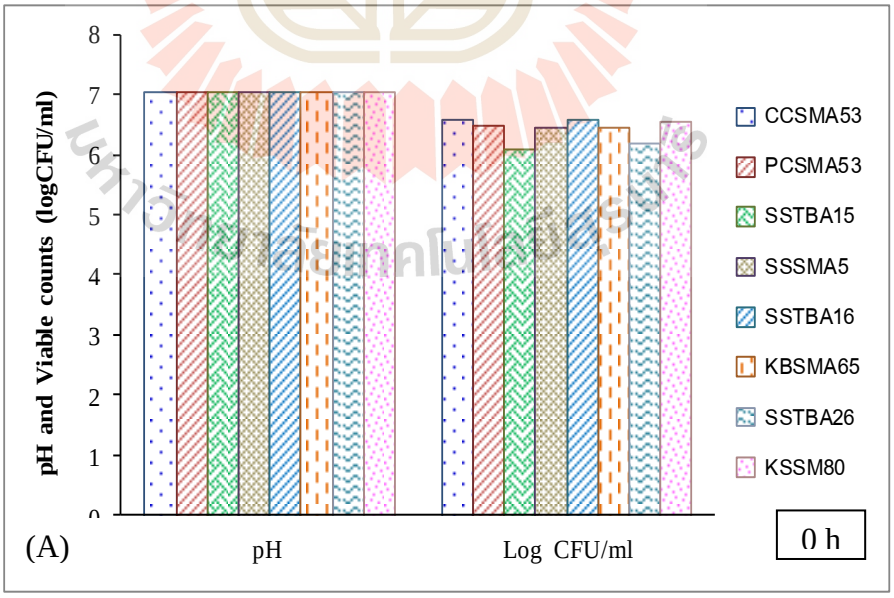


Figure 4.10 Number of cell bacteria (CFU/ml) and pH in mealy bug broth (MBB) at 35 °C for 0 h (A), before incubation.

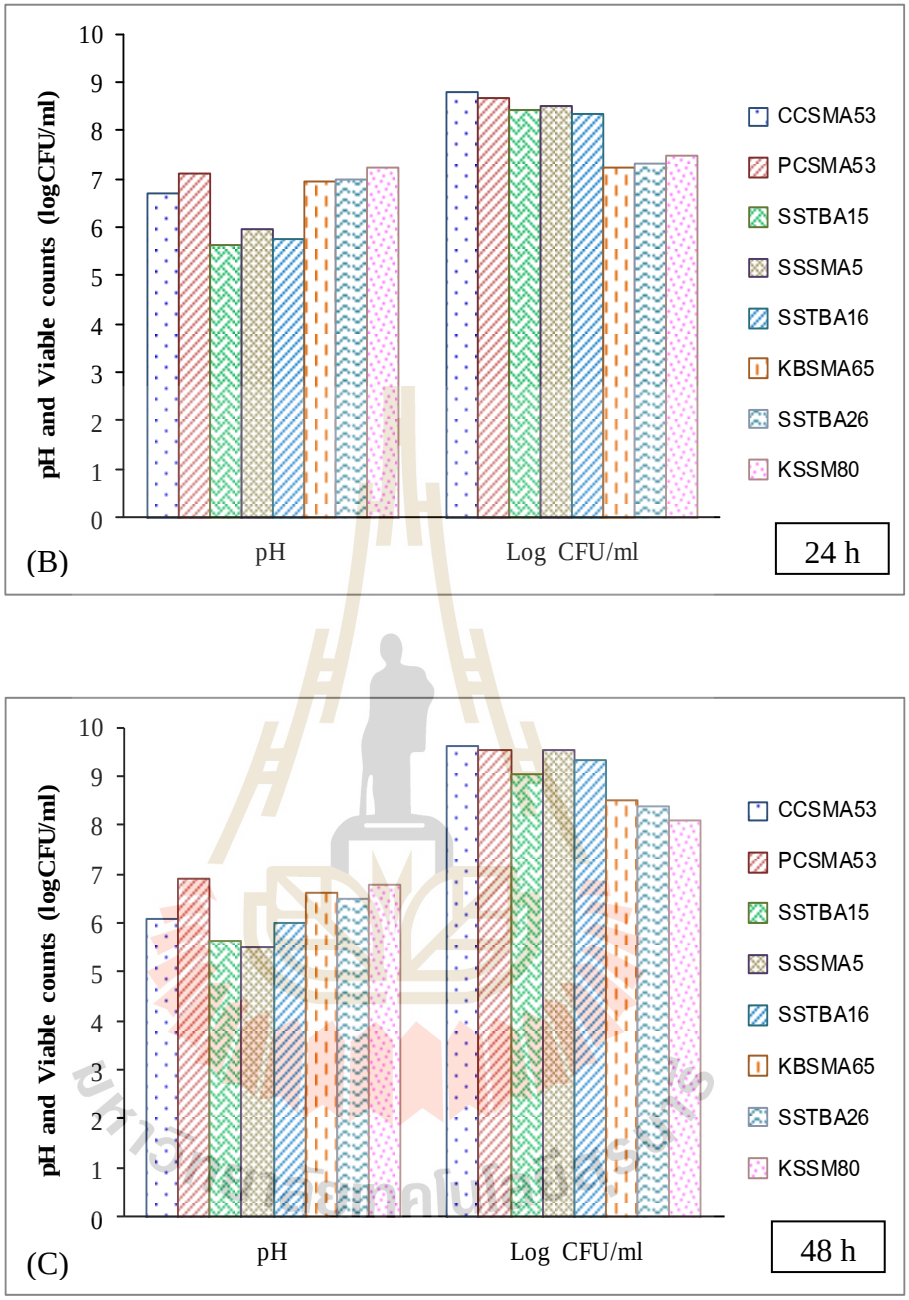


Figure 4.10 (Continued) Number of cell bacteria (CFU/ml) and pH in mealy bug broth, (MBB) at 35 °C for 24 h (B) and 48 h (C), after incubation.

Table 4.6 Number of cell bacteria (CFU/ml) in mealy bug broth at 35 °C for 48 h.

Bacterial code	Period of time (h) for incubation	pH	Number of cell bacteria (CFU/ml)	Log CFU/ml
CCSMA59	0	7.02± 0.057	$3.60x10^{6}$	6.56^{b}
	24	$6.69± 0.014^{d}$	$6.20 x10^{8}$	8.79^{a}
	48	$6.09± 0.014^{c}$	$3.89 x10^{9}$	9.59^{a}
PCSMA53	0	7.02± 0.057	$2.95 x10^{6}$	6.47^{d}
	24	$7.10± 0.028^{b}$	$4.68 x10^{8}$	8.67^{a}
	48	$6.89± 0.007^{a}$	$3.31 x10^{9}$	9.52^{b}
SSTBA15	0	7.02± 0.057	$1.20 x10^{6}$	6.08^{h}
	24	$5.62± 0.007^{g}$	$2.71 x10^{8}$	8.43^{bc}
	48	5.63± 0.007	$1.05 x10^{9}$	9.02^{d}
SSSMA5	0	7.02± 0.057	$2.80 x10^{6}$	6.45^{e}
	24	$5.97± 0.028^{e}$	$3.20 x10^{8}$	8.51^{b}
	48	$5.49± 0.014^{h}$	$3.20 x10^{9}$	9.51^{b}
SSTBA16	0	7.02± 0.057	$3.76 x10^{6}$	6.58^{a}
	24	$5.76± 0.014^{f}$	$2.16 x10^{8}$	8.33^{c}
	48	$5.98± 0.021^{f}$	$2.09 x10^{9}$	9.32^{c}
KBSMA65	0	7.02± 0.057	$2.74 x10^{6}$	6.44^{f}
	24	$6.95± 0.021^{c}$	$1.70 x10^{7}$	7.23^{e}
	48	$6.59± 0.007^{d}$	$3.12 x10^{8}$	8.49^{e}
SSTBA26	0	7.02± 0.057	$1.50 x10^{6}$	6.18^{g}
	24	$6.96± 0.021^{c}$	$2.06 x10^{7}$	7.31^{e}
	48	$6.49± 0.028^{e}$	$2.44 x10^{8}$	8.39^{f}
KSSM80	0	7.02± 0.057	$3.32 x10^{6}$	6.52^{c}
	24	$7.21± 0.035^{a}$	$3.07 x10^{7}$	7.49^{d}
	48	$6.79± 0.014^{b}$	$1.27 x10^{8}$	8.10^{g}

Data are mean± standard deviation (SD) for triplicate data, letters indicate significant difference within the same column (P<0.05).

4.5 The possibility of selected bacteria to destroy mealy bugs

From the screening step and identification, selected isolates eight for possibility for destroying cassava mealy bug. Bacterial isolates, CCSMA59, PCSMA53, SSTBA15, SSSMA5, SSTBA16, SSTBA26, KSSMA80, and KBSMA65, respectively. The bacterial isolates against the nymphs and adults of the pest as a possible biological control agent. For 5 days after application of the eight bacterial suspension. The isolates of CCSMA59, PCSMA53, SSTBA15, SSTBA16, SSSMA5, SSTBA26, KBSMA65, KSSMA80, and 90.83, 92.5, 80.0, 64.17, 57.5, 50.85, 44.18, and 27.50 % mortalities when placed the stem piece of cassava on soil and compared with tissue paper, 70.0, 81.67, 38.33, 35.84, 40.0, 23.35, 28.33, and 18.38 % motilities, respectively. However, these were significantly higher than the control ($p>0.05$). Bacterial isolates, CCSMA59 (*Serratia sp.*), PCSMA53 (*Pseudomonas sp.*), and SSTBA15 (*Chromobacterium sp.*) caused the high mortality with the control ($p > 0.05$). This study need to stimulated considerable interest in using bacteria as biological control agent cassava mealy bug, especially, habitat stage in soil. So, the survival of bacteria isolates when sprayed on soil was studied. However, this studied showed high motility of pink cassava mealy bugs ($p > 0.05$) when sprayed suspension of bacteria isolates on soil more than tissue paper (Figure 4.11). Cassava mealy bugs mortality was recorded for 5 days (no movement or response to stimulus and changed color). Three replicates for each treatment were established and investigate under light microscopy (LM), stereo microscope and scanning electron microscope (SEM) (Figure 4.12-4.14, respectively).

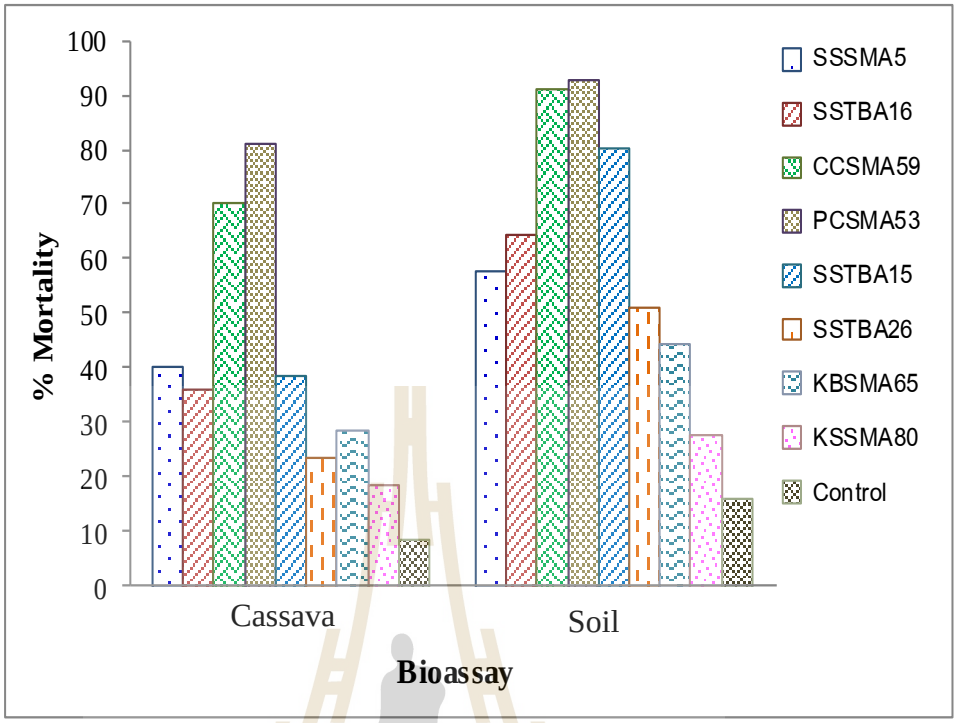


Figure 4.11 Percent mortality of larvae and adult of cassava mealy bugs (*Phenacoccus manihoti* Matile-Ferrero) within 5 days after application compared with stem piece of cassava on cassava and soil. SSSMA5: *Chromobacterium* sp., SSTBA16: *Chromobacterium* sp., CCSMA59: *Serratia* sp., PCSMA53: *Pseudomonas* sp., SSTBA15: *Chromobacterium* sp., SSTBA26: *Chryseobacterium* sp., KBSMA65: *Pseudomonas* sp., KSSMA80: *Chryseobacterium* sp., and Control: 0.85% normal saline.

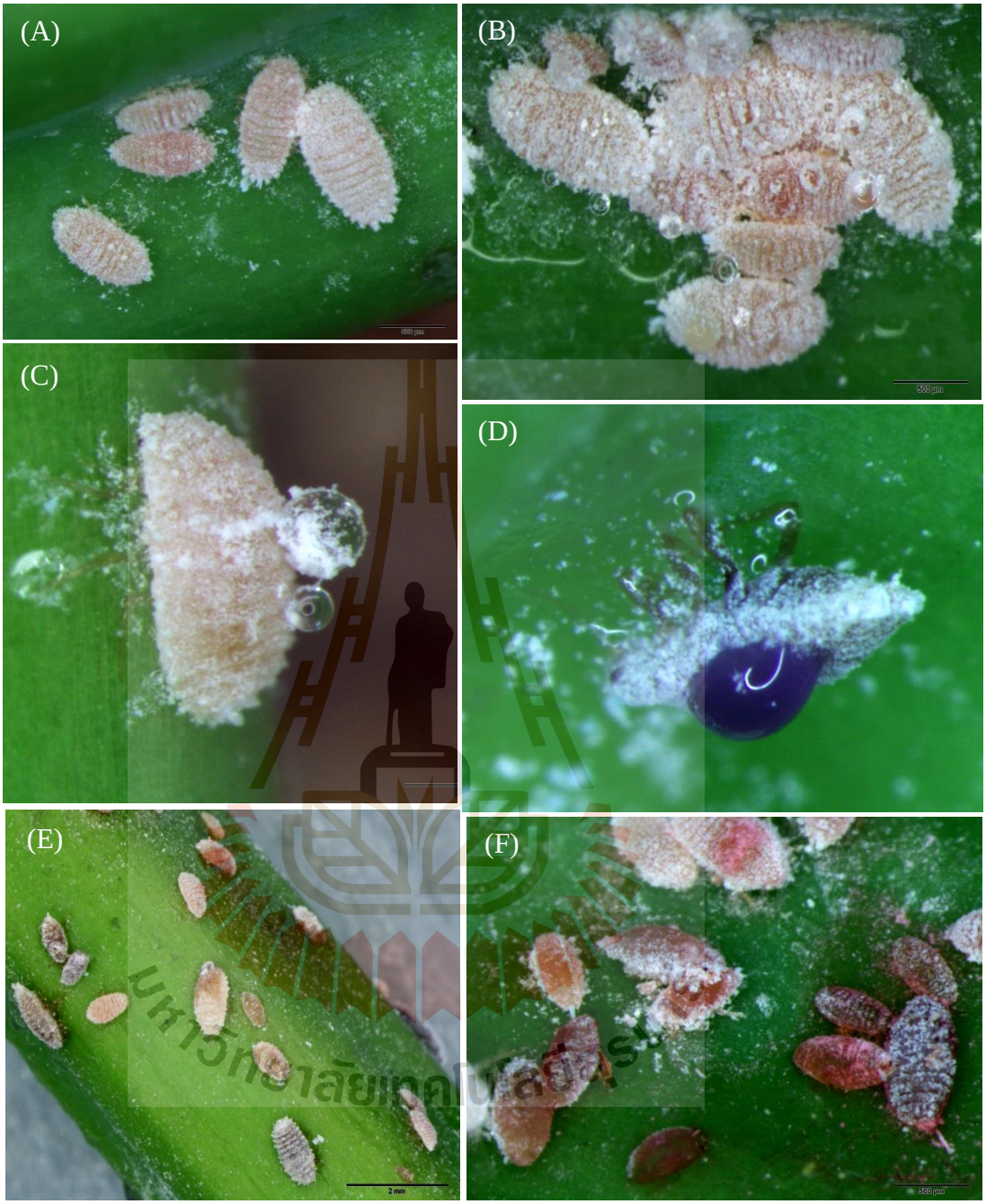


Figure 4.12 Effect of bacterial treatments on cassava mealy bugs, *Phenacoccus manihoti* Matile-Ferrero, no spray (A) spray with 0.85% NSS, for control (B), position of infection, before spray (C) and after spray (D), effect of bacterial isolates on cassava mealy bugs after spray (E and F).

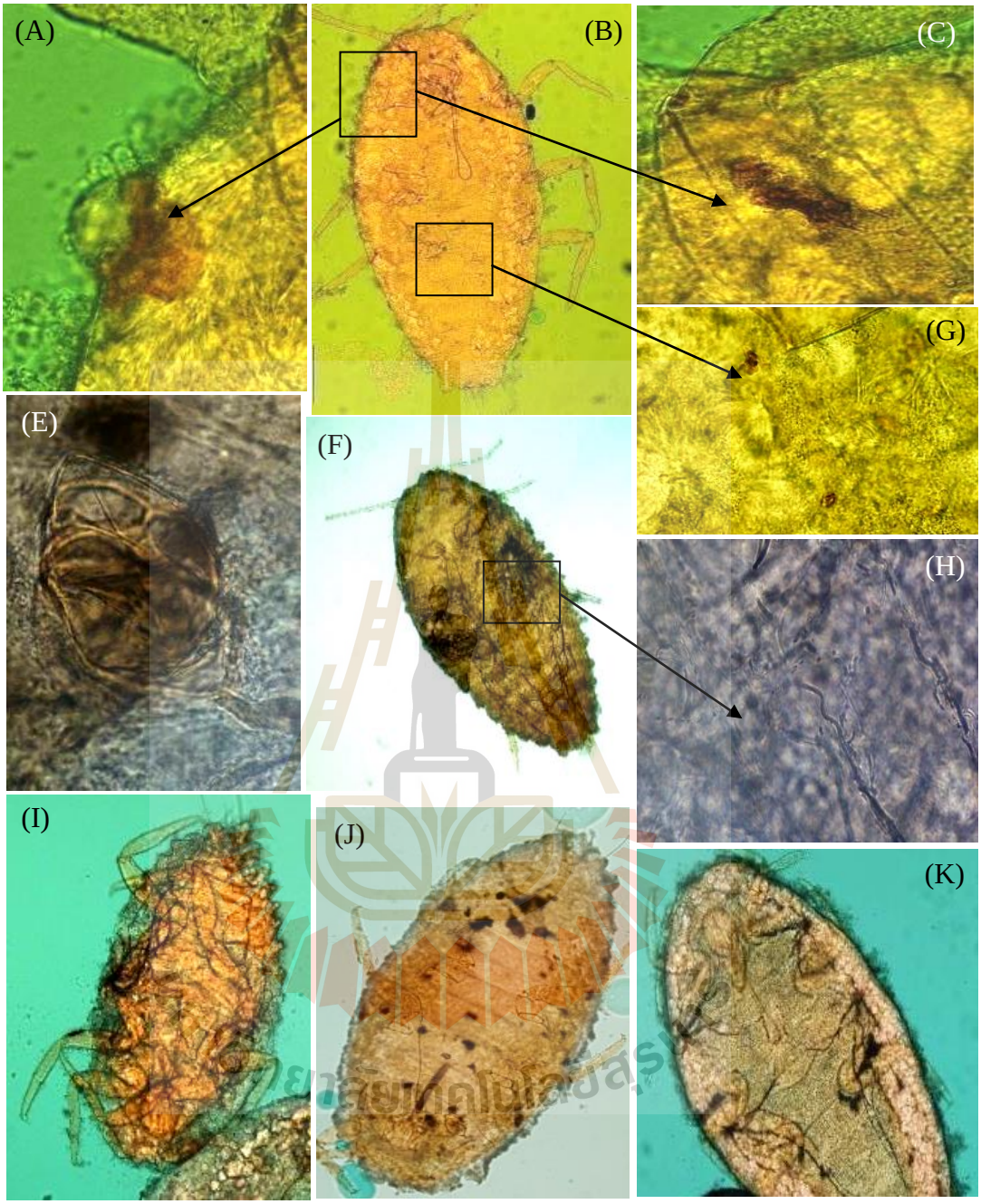


Figure 4.13 Effect of bacterial treatments on cassava mealy bugs, *Phenacoccus manihoti* Matile-Ferrero, positions of the infection, eye (A, B and E), ostioles, (B and C), pore on body (G), circulus (J) cells into the body of cassava (F, H, I and K).

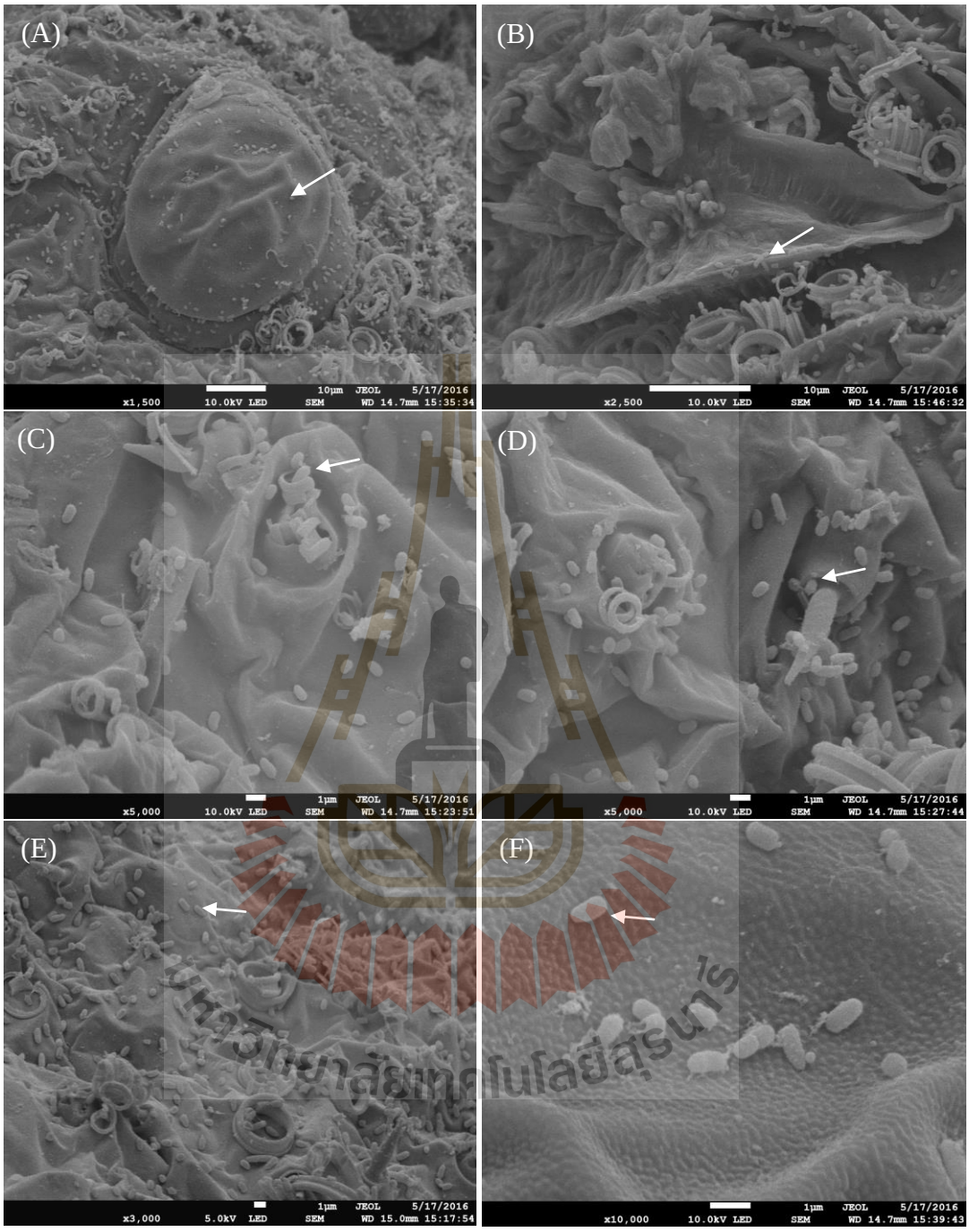


Figure 4.14 Effect of bacterial treatments on cassava mealy bugs, *Phenacoccus manihoti* Matile-Ferrero, observed under scanning electron microscope (SEM) area of eye, (A), Ostiole, (B), and body, (C, D, E and F).

4.6 Identification of bacterial isolates

4.6.1 Morphological characterization

For morphological characterization of bacterial isolates, cell morphology of the bacteria, was examined. Bacterial isolates according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). All isolates were Gram-negative rod, and no endospore forming (Figure 4.10).

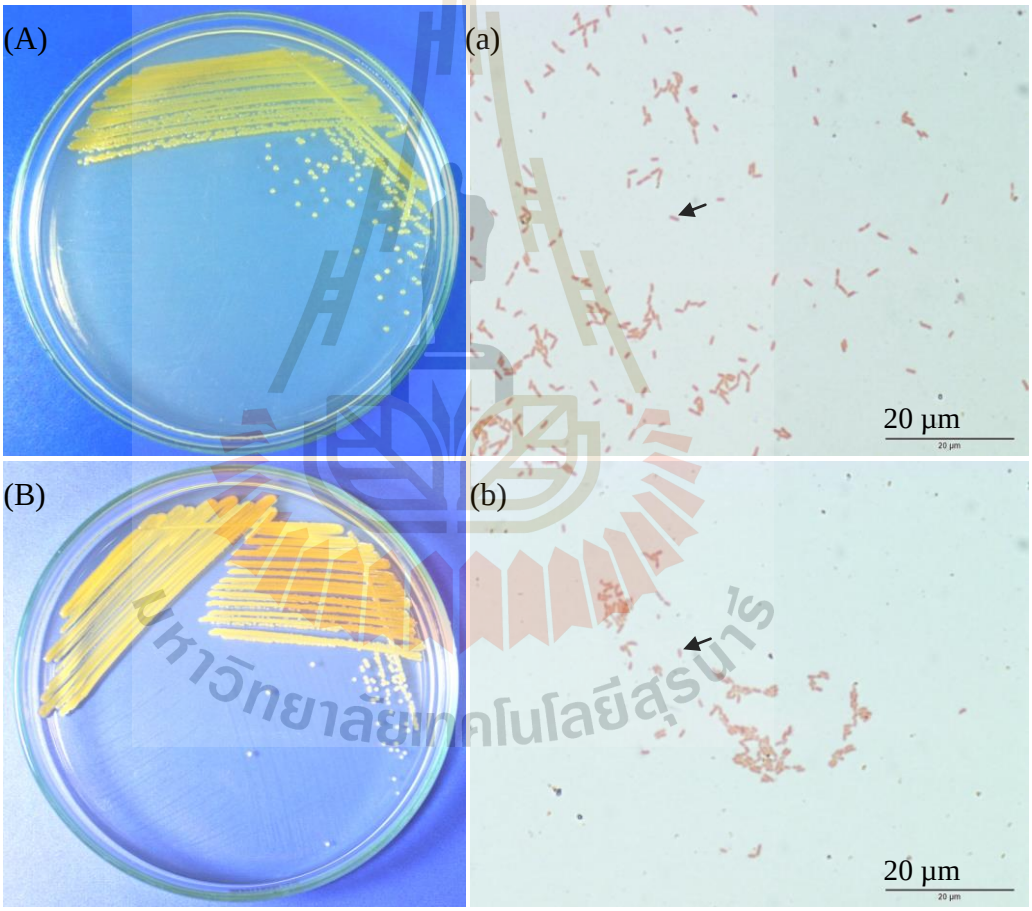


Figure 4.15 Examples of colony and Gram-staining of Gram-negative, (100x) rod shape of isolate SSTBA26 (Aand a) and KSSMA80 (B and b), respectively.

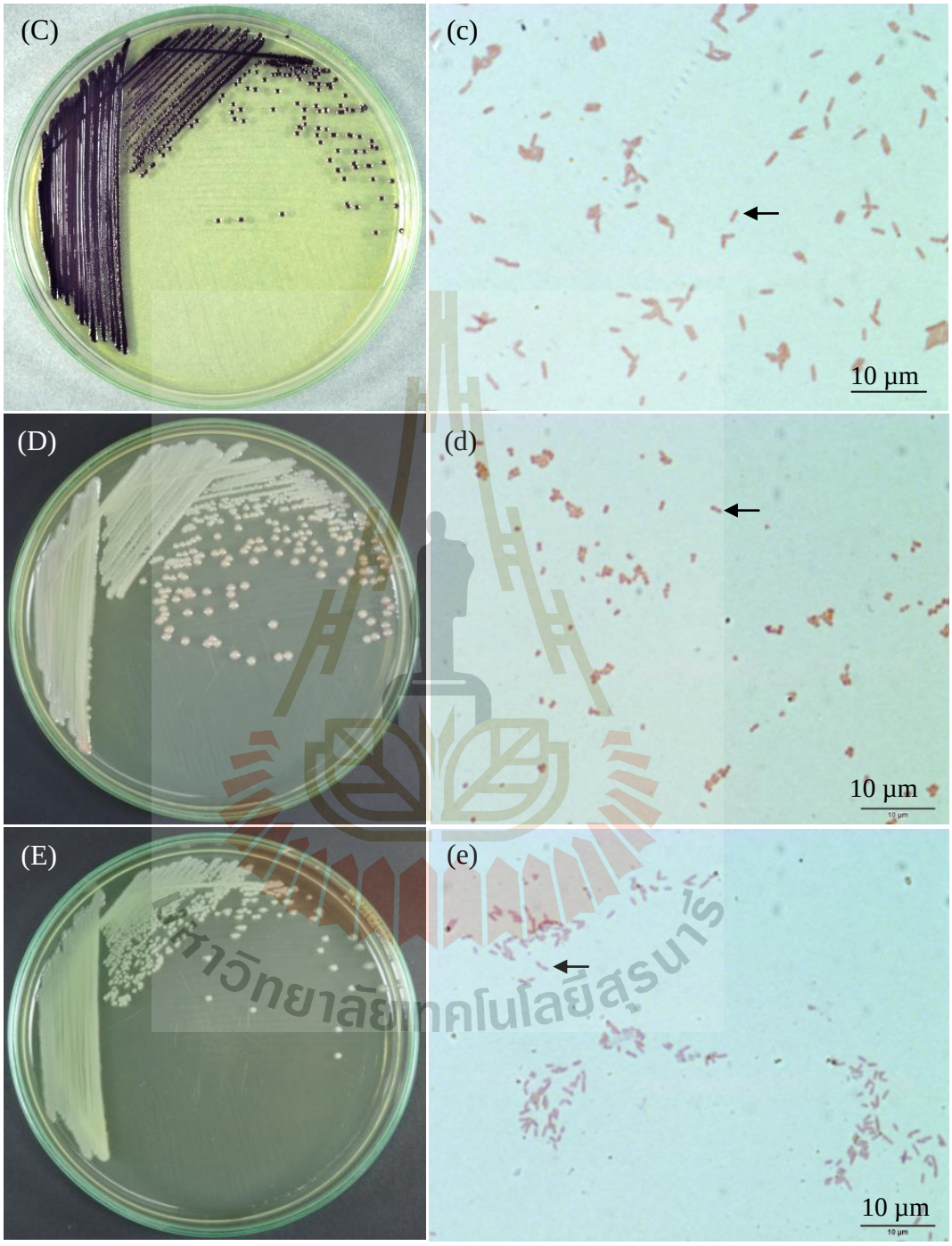


Figure 4.15 (Continued) Examples of colony and Gram-staining of Gram-negative, (100x) rod shape of isolate SSTBA15 (C and c), CCSMA59 (D and d), and PCSMA53 (E and e), respectively.

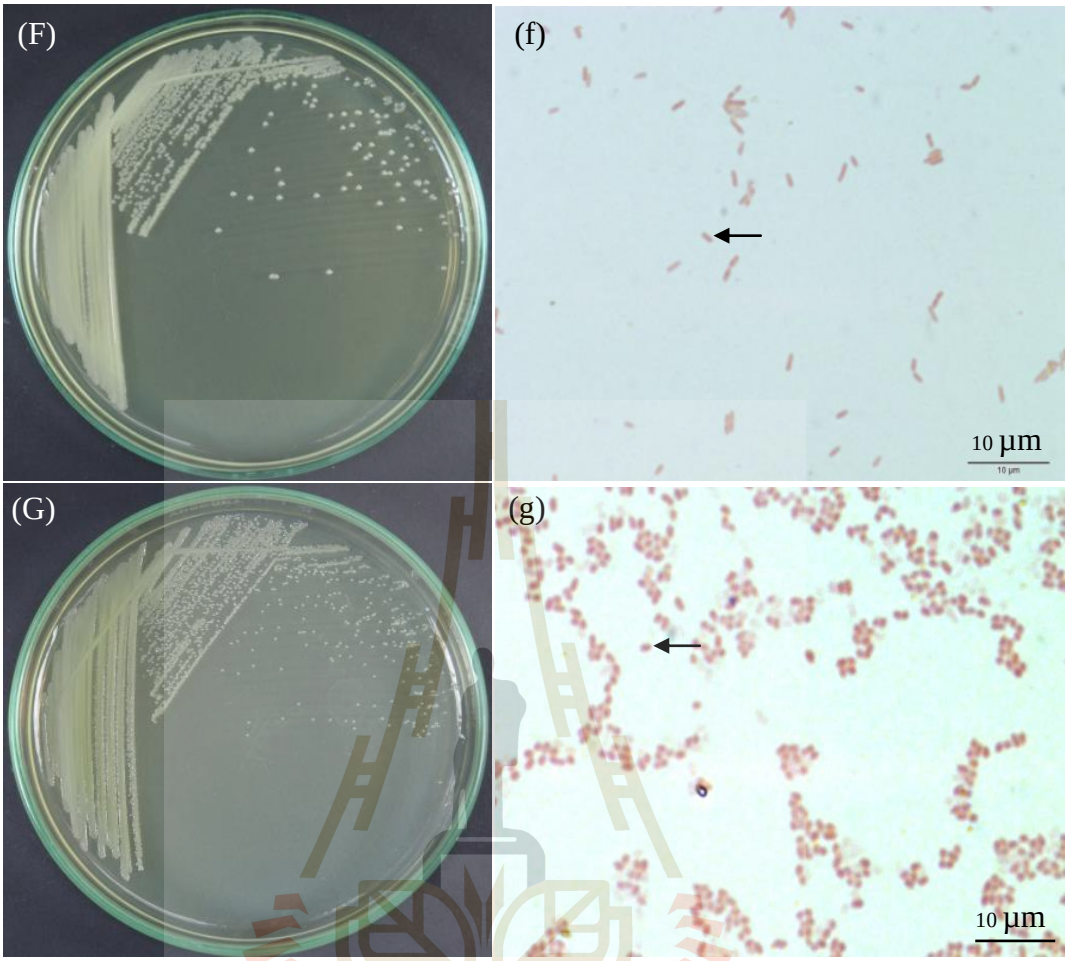


Figure 4.15 (Continued) Example of colony and Gram-staining of Gram-negative, rod shape (100x) of isolate SSSMA5 (F and f) and KBSMA65 (G and g), respectively.

4.6.2 Physiological characterization

Bacteria isolated from soil sample were found to have different physiological characteristics (Tables 4.7). Results from API identification system (bioMérieux, Inc., France) (Figures 4.16) were compared with known species in API online data base. Isolate CCSMA59, PCSMA53, SSTBA26, KSSMA80, and KBSMA65, had 96.9, 99.9, 99.1, 96.1, and 93.6% identity to *Serratia macescens*,

Pseudomonas aeruginosa, *Chromobactrium violaceum*, *Chryseobacterium indologenes*. Isolates SSTBA15, SSSMA5 and SSTBA16, had 99.9, 99.1, and 99.1% identity to *Chromobactrium violaceum*, respectively.

Table 4.7 Physiological characteristics of bacterial isolates from soil samples. biochemical test results using API 20 E (bioMérieux, Inc., France).

Characteristics	Bacterial isolates No.							
	1	2	3	4	5	6	7	8
Gram reaction	-	-	-	-	-	-	-	-
Cell shape	R	R	R	R	R	R	R	R
Endospore-produced	-	-	-	-	-	-	-	-
Cell diameter < 1.0 μm	+	+	+	+	+	+	+	+
Oxidase	+	-	+	+	+	+	+	+
2-nitrophenyl-BD-galactopyranoside	+	-	-	-	-	-	-	-
Arginine dihydrolase	-	+	+	+	-	-	+	+
Lysine decarboxylase	+	-	-	-	-	-	-	-
Ornithine decarboxylase	+	-	-	-	-	-	-	-
Citrate utilization	+	+	-	-	+	-	+	-
H_2S production	-	-	+	-	-	-	-	-
Urea hydrolysis	-	-	-	-	+	+	-	-
Trytophane hydrolysis	+	+	+	+	-	-	-	+
Indole production	-	-	+	-	+	+	-	-
Voges-Proskauer	+	-	-	-	-	-	+	-
Gelatin hydrolysis	+	+	+	+	+	+	+	+
D-glucose	+	+	-	-	-	-	-	-
D-mannitol	+	-	-	-	-	-	-	-
D-sorbitol	+	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-
D-sucrose	+	-	+	-	-	-	-	-
D-melibiose	+	-	-	-	-	-	-	-
Amygdalin	+	-	-	-	-	-	-	-
L-arabinose	-	-	-	-	-	-	-	-
NO_2 production	+	-	+	+	-	-	-	+
Reduction to N_2 gas	-	+	-	-	-	+	-	-
Molility	+	+	+	+	+	+	+	+
MacConkey medium	+	+	+	+	+	+	+	+
D-Glucose fermentation	+	+	+	+	-	-	+	+
D-Glucose oxidation	+	-	+	+	-	-	-	+

Symbols: - = negative; + = positive; C = cocci; ND = not detected; and R = rod Isolates:1= CCSMA59; 2= PCSMA53; 3=SSTBA15; 4=SSSMA5; 5=SSTBA26; 6=KSSMA80; 7= KBSMA65 and 8=SSTBA16

Figure 4.16 Patterns form API (bioMérieux, Inc., France), API 20E of bacterial isolates SSTBA15 (A), KSSMA80 (B), SSTBA26 (C), PCSMA53 (D), CCSMA59 (E), SSSMA5 (F), KBSMA65 (G), and SSTBA16 (H) when incubated at 35 °C for 24 h.

4.6.3 16SrRNA gene characterization

For 16S rRNA gene characterization of bacterial isolates, genomic DNA was extracted from 7 bacterial isolates. 16S rDNA was amphified using primers fD1, and rP2. The size of amplified DNA fragment was about 1,500 bp. Phylogenetic tree of these bacteria were contributed (Figures 4.17-4.20). These isolates had high similarity to type strains (Tables 4.21-4.23). When compared these sequences to sequences from GenBank database, it was found that 8 isolates of Gram-negative rods had 99%, 99% 95%, 98%, 97%, and 96% similarity to *Serratia symbiotica* CWBI-2.3 gu394001, *Chromobacterium pseudoviolaceum* LMG 3953 (NR_114954) (99%), *Chromobacterium subtsugae* PRAA4-1 NR_042853, *Chryseobacterium* sp. S8 HE662644 *Pseudomonas* sp. 271 KT034417, *Chryseobacterium arthrosphaerae* CC-VM-7 NR_116977, respectively. For bacterial isolate PCSMA53 and SSTBA16 not detected for sequence because when compared these sequences to sequences not similarity to type strains in GenBank but AIP test confirm PCSMA53 and SSTBA16 at 99.9% and 99.1% identity to *Pseudomonas aeruginasa and Chromobacterium violaceum*, respectively. Identification results of selected bacterial isolates based on API identification system (bioMérieux, Inc., France) compared to 16S rRNA gene sequence and similarity of 16S rRNA gene sequence of selected bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI) were concluded in (Table 4.12).

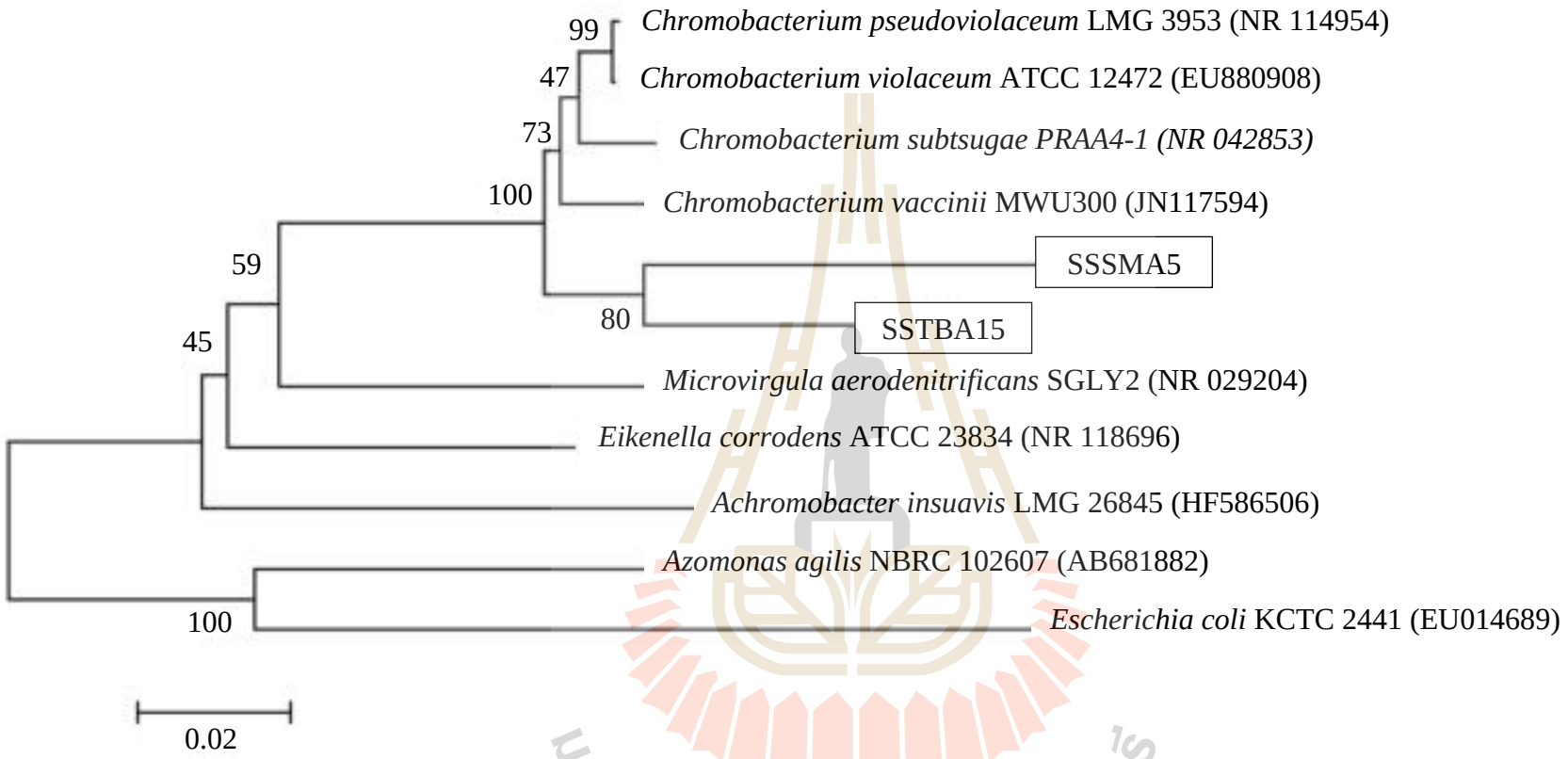


Figure 4.17 Phylogenetic tree of bacteria isolates SSTBA5 and SSSMA5, Gram-negative rods based on 16S rRNA gene sequence data using the neighbourjoining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

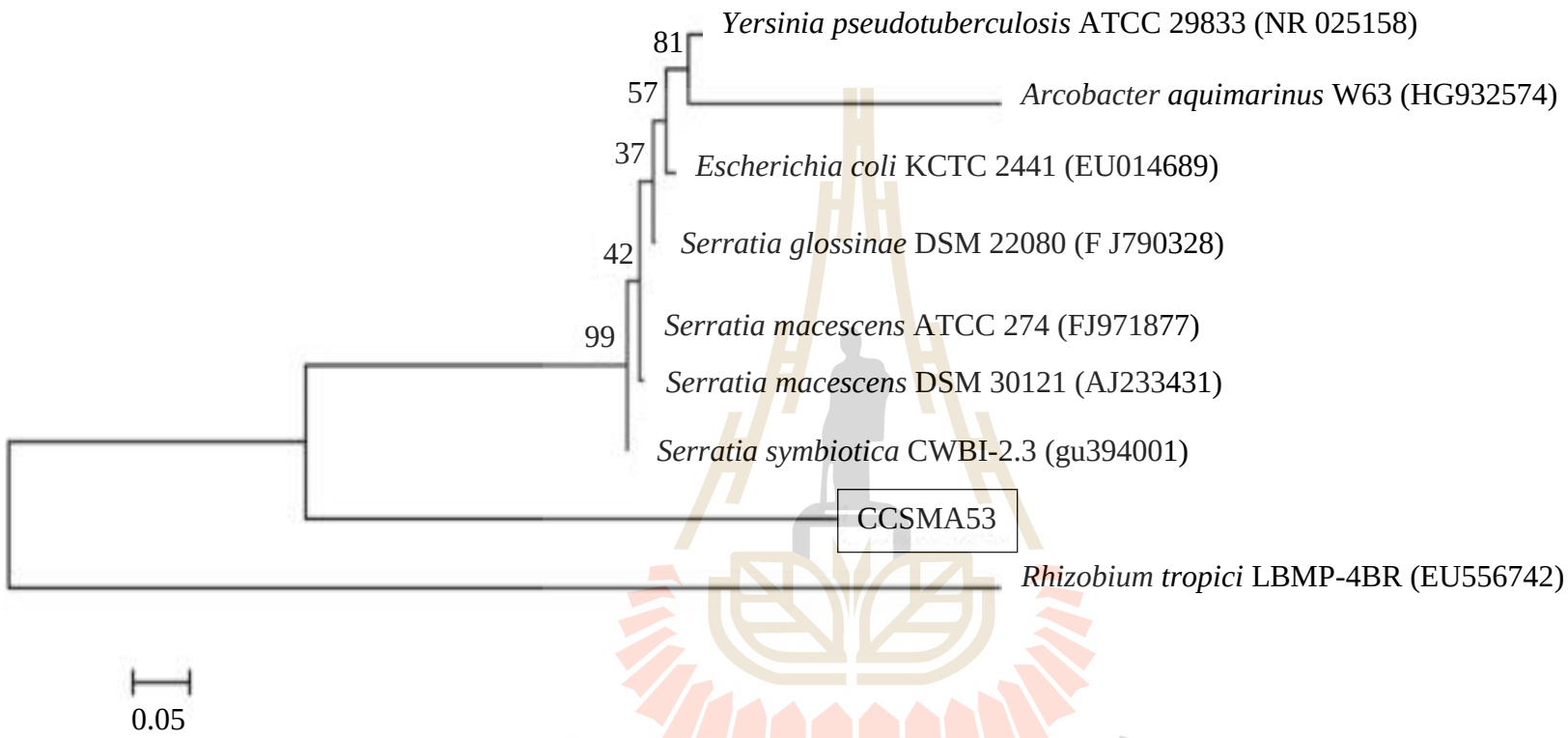


Figure 4.18 Phylogenetic tree of bacterial isolate CCSMA5, Gram-negative rods based on 16S rRNA gene sequence data using the neighbourjoining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

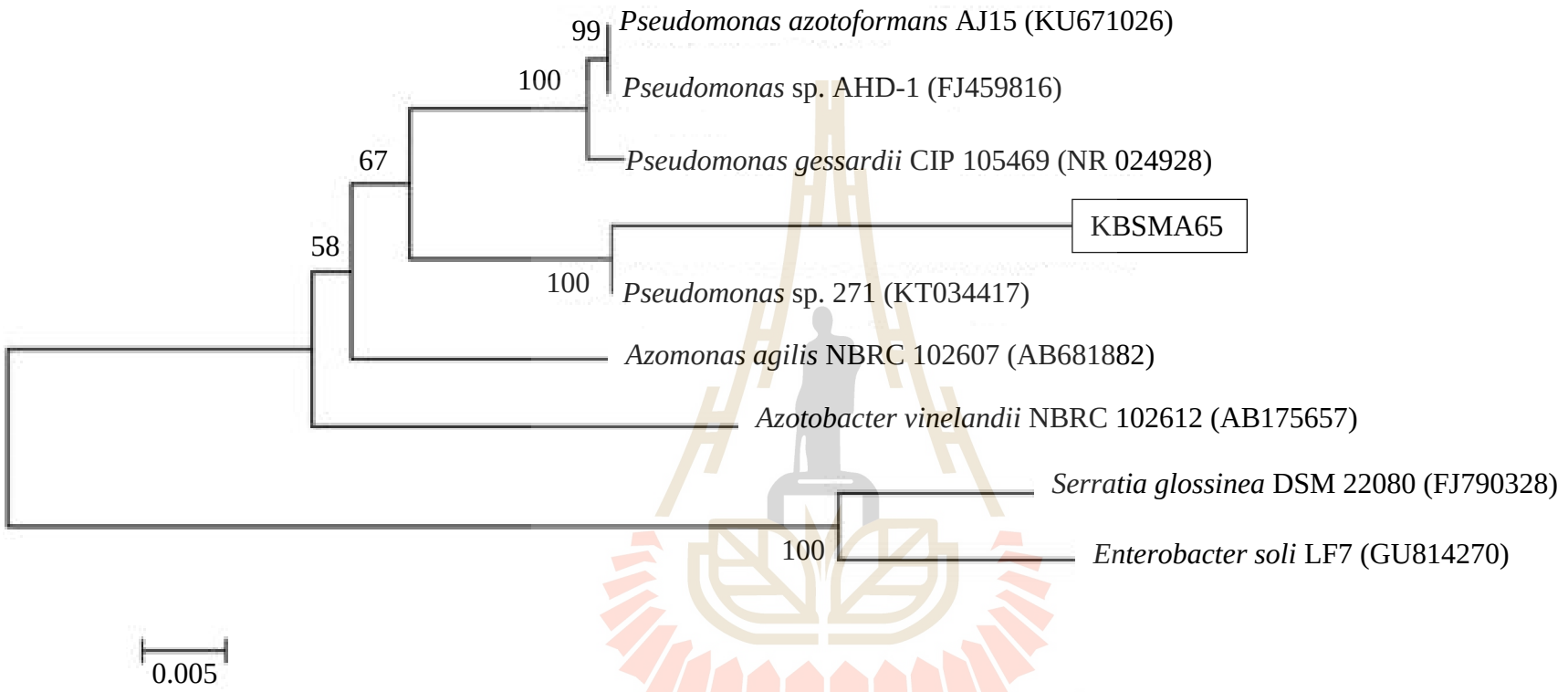


Figure 4.19 Phylogenetic tree of bacterial isolate KBSMA65, Gram-negative rods based on 16S rRNA gene sequence data using the neighbourjoining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

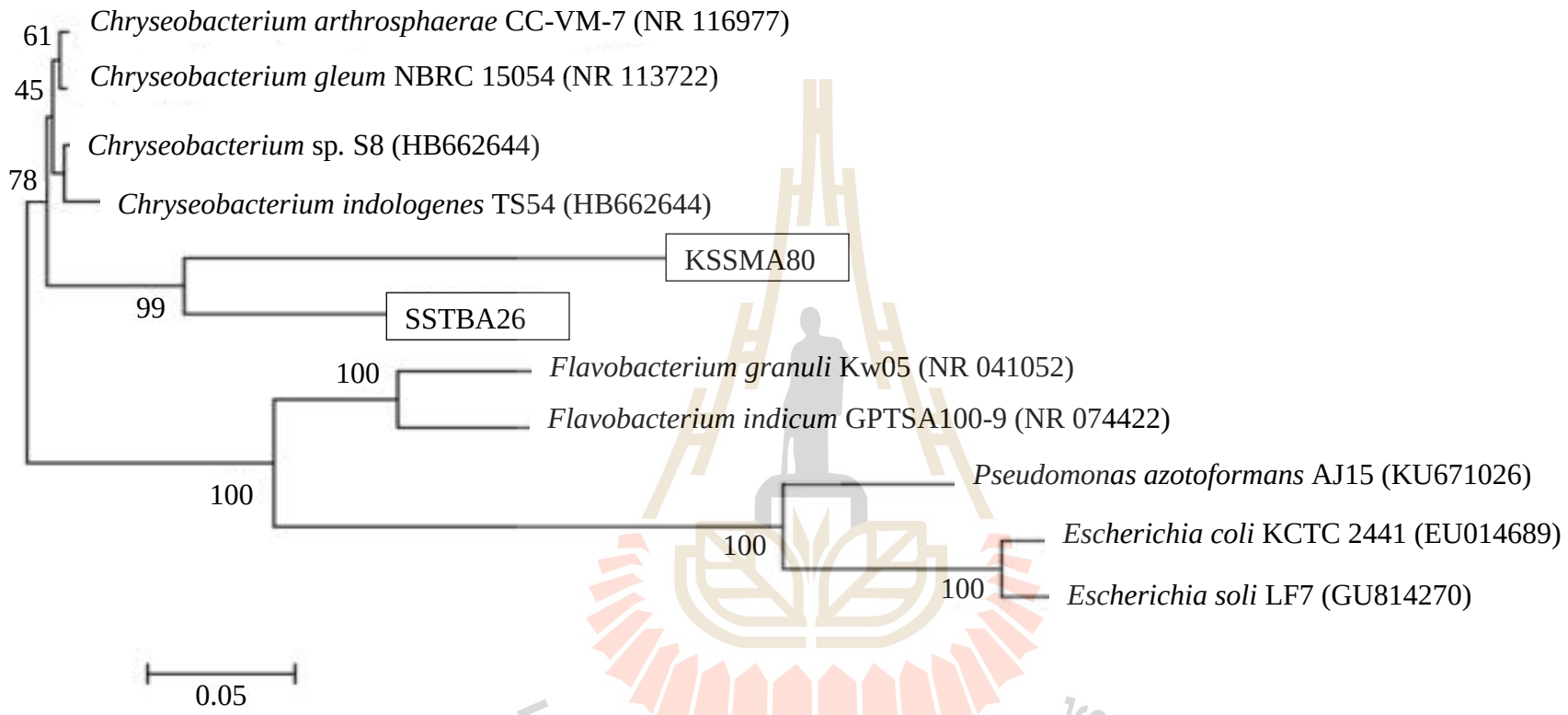


Figure 4.20 Phylogenetic tree of bacteria isolates KSSMA80 and SSTBA26, Gram-negative rods based on 16S rRNA gene sequence data using the neighbourjoining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

Table 4.8 16S rRNA gene sequence similarity of bacterial isolate, KBSMA65 and related species.

Bacterial strain	KBSMA65	1	2	3	4	5	6	7	8
KBSMA65	100								
1	97	100							
2	95	97	100						
3	95	96	99	100					
4	95	97	99	99	100				
5	91	97	95	95	96	100			
6	92	94	94	94	94	94	100		
7	84	87	87	87	87	86	86	100	
8	84	86	86	86	86	85	86	96	100

1: *Pseudomonas* sp. 271 (KT034417), 2: *Pseudomonas azotoformans* AJ15 (KU671026), 3: *Pseudomonas* sp. AHD-1 (FJ459816), 4: *Pseudomonas gessardii* CIP 105469 (NR_024928), 5: *Azomonas agilis* NBRC 102607 (AB681882), 6: *Azotobacter vinelandii* NBRC 102612 (AB175657), 7: *Serratia glossinae* DSM 22080 (FJ790328), 8: *Enterobacter soli* LF7 (GU814270).

Table 4.9 16S rRNA gene sequence similarity of bacterial isolates SSSMA5, SSTBA15 and related species.

Bacterial strain	SSSMA5	SSTBA15	1	2	3	4	5	6	7	8	9
SSSMA5	100										
SSTBA15	94	100									
1	95	97	100								
2	95	97	98	100							
3	94	99	97	98	100						
4	94	99	98	99	99	100					
5	89	90	91	90	93	91	100				
6	87	88	88	89	92	89	89	100			
7	86	88	88	89	94	89	87	86	100		
8	83	84	84	84	91	85	85	83	81	100	
9	82	85	83	82	83	82	83	82	82	86	100

1: *Chromobacterium vaccinii* MWU300 (JN117594), 2: *Chromobacterium subtsugae* PRAA4-1 (NR_042853), 3: *Chromobacterium violaceum* ATCC 12472 (EU880908), 4: *Chromobacterium pseudoviolaceum* LMG 3953 (NR_114954), 5: *Microvirgulaae rodenitrificans* SGLY2 (NR_029204), 6: *Achromobacter insuavis* LMG 26845 (HF586506), 7: *Eikenella corrodens* ATCC 23834 (NR_118696), 8: *Azomonas agilis* NBRC 102607 (AB681882), 9: *Escherichia coli* KCTC 2441 (EU014689)

Table 4.10 16S rRNA gene sequence similarity of bacterial isolates KSSMA80, SSTBA26 and related species.

Bacterial strain	KSSMA80	SSTBA26	1	2	3	4	5	6	7	8
KSSMA80	100									
SSTBA26	96	100								
1	98	94	100							
2	97	95	98	100						
3	97	96	98	99	100					
4	95	93	98	97	97	100				
5	82	83	84	85	85	86	100			
6	81	82	84	84	84	86	91	100		
7	76	76	77	76	76	77	77	77	100	
8	76	75	76	76	76	76	77	77	96	100

1: *Chryseobacterium* sp. S8 (HE662644), 2: *Chryseobacterium gleum* NBRC 15054 (NR_113722), 3: *Chryseobacterium arthrosphaerae* CC-VM-7 (NR_116977), 4: *Chryseobacterium indologenes* TS54 (HQ647283), 5: *Flavobacterium indicum* GPTSA100-9 (NR_074422), 6: *Flavobacterium granuli* Kw05 (NR_041052, 7: *Escherichia coli* KCTC 2441 (EU014689), 8: *Enterobacter soli* LF7 (GU814270)

Table 4.11 Identification results of selected bacterial isolates based on API identification system (bioMérieux, Inc., France) compared to 16S rRNA gene sequence.

Bacterial isolate code	Cell shape/arrangement	Cell size (μm)	Identification (% identity)	
			API system (bioMérieux, Inc., France)	16S rRNA gene sequence (GenBank, U.S.A.)
CCSMA59	Rods, singly short chain	0.3-0.5×2.0-2.4	*Serratia macescens* (96.9%)	*Serratia symbiotica* CWBI-2.3 (GU394001) (99%)
PCSMA53	Rods, singly, short chain	0.2-0.5×1.2-1.3	*Pseudomonas aeruginosa* (99.9%)	ND
SSTBA15	Rods, singly, short chain	0.2-0.6×1.0-1.5	*Chromobacterium violaceum* (99.9%)	*Chromobacterium pseudoviolaceum* LMG 3953 (NR_114954) (99%)
SSSMA5	Rods, singly, short chain	0.2-0.5×1.0-1.5	*Chromobacterium violaceum* (99.1%)	*Chromobacterium subtsuga*PRAA4-1 (NR_042853) (95%)
SSTBA16	Rods, singly, short chain	0.2-0.5×1.0-1.5	*Chromobacterium violaceum* (99.1%)	ND
KSSMA80	Rods, singly, short chain	0.2-0.5×1.0-3.0	*Chryseobacterium indologenes* (98%)	*Chryseobacterium* sp. S8 (HE662644) (98%)
KBSMA65	Rods, singly, short chain	0.2-0.6×0.7-1.5	*Pseudomonas fluorescens* (96.1%)	*Pseudomonas* sp. 271 (KT034417) (97%)
SSTBA26	Rods, singly, short chain	0.2-0.5×1.2-2.5	*Chryseobacterium indologenes* (93.6%)	*Chryseobacterium arthrosphaerae* CC-VM-7 (NR_116977) (96

ND; Not detected.

Table 4.12 Similarity of 16S rRNA gene sequence of selected bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI).

Bacterial isolate code	Length of sequence (bp)	Nucleotide sequence comparison, identification result and details			
		Closest relative	Length of sequence (bp).	Sequence homology (%)	Isolation source / remark of closest relative
KBSMA65	1,418	*Pseudomonas* sp. 271 (KT034417)	1,470	97	Sediment
		Pseudomonas azotoformans AJ15 (KU671026)	1,233	95	Petroleum contaminated soil
		Pseudomonas sp. AHD-1 (FJ459816)	1,395	95	Soil
		Pseudomonas gessardii CIP 105469 (NR_024928)	1,516	95	Natural mineral waters
KSSMA80	1,471	*Chryseobacterium* sp. S8 (HE662644)	1,425	98	Soil
		Chryseobacterium gleum NBRC 15054 (NR_113722)	1,442	97	Data not show
		Chryseobacterium arthrosphaerae CC-VM-7 (NR_116977)	1,364	97	Faeces of the pill millipede
		Chryseobacterium indologenes TS54 (HQ647283)	1,420	95	Rhizosphere soil (wild rice)
SSTBA26	1,322	*Chryseobacterium* sp. S8 (HE662644)	1,425	94	Soil
		Chryseobacterium gleum NBRC 15054 (NR_113722)	1,442	95	Data not show
		Chryseobacterium arthrosphaerae CC-VM-7 (NR_116977)	1,364	96	Faeces of the pill millipede
		Chryseobacterium indologenes TS54 (HQ647283)	1,420	93	Rhizosphere soil (wild rice)
SSSMA5	1,464	*Chromobacterium vaccinii* MWU300 (JN117594)	1,527	95	Rhizosphere
		Chromobacterium subtsugae PRAA4-1 (NR_042853)	1,440	95	Soil
		Chromobacterium violaceum ATCC 12472 (EU880908)	987	95	Soil
		Chromobacterium pseudoviolaceum LMG 3953 (NR_114954)	1,416	94	Environmental samples
SSTBA15	1,395	*Chromobacterium vaccinii* MWU300 (JN117594)	1,527	97	Rhizosphere soil
		Chromobacterium subtsugae PRAA4-1 (NR_042853)	1,440	97	Environmental samples
		Chromobacterium violaceum ATCC 12472 (EU880908)	987	99	Soil
		Chromobacterium pseudoviolaceum LMG 3953 (NR_114954)	1,416	99	Environmental samples

Note: ATCC, American Type Culture Collection; CIP, Collection of Institut Pasteur; MWU, Midwestern University and NBRC, National Board for Respiratory Care.

All eight bacterial isolates showed different potentials to affect the pink cassava mealy bugs (*P.manihoti)*. Among the eight isolates, comparatively the motility of pink cassava mealy bugs was observed. Three isolates better efficacy of *S. symbiotica*, *P. aeruginosa* and *C. violaceum* in reduction of number of pink cassava mealy bugs can also be attributed to its trait to produce variety of hydrolases nutrients such as glucose, liquid inside the body of pink mealy bug including protease, lipase and chitinase which contribute to virulence or are thought to play a role in the nutrition of the bacterium (Petra *et al*., 2010). The colored pigment is one of the most important bioactive factors produced with different biological activities (Marks and Bogorad 1960; Bennett and Bentley, 2000). The effective management of pink cassava mealy bugs can be achieved through understanding of life history parameters (Chong *et al*., 2008; Patil *et al*., 2011; Aristizabal *et al*., 2012) and the use of biological control agents such as natural enemies like the parasitoid *Anagyrus kamali* and the ladybeetle *Cryptolaemus montrouzieri* Mulsant (Kairo *et al*., 2000). However, to our knowledge there is no report on bacteria to control the pink cassava mealy bug.

Serratia spp. strains have been reported to be lethal to insect pests when ingested in high doses. However, in some cases, the strains can be highly virulent at low doses, killing the larvae in 2-3 days with symptoms similar to a virus infection (Steinhaus, 1959; Lysyk *et al*., 2002). *Serratia* spp. have also been reported to control effectively the diamondback moth, *Plutella xylostella* (L) (Lepidoptera: Plutellidae) (Jeong *et al*., 2010), the grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae) in lawns (Jackson *et al*., 1991) and the fourth instar mosquito larvae (Diptera: Culicidae) of *Aedes aegypti*, *Anopheles steph*ensi and *Culex quinquefasciatus* (Patil *et al*., 2012). *P. aeruginosa* are important as they are involved in biocontrol of plant

diseases (Bano and Musarrat 2003; Minaxi and Saxena, 2010). However, according to our results they become a very promising group of bacteria to control insect pests. A strain of *Chromobacterium subtsugae*, first isolated from a soil sample in Maryland (USA), was discovered to show high insecticidal activity against insect species in different orders, including the Diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae), the Sweet potato whitefly *Bemisia tabaci* Gennadium (Rhynchota: Aleyrodidae), the Southern green stink bug *Nezara viridula* L. (Rhynchota: Pentatomidae), the Southern corn rootworm *Diabrotica undecimpunctata* Mannerheim (Coleoptera:Chrysomelidae spectrum), the Western corn rootworm *Diabrotica virgifera* Le Conte (Coleoptera: Chrysomelidae), the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), and the Small hive beetle *Aethina tumida* Murray (Coleoptera: Nitidulidae) (Salunkhe *et al*., 2013). The broad spectrum activity of this strain is related to multiple modes of action probably involving different chemical compounds produced by the bacterium. Among the bacterial metabolites, *C. subtsugae* synthesizes the tryptophan derivative violacein, which confers a typical violet color to its colonies. In addition to this, various molecules produced by this species have been characterized and associated with the insecticidal action. Bioactive compounds were reported to be associated to the stationary growth phase, and the heat-stability of insecticidal toxins was also demonstrated. The active ingredient of available commercial formulations is represented by *C. subtsugae* strain PRAA4-1T and spent fermentation media (Ruiu, 2015). More recently, a new strain identified as Chromobacterium, isolated from the midgut of *Aedes aegypti* L. (Diptera: Culicidae) was shown to be able to colonize the

insect midgut and to display entomopathogenic and anti-pathogen properties (Ruiu, Satta, and Floris, 2013; Ruiu, 2015).

In our study, the bacterial strains, especially *S. symbiotica*, *P. aeruginosa* and *C. violaceum* caused the most adverse effects on the pink mealy bug. Pesticides cannot penetrate the heavy layers of wax that shield the body and waxy ovisac (Kairo *et al.*, 2000; Meyerdirk *et al.*, 1998) of pink mealy bug. Therefore, small cells of bacteria and grown inside of mealy bug by use nutrients in mealy bugs could be one of the potential tools to control them. These results suggest that bacterial strains might be useful as biocontrol agents for mealy bugs. However, more studies are needed to determine if these bacteria can be used in integrated pest management programs.

CHAPTER V

CONCLUSION

Cassava mealy bug in Northeastern Thailand from different plantation area during February 2012 to March 2013, found four species, Pink mealy bug (*Phenacoccus manihoti* Matile-Ferrero) has a pink oval shape with short wax filaments and high found in cassava plantation area. Green mealy bug (*Phenacoccus maderiensis* Green) has yellowish green, oval shape with short wax filament. Jackbeard mealy bug (*Pseudococcus jackbeardsleyi* Gimpel & Miller) has a long and oval body, and wax filaments around the periphery, and (*Ferrisia virgata* Cockerel) has also a long and oval body with one pair of the caudal filaments. Pink mealy bugs were studied using light microscope (LM), paraffin section technique by rotary microtome, found cuticle layers (3-4 µm) and wax gland, (6-10 µm) to produce wax filament under cuticle layer. After that pink mealy bug were studied under scanning electron microscope (SEM), found several structure such as spiracles, (9-30 µm), ostioles (2-59 µm), circulus (17-20 µm), quinquelocular pores (5-7 µm), trilocular pore (3-5 µm), multilocular pores (4-6 µm), and discoidal pores (2-3 µm). This data could be used to find the way to protect and eradicate cassava mealy bug by using bacteria isolates.

A total of 416 bacteria isolates from soil samples were collected and screened for possibility of destroying structure of cassava mealy bugs. Eight isolates from different area of soil samples were selected based on their small cells sizes, no

spore forming, produce of extracellular enzymes, protease, lipase and chitinase and grown on mealy bugs broth (MBB). Eight isolates, namely CCSMA59, PCSMA53, SSTBA15, SSSMA5, SSTBA16, SSTBA26, KSSMA80, and KBSMA65 have properties for test possibility to destroy cassava mealy bugs. All isolates were identified as *Serratia macescen*, *Pseudomonas aeruginosa*, *Chromobacterium violaceum*, *Chromobacterium subtsugae*, *Chryseobacterium* sp. *Pseudomonas* sp., and *Chryseobacterium arthrosphaerae*, according to their morphological and physiological characteristics, and 16S rRNA gene sequence.

Several tactics are used in the control of cassava mealy bugs, such as: resistant cultivars, cultural practices, and entomopathogens, such as the fungus, *Beauveria bassiana* and *Metarhizium flavoviride* have the potential use as biological control agents against mealy bugs because they were relatively safe on non target insects, such as natural enemies and beneficial soil insects, but fungus have specific condition, they slowly destroy insect pest and require knowledge as using. There is no report on bacteria to control cassava mealy bug. This research aims to investigate soil bacteria that can destroy structure of cassava mealy bugs. These bacteria will be useful for further application in agriculture. This studied also tested these bacteria against the nymphs and adults of pink cassava mealy bugs. Among the bacteria tested in genus, *Serratia*, *Pseudomonas*, *Chromobacterium*, and *Chryseobacterium* showed motility of cassava mealy bug significantly higher than the control ($p>0.05$) in particular may be a good candidate for further investigation as a possible biocontrol agent. However, more studies are needed to determine if these bacteria can be used in integrated pest management programs. Environmental safe methods for controlling cassava mealy bugs are still needed.

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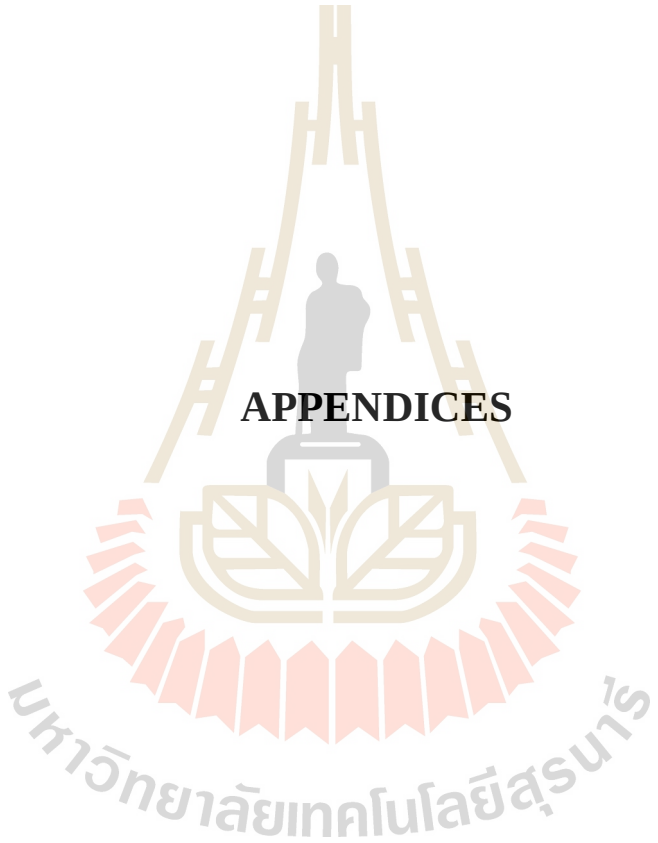
APPENDICES



APPENDIX A

CULTURE MEDIUM AND REAGENT PREPARATION

A.1 Culture media for isolation soil bacteria

All components of each medium were added to distilled water mixed thoroughly and gently heated until dissolved, and brought volume up to 1.0 L. The media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches. Except, Skim milk agar sterilization was done by autoclaving for 10 min at 115 °C, 10 lb/square inches.

A.1.1 Chitin agar (CCA)

Chitin, precipitated	3.00	g
$(NH_4)_2SO_4$	2.00	g
Na_2HPO_4	1.10	g
KH_2PO_4	0.70	g
$MgSO_4.7H_2O$	0.20	g
$FeSO_4$	1.00	mg
$MnSO_4$	1.00	mg
Agar	15.00	g

pH 7.0 ± 0.2 at 25 °C

Chitin, precipitated: composition per 2.5L

Chitin	40.00	g
HCl	400.00	ml

Preparation of chitin, precipitated: Add chitin to 400.0 ml of cold concentrated HCl. Add this solution to 2.0 L of distilled/deionized water at 4 °C. Filter the solution through Whatman#1 filter paper. Dialyze the precipitated chitin against tap water for 12 hr. Adjust the pH to 7.0 with KOH.

A.1.2 Tributyrin agar (TBA)

Tributyrin	10.00	ml
Peptone	10.00	g
Yeast extract	3.00	g
Agar	15.00	g

pH 7.0 ± 0.2 at 25 °C

A.1.3 Skim Milk Agar (SMA)

Skim milk	10.00	g
Prancreatic digest of casein	5.00	g
Yeast extract	2.50	g
Glucose	1.00	g
Agar	15.00	g

pH 7.0 ± 0.2 at 25 °C

A.1.4 Trypticase soy broth (TSB)

Tributyrine	10.00	ml
Peptone	5.00	g
Yeast extract	3.00	g
Agar	15.00	g

pH 7.0 ± 0.2 at 25 °C

A.1.5 Trypticase soy agar (TSA)

Pancreatic digest of casein	17.00	g
Phytone (Papaic digest of soya meal)	3.00	g
NaCl	5.00	g
KH_2PO_4	2.50	g
Glucose	2.50	g
Agar	15.000	g

pH 7.0 ± 0.2 at 25 °C

A.1.6 Triple sugar iron (TSI)

Peptic digest of animal tissue	10.00	g
Casein enzyme hydrolysate	10.00	g
Yeast extract	3.00	g
Beef extract	3.00	g
Lactose	10.00	g
Sucrose	10.00	g
Dextrose	1.00	g
NaCl	5.00	g
Ferric sulfate	0.20	g
Sodium thiosulfate	0.30	g
Phenol red	24.00	mg
Agar	12.00	g

pH = 7.4± 0.2 at 25 °C

All ingredients except carbohydrates were added to distilled water and gently heated until dissolved. Then, the media were sterilized by autoclaving for 15 min at 121 °C, 15 lb/square inches. The medium was allowed to cool down to 45-50 °C and added the sterile carbohydrates into medium.

A.1.7 Lysine indole motile (LIM) medium

Peptone	10.00	g
Tryptone	10.00	g
Yeast extract	3.00	g
L-lysine hydrochloride	10.00	g
Dextrose	1.00	g
Ferric ammonium citrate	0.50	g
Bromcresol purple	0.02	g
Agar	2.00	g

pH = 6.6 ± 0.2 at 25 °C

A.1.8 Methyl Red Voges-Proskauer (MR-VP) medium

Peptone	5.00	g
Glucose	5.00	g
K_2PO_4	5.00	g

pH = 7.5± 0.2 at 25 °C

A.1.9 Simmons citrate agar

$MgSO_4$	0.20	g
$(NH4)_2PO_4$	1.00	g
K_2PO_4	1.00	g
Sodium citrate	2.00	g
NaCl	5.00	g
Agar	15.00	g
Bromthymol blue	0.05	g

pH = 6.8 ± 0.2 at 25°C

A.1.10 Nitrate reduction broth

Peptone	5.00	g
Beef extract	3.00	g
KNO_3	1.00	g

pH 6.9 ± 0.2 at 25°C

A.1.11 Oxidation-Fermentation medium

NaCl	5.00	g
Pancreatic digest of casein	2.00	g
K_2HPO_4	0.30	g
Bromthymol Blue	0.03	g
Agar	2.50	g
Glucose solution	100.0	g

pH 7.2 ± 0.2 at 25°C

Preparation of glucose solution: glucose powder (10.0 g) was added into 100.0 mL of distilled water. The solution was sterile by filtration. Medium preparation: all gradients were added into 900.0 ml of distilled water. The medium was mixed thoroughly and gently heated until dissolved, then sterilized by autoclaving for 15 min at 121 °C, 15 lb/square inches. The medium was allowed to cool down to 45-50 °C and added with 100.0 ml of glucose solution.

A.1.12 Lauryl sulfate tryptose (LST) broth

Tryptose	20.00	g
Lactose	5.00	g
NaCl	5.00	g
Lauryl sulfate sodium salt	0.10	g
K_2HPO_4	2.75	g
KH_2PO_4	2.75	g

pH 6.8 ± 0.2 at 25 °C

A.1.13 Motility test medium

Tryptone	10.00	g
Agar	5.00	g
NaCl	5.00	g

pH 7.2 ± 0.2 at 25 °C

A.1.14 Nutrient gelatin

Peptone	5.00	g
Glucose	5.00	g
K_2PO_4	5.00	g
Gelatin	120.00	g

pH 7.5 ± 0.2 at 25 °C

A.1.15 Phenol red broth

Proteose peptone	10.00	g
Phenol red	18.00	mg

pH 7.4 ± 0.2 at 25 °C

Proteose peptone was added in distilled water, then phenol red was added. The medium was sterilized by autoclaving for 15 min at 121 °C, 15 lb/square inches after preparation.

A.2 Chemicals and reagents for microbiological analysis

The reagents for conventional method of bacterial characterization were prepared as described by Cappuccino and Sherman (1999).

A.2.1 Tetramethyl-p-phenylenediamine dihydrochloride (1% solution)

Tetramethyl-p-phenylenediamine dihydrochloride	1.00	g
Distilled water	100.00	ml

Tetramethyl-p-phenylenediamine dihydrochloride was dissolved in 100.0 ml. This reagent was kept in the refrigerator.

A.2.2 **Crystal violet** (Gram stain)

Crystal violet	2.00	g
95% Ethanol	20.00	ml
1% ammonium oxalate	80.00	ml

Crystal violet (2.0 g) was dissolved in 20.0 mL of ethanol (95%). Then, the mixture was transferred to 80.0 mL of 1% ammonium oxalate solution and mixed throughly.

A.2.3 **Safranin O** (Gram stain)

Safranin O	0.25	g
95% Ethanol	10.00	ml
Distilled water	90.00	ml

Safranin O (0.25 g) was dissolved in 10.0 mL of 95% ethanol. Then, the mixture was diluted with 90.0 mL of distilled water.

A.2.4 **Iodine solution**

Iodine	2.00	g
Potassium iodide	4.00	g
Distilled water	600.00	ml

Iodine (2.0 g) and potassium iodide (4.0 g) were dissolved in distilled water and made up volume to 600.0 mL. This solution was kept in the dark place.

A.2.5 95% Ethyl alcohol

Absolute ethanol	95.00	ml
Distilled water	5.00	ml

Absolute ethanol was added to distilled water, and mixed thoroughly.

A.2.6 Hydrogen peroxide diluted (3%)

40% H_2O_2	7.50	ml
Distilled water	100.00	ml

The 40% H_2O_2 7.5 mL was diluted with distilled water to 100.0 mL.

A.2.7 Kovacs' reagent

p-Dimethylaminobenzaldehyde	5.00	g
Amyl alcohol	75.00	ml
Concentrated hydrochloric acid	25.00	ml

p-Dimethylaminobenzaldehyde (5.0 g) was dissolved in 75.0 mL of amyl-alcohol at 50 °C. The mixture was added to concentrated hydrochloric acid (25.0 mL). This reagent was kept in the dark bottle and stored in a refrigerator.

A.2.8 Methyl red solution

Methyl red	0.10	g
95% Ethanol	300.00	ml

Methyl red (0.1 g) was dissolved in 300.0 mL of 95% ethanol, and the total volume to 500.0 mL with distilled water.

A.2.9 Nitrate test reagents

Sulfanilic acid	0.80	ml
5 N acetic acid	2.00	ml
Dimethyl-α-napthylamine	5.00	ml

Nitrate test reagents composed of solution A and B. For the preparation of solution A, sulfanilic acid (0.8 g) was dissolved in 1.0 L of 5 N acetic acid (1 part of glacial acetic acid in 2.5 parts of distilled water). For preparation of solution B, dimethyl-α-napthylamine (5.0 g) was dissolved in 1.0 L of 5 N acetic acid.

A.2.10 Voges-Proskauer test solution

Alpha-napthol	10.00	g
95% Ethanol	100.00	ml
KOH	20.00	g
Distilled water	100.00	ml

This solution was composed of two solutions: solution A and B. Solution A was prepared by diluting 10.0 g of alpha-napthol in 100.0 mL of 95% ethanol. Solution B was prepared by diluting 20.0 g of KOH in 100.0 mL of distilled water

A.2.11 Phosphate buffer (pH 7.2)

Na_2HPO_4, anhydrous	724.00	mg
KH_2PO_4	210.00	mg
Distilled water	1.00	ml

Dissolve ingredients in distilled water. Adjust pH to 7.2 (with 1 N NaOH). Thebuffer was sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches

A.3 Chemical preparation for API identification system (bioMérieux,Inc., France)

A.3.1 McFarland turbidity standards

1% solution of anhydrous $BaCl_2$ and a 1% solution of H_2SO_4 were prepared and mixed together in various proportions as detailed in Table 1A to form a turbid suspension.

Table 1A Preparation of McFarland turbidity standards

McFarland scale number	Amount of 1% $BaCl_2$ (mL)	Amount of 1% H_2SO_4 (mL)	Corresponding approximate density of bacteria (million/mL)
1	2	3	4
5	6	7	8
9	10	0.1	0.2
0.3	0.4	0.5	0.6
0.7	0.8	0.9	1.0
9.9	9.8	9.7	9.6
9.5	9.4	9.3	9.2
9.1	9.0	300	600
900	1,200	1,500	1,800
2,100	2,400	2,700	3,000

Source: Cappuccino and Sherman (1999).

A.3.2 Normal saline 0.85%

NaCl	0.85	g
Distilled water	100.00	ml

Dissolve ingredients in distilled water. This solution was sterilized by autoclaving for 15 min at 121 °C, 15 lb/square inches

A.3.3 Sterile emulsion oil

Emulsion oil or parafilm oil	10.00	g

The oil solution was poured in glass vials with screw cap, and sterilized by autoclaving for 15 min at 121 °C, 15 lb/square inches before use.

A.4 Reagents for 16S rRNA gene

A.4.1 STE buffer

NaCl	2.92	g
Tris base	1.21	g
$EDTA{\cdot}2H_2O\text{-}Na_2$	1.86	g

The final concentration of solution per litter was 100 mM NaCl, 10 mM Tris/HCl, and 1 mM EDTA. The solution pH was adjusted to 8.0 with NaOH, then autoclaved at 121 °C, 15 lb/square inches for 15 min.

A.4.2 Saline-EDTA (0.5M NaCL+ 0.1M EDTA, pH8.0)

Sodium chloride	8.76	g
EDTA (di-sodium salt)	37.22	ml

Added distilled water and brought volume up to 1000.00 ml

A.4.3 TE buffer

Tris Base	1.21	g
EDTA ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$)	0.37	ml

The ingredients were dissolved. The volume was adjusted to 1.0 L with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121 °C, 15 lb/square inches.

A.4.4 Tris-saturated phenol (pH 8.0)

Dissolved phenol was transferred to the mixture of 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0

A.4.5 Loading buffer

Bromophenol blue	5.00	g

The dye was dissolved and adjusted the volume to 10.0 mL with 40% sucrose in water.

A.4.6 Ethidium bromide (10 mg/mL)

Ethidium bromide	1.00	g

The chemical was dissolved in sterilized deionized water and the volume was adjusted to 100.0 mL, pH 8.0.

A.4.7 Lysozyme (0.75 mg/ml)

Lysozyme	7.50	g
Added distilled water and brought volume up to	10.00	mL

A.4.8 Tris-HCl buffer (0.1M, pH 9.0)

Tris	1.21	g

Adjust to pH 9.0 using 1N HCl

Added distilled water and brought volume up to 100.00 ml

A.4.9 SDS (10% w/v)

Sodium dodecylsulfate (SDS)	100.00	mg

Added distilled water and brought volume up to 1000.00 ml

A.4.10 Phenol:chloroform (1:1 v/v)

Crystalline phenol was liquidified in water bath at 65 °C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a tight bottle.

A.4.11 RNAase (10 mg/ml)

RNAase	10.00	mg

The RNAase was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl and stored at -20 °C.

A.4.12 Proteinase K

Proteinase K	4.00	mg
50 mM Tris-HCl (pH 7.5)	1.00	ml

Use freshly prepared solution.

A.4.13 Saline-sodium citrate (20X)

NaCl	175.30	g
Sodium citrate	88.20	ml

Add distilled water and bring volume up to 1,000.00 ml

A.4.14 Phosphate buffer (0.2125 M, pH 8.2)

di-Sodium hydrogen phosphate (Na_2HPO_4)	30.17	g

Add distilled water and bring volume up to 1,000.00 ml

A.4.15 TNBS (Picrylsulfonic acid solution) (0.05% v/v)

TNBS (5% v/v)	1.00	ml

Add distilled water and bring volume up to 100.00 ml

A.4.16 HCl (0.1 N)

HCl (5% v/v)	9.00	ml

Add distilled water and bring volume up to 1,000.00 ml

A.5 Chemicals and reagents for structure analysis of mealy bugs

A.5.1 OsO_4 (1%)

OsO_4	1.00	g
Distilled water	25.00	ml

One gram of OsO_4 was added into 25.0 mL of distilled water in the dark bottle and seal with parafilm, and then kept overnight in the dark place. OsO_4 solution (4%) was filtrated by filter paper. For preparation

of 1% OsO_4, pipette one part of 4% OsO_4 was mixed with 3 parts of phosphate buffer. The solution kept in double dark bottle in flume hood.

A.5.2 Glutaraldehyde (5%)

50% Glutaraldehyde 10.00 ml

Pipette 10.0 mL of 50% glutaraldehyde into 100.0 mL volume metric flask and adjusted the volume using distilled water.

A.5.3 Series of dehydration reagent

Table 2A Preparation of acetone series

Acetone concentration (%)	100% Acetone (mL)	Distilled water (mL)
20	20.0	80.0
40	40.0	60.0
60	60.0	40.0
80	80.0	20.0

A.5.4 0.2 M Phosphate buffer

Preparation of stock solution A and B

Solution A $NaH_2PO_4 \cdot H_2O$ 27.60 g

Weighing of $NaH_2PO_4 \cdot H_2O$ 27.60 g added in distilled water and adjusted the volume to 1.0 L kept in dark bottle.

Solution B Na_2HPO_4 28.40 g

Weighing of Na_2HPO_4 28.40 g added in distilled water and adjusted the volume to 1.0 L kept in dark bottle.

Table 3A Working solution of 0.2 M phosphate buffers.

Solution A (mL)	Solution B (mL)	pH
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5

A.5.5 Carbol fuchsin staining

Basic fuchsin	2.50	g
Distilled water	250.00	ml
100% alcohol	25.00	ml
Phenol crystals, melted	12.50	ml

Solution A: 0.2 g basic fuchsin and 10 ml 95 % ethanol

Solution B: 5 g phenol and 90 ml distilled water.

Prepare the carbol fuchsin stain by mixing the following 2 solutions. Mix well, filter into brown bottle. Label bottle with date and initials, solution is stable for 1 year. CAUTION: Carcinogen, toxin.

A.5.6 Methylene blue staining

Stock Solution		
Methylene blue	0.70	g
Distilled water	50.00	ml
Working Solution		
Methylene Blue Stock	5.00	ml
Distilled water	45.00	ml

Mix well, filter into brown bottle. CAUTION: Carcinogen, toxin.

APPENDIX B

COLLECTION AND CHARACTERIZATION

OF CASSAVA MEALY BUGS

Table 1B Examples of cassava mealy bugs found from natural habitats in Northeastern, Thailand.

Figures	Morphological characteristics							Preliminary species identification
	Color	Body	Sizes	Dorsum of body	Antenna	Lateral of body	Venter of body	
	Gray	Body oval; slightly rounded in lateral view	Long 2.8-3.0 mm width1.8-2.0 mm	The covered with powdery white wax, without stripes on back	Antenna 8 segmented	Thin filaments around body, with 17 pairs of lateral filaments	Caudal pair about one-half length of body or more	*Pseudococcus jackbeardsley i* Gimpel & Miller
	Dark gray	Body elongate oval	long 2.20-4.10 mm, width 0.90-2.50 mm	With wax removed with 2 stripes on back, with a few filamentous glassy strands of wax protruding from the body	Antenna 8 segmented	Non have waxy filaments on the lateral	only 1 pair of conspicuous anal wax filaments present, hind filament one-half length of body	*Ferrisia virgata* Cockerell
	Gray	Body elongate oval	long 0.20-2.10 mm, width 0.70-1.50 mm	With wax removed with 2 stripes on back, with a few filamentous glassy strands of wax protruding from the body	Antenna 8 segmented	Non have waxy filaments on the lateral	only 1 pair of conspicuous anal wax filaments present, hind filament one-half length of body	*Ferrisia virgata* Cockerell

Table 1B (Continued) Examples of cassava mealy bugs found from natural habitats in Northeastern, Thailand.

Figures	Morphological characteristics							Preliminary species identification
	Color	Body	Sizes	Dorsum of body	Antenna	Lateral of body	Venter of body	
	Pale green	Body oval	Long 0.88-2.30mm, Width 0.74-1.38 mm	covered with mealy white wax, which is not thick enough to hide the yellow body, no markings on back	Antenna 9 segmented	with 15 to 17 pairs of short marginal wax filaments around body	Wax filament, the posterior pair is conspicuously longer than the others being about 1/8 of the length of the body	*Phenacoccus maderiensis* Green
	Pink	Body oval	Long 0.60-2.50 mm, Width 1.00-1.50mm	Dusted with white mealy powder, without stripes on back	Antenna 9 segmented	with 15 to 17 pairs of short marginal wax filaments around body	only 1 pair of conspicuous anal wax filaments present, hind filament one-half length of body	*Phenacoccus manihoti* Matile-Ferrero
	Pink	Body oval	Long 0.60-2.50 mm, Width 1.00-1.50mm	Dusted with white mealy powder, without stripes on back	Antenna 9 segmented	with 15 to 17 pairs of short marginal wax filaments around body	only 1 pair of conspicuous anal wax filaments present, hind filament one-half length of body	*Phenacoccus manihoti* Matile-Ferrero

Table 2B Morphological characteristics of cassava mealy bugs in Northeastern, Thailand under stereo microscope.

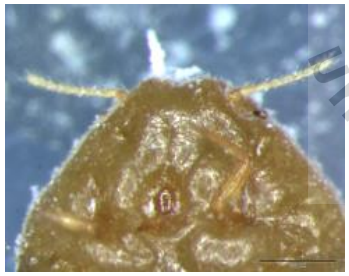
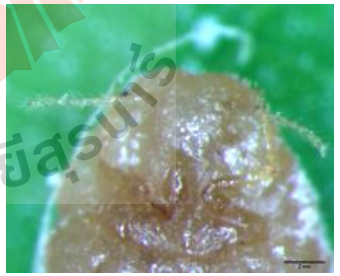
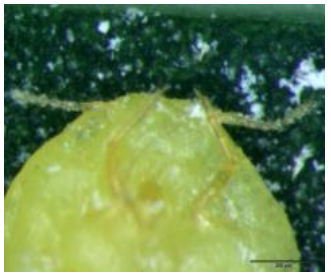
Characteristics	*Pseudococcus jackbeardsleyi* Gimpel & Miller	*Ferrisia virgata* Cockerell	*Phenacoccus manihoti* Matile-Ferrero	*Phenacoccus maderiensis* Green
Color and body	Color gray ; Body oval; slightly rounded in lateral view	Color dark gray; Body elongate oval	Color pink; Body oval	Color pale green in color; Body oval
				
Antenna	Antenna 8 segmented	Antenna 8 segmented	Antenna 9 segmented	Antenna 9 segmented
				

Table 2B (Continued) Morphological characteristics of cassava mealy bugs in Northeastern, Thailand under stereo microscope.

Characteristics	*Pseudococcus jackbeardsleyi* Gimpel & Miller	*Ferrisia virgata* Cockerell	*Phenacoccus manihoti* Matile-Ferrero	*Phenacoccus maderiensis* Green
Lateral of body	Thin filaments around body, with 17 pairs of lateral filaments	Non have waxy filaments on the lateral	with 15 to 17 pairs of short marginal wax filaments around body	with 15 to 17 pairs of short marginal wax filaments around body
				
Venter of body	only 1 pair of wax filaments	only 1 pair of wax filaments	Caudal filaments	Caudal filaments
				

APPENDIX C

DEPOSITION OF BACTERIAL RIBOSOMAL SEQUENCES IN GENBANK (U.S.A.)

Table 1C 16S rRNA genes (partial sequences) of isolated bacteria deposited in GenBank (U.S.A.).

Isolation source	**Bacterial strain**	**Nucleotide sequence**	
		Length	**NCBI accession no.**
Soil sample	CCSMA59	1478	GU394001
Soil sample	SSTBA15	1,416	NR_114954
Soil sample	SSSMA5	1,440	NR_042853
Soil sample	KBSMA65	1,470	KT034417
Soil sample	KSSMA80	1,425	HE662644
Soil sample	SSTBA26	1,364	NR_116977

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