

SELECTION OF SOIL BACTERIA FOR CONTROLLING CASSAVA MEALYBUGS

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Abstract

Soil bacteria are the key living organisms in several kinds of biogeochemical processing due to their production of potential hydrolytic enzymes, which have been attractive for several applications including bio-pesticides. In this study, bacteria capable of producing chitinase, proteinase, and lipase, were enumerated and isolated from soil collected from different cassava plantation areas, to obtain the dominant bacteria having small cells suitable for invading some pores of the cassava mealybugs collected from the same locations as the soil. Seven Gram-negative, non-spore forming bacterial isolates capable of producing potential hydrolytic enzymes and with small cell sizes (0.2-1.0×1.0-3.0 μm) occurring singly or in pairs, were selected. These isolates could still not be identified into species by their morphological, cultural, and biochemical characteristics, as well as 16S rRNA gene sequences. When compared to their closest relatives, the 7 isolates designated as SUTT 15 and SUTT 16, SUTT 26 and SUTS 80, SUTS 53 and SUTS 65, and SUTS 59, belonged to genera *Chromobacterium*, *Chryseobacterium*, *Pseudomonas*, and *Serratia*, respectively. Cassava mealybugs, particularly the nymph and adult of the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero), as the bacterium-invading targets were mainly found on cassava plants and accumulated in the soil under the plants to be the nutrients

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for the soil bacteria. The pink mealybug body is covered with a 3-4 μm thick cuticle layer having several pores, particularly ostioles, cerarii, and spiracles with 2-59, 2-7, 17-20, and 9-30 μm diameters, respectively. The 7 bacterial isolates had small cells suitable for invading the pores on the insect's body wall, and exhibited very good growth in the medium prepared from the collected cassava mealybugs. The facultative anaerobe *Serratia* sp. SUTS 59, showing the highest growth in the mealybug broth, was selected for the preparation of the cell suspension (10^8 CFU/mL at the late log phase of growth) to be sprayed onto 40 adult female mealybugs, compared with a sterile normal saline solution as the negative control. The insect mortality rate of 52% and 70% was detected after 4- and 5-day treatments, respectively. The soil bacterium revealed its capability to be further developed for application to control cassava mealybugs.

Keywords: Soil bacteria, protease, lipase, chitinase, cassava mealybugs

Introduction

Soil bacteria are the most abundant microbes in the soil compared to other living organisms. Various species of bacteria live in the microenvironment of the soil. The roles of soil bacteria are mainly important for involvement in biogeochemical processes such as the decomposition, mineralization, and release of carbon dioxide into the atmosphere (Kaiser *et al.*, 2016). All processing occurring in the soil is part of several cycles - the carbon cycle, sulfur cycle, and nitrogen cycle (Wang *et al.*, 2018; Ciesla *et al.*, 2016). For some other roles, for instance soil bacteria mutualizing with nearby organisms by acting as growth promoting factors, *Rhizobium* could be included in this group (Ciesla *et al.*, 2016). *Pseudomonas* sp. could act as a disease suppressor in soil because of its ability to secrete fungicide to prevent the fungal infection of plants (Otieno *et al.*, 2015). Good soil and suitable bacteria induce the opportunities for plant growth. The bacteria inhabiting the soil also have a variety of potential enzymes in their metabolisms, especially hydrolytic enzymes such as protease, lipase, chitinase, and amylase, causing them to become established in various kinds of environments. Some of them can produce other valuable metabolites, particularly antibiotics. Hence, soil bacteria are getting more attention, especially with their ability to produce several kinds of extracellular enzymes. The chitinase enzyme, which catalyzes the hydrolysis of

chitin into its monomers, could play a major role in the separation and hydrolysis of insect structures and cause damage to the insects (Sharma *et al.*, 2016).

Chitinase is produced by a number of microorganisms including: (a) fungi such as *Metarhizium anisopliae*; (b) bacteria, particularly *Bacillus cereus*, *Bacillus pumilus*, and *Serratia marcescens*; (c) actinomycetes such as *Streptomyces* spp.; and (d) yeasts such as *Tilletiopsis albescens* and *Tilletiopsis washingtonensis* (Aggarwal *et al.*, 2015). The use of chitinase-producing entomopathogenic fungi in pest control has been reported (Aggarwal *et al.*, 2015, Veliz *et al.*, 2017). However, they have not been commercially successful due to their slow killing rates and more sustainable pesticides in the field.

Lipase is another enzyme which could be produced by soil bacteria. It has the ability to hydrolyse lipids such as releasing free fatty acids and glycerol. Currently, bacterial lipases are in great demand because of the potential industrial applications in the food, textile, and paper industries. The synthesis of biodiesel has also included lipase usage (Sirisha *et al.*, 2010).

Protease is the enzyme that breaks protein into smaller peptides or amino acids. Bacteria, especially the genera *Bacillus* and *Pseudomonas*, are preferred to be used for industrial production of protease because of their ability to produce large amounts of the

enzyme with a significant proteolytic activity and stability at a high pH and temperature (Kumar and Vats, 2010).

The cassava mealybug is an insect with a waxy secretion covering its body and is an important pest of the cassava plant. More than 15 species of the insect attack cassava in Asia, America, and Africa (Sattayawong *et al.*, 2016). The mealybug is usually found on the underside of leaves in the plant canopy, especially around the main veins (Calatayud and Le Rü, 2006). Five species of mealybugs have been reported to attack cassava plants in Thailand. These species are the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero), Madeira mealybug (*Phenacoccus madeirensis* Green), Jack Beardsley mealybug (*Pseudococcus jackbeardsleyi* Gimpel & Miller), striped mealybug (*Ferrisia virgate* Cockerell), and solenopsis mealybug (*Phenacoccus solenopsis* Tinsley), causing yield losses in large cassava field areas (Neuenschwander, 2003; Soroush *et al.*, 2012). The control of cassava mealybugs is rather difficult due to the waxy secretion covering the whole body (Salunkhe *et al.*, 2013). The insect's cuticle is another complex structure composed of protein (nearly 50-80% of the total mass), chitin, and fatty acid varying considerably depending upon the insect species. Currently, the control of cassava mealybug can be by the application of the *Trichogamma* wasp and fungi, *Beauveria bassiana* (Balsamo) Vuillemin (Greenfield *et al.*, 2016) and *Metarhizium anisopliae* (Metschn) Sorokin (Taluengphol and Thaochan, 2015). However, the fungus *Beauveria bassiana* was scarce in live mealybugs at 1-5 days, and growth was limited to parts of dead hosts at 6-7 days, which was similar to *Metarhizium anisopliae* for the time to attain the insect target's mortality.

Microorganisms could produce potential extracellular enzymes, particularly chitinase, lipase, and protease, as a lethal component against insect pests. The lipase enzyme plays a major role in the separation and hydrolysis of the insect waxy mass making the cuticle accessible to degradation by chitinases and proteases (Sharma *et al.*, 2016). Microorganisms producing these enzymes have received much attention regarding their potential development

as biopesticides. The aim of this study was to isolate dominant soil bacteria at different cassava plantation areas in Thailand, and to collect cassava mealybugs from the same locations as the soil collection. The cassava mealybug structure was then investigated to evaluate the capability of the selected dominant soil bacteria to invade the mealybug target body by spraying the selected bacterial cell suspension (approximately 10^8 CFU/mL) on the bodies of adult female pink mealybugs. The mortality rate of the mealybugs was recorded and the dead insects were observed under a stereomicroscope and scanning electron microscope (SEM). The positive results reveal that these bacteria could be useful for further application for controlling cassava mealybugs and other bugs having similar structures.

Materials and Methods

Soil Samples and Insect Specimens

Soil samples were collected from at least 10 locations in cassava plantation areas in Northeastern Thailand, where cassava devastation by mealybugs has been reported. Five hundred grams of soil samples were taken from the soil surface and below the surface at a depth of about 10-15 cm, in triplicate at each location, and put into a clean plastic bag in an ice box. The collected soil samples were immediately transported to the laboratory, then determined for their pH and moisture contents according to the Official Methods of Analysis Method 934.01 (AOAC International, 2006) and further applied for soil bacterium enumeration and isolation. The mealybugs inhabiting the cassava leaves were also collected from the plantation areas. The insect specimens on the plant leaves were collected as they were, then kept in 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. The boxes were immediately transported to the laboratory for further classification and identity of the insect specimens.

Enumeration, Screening, and Selection of Extracellular Enzyme-Producing Bacteria from Soil

Bacteria able to produce the extracellular enzymes, chitinase, lipase, and proteinase - considering the main components chitin, lipid, and protein of the insect as substrates - were enumerated and isolated from soil samples using the standard pour plate technique (Beishier, 1991). Ten grams of each soil sample were mixed with 90 mL of sterile normal saline solution. The suspension was mixed, processed into the serial dilution ($1:10^6$), and poured in triplicate into petri dishes containing colloidal chitin agar (CCA) composed of colloidal chitin, Na_2HPO_4 , KH_2PO_4 , NH_4Cl , NaCl , yeast extract, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and agar at concentrations of 1.0, 0.6, 0.3, 0.1, 0.05, 0.005, 0.02, and 1.5%, respectively (Nurderbyandaru *et al.*, 2010), tributyrin agar (TBA) containing tributyrin, peptone, yeast extract, and agar of 1.0, 0.5, 0.3, and 1.5%, respectively (Atlas, 2010), and skim milk agar (SMA) containing pancreatic digestion of casein, yeast extract, glucose, skim milk, and agar of 1.5, 0.5, 0.25, and 1.0, and 1.5%, respectively (Atlas, 2010). The CCA, TBA, and SMA media were used to detect the activity of chitinase, lipase, and protease, respectively, which were represented by producing clear zones around their colonies. The plates were aerobically incubated at 35°C for 24 h. Results were recorded for the number of colonies that were counted as a colony-forming unit (CFU) per gram of soil. The non-mucoid colonies having different morphological characteristics and exhibiting wide clear zones were selected for pure culture preparation and further investigation. The mucoid colonies could contain slime that might interfere with the bacterial cells passing through some pores on the mealbug's body wall into the insect's body. The pure bacterial colony of the first selection was grown on trypticase soy agar (TSA) composed of pancreatic digest of casein, pancreatic digest of soybean meal, glucose, sodium chloride, di-potassium hydrogen phosphate, and agar at concentrations of 1.7, 0.3, 0.25, 0.5, 0.25, and 1.5%, respectively (Atlas, 2010) and kept at 4°C until use.

Screening and Selection of Gram-Negative, Non Spore-Forming Soil Bacteria

The pure colonies of soil bacteria from the first selection were examined by Gram- and endospore staining. The cell size and arrangement, the endospore formation, were observed under a light microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The Gram-negative isolates having a single or pair of cells arrangement, as well as a smaller size than the main pores on the collected mealbug body wall, were selected. The selected bacteria were further characterized and identified by their physiological characteristics and 16S ribosomal RNA sequences similarity.

Characterization and Identification of the Selected Bacteria

Morphological, cultural, and biochemical characteristics (Garrity *et al.*, 2005) and molecular data based on 16S ribosomal RNA gene sequences, were used for characterization and identification of the selected bacterial isolates (Dorsch *et al.*, 1994). Partial sequencing of the 16S rRNA gene was performed by extracting the total genomic DNA from bacterial cells using the method as described by Sambrook and Russell (2001). The 16S rRNA gene of each bacterium was amplified from the genomic DNA by polymerase chain reaction (PCR) using the following universal primers: - UNI16S-L: 5'-ATTCTAGAGTTTG ATCATGGCTCA-3' as forward and UNI16S-R: 5'-ATGGTACCGTGTGACGGGCGGTG TGTA-3' as reverse to obtain approximately 1,500 bp (Dorsch *et al.*, 1994). The PCR was performed in a thermal cycle (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, CA, USA) with the amplification conditions of an initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 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, then holding at 4°C . The amplified products were examined by gel electrophoresis, and sequenced using a high throughput applied biosystem 3730XL sequencer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, CA, USA). The 16S rRNA gene sequences were

compared to a local alignment search of the GenBank database using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnological Information (NCBI, Bethesda, MD, USA). A phylogenetic tree was constructed using the neighbour-joining method with software MEGA 5.0 program (Tamura *et al.*, 2011). Alignment gaps were treated as missing data. The stability relationships were evaluated by a boot strap analysis of 1,000 replications.

Classification and Identification of the Collected Cassava Mealybugs

The collected mealybugs inhabiting cassava leaves were classified, identified, and studied for their body and wall morphology and structures. The fresh insect specimens were firstly observed under a stereo-microscope (Olympus Optical Co., Ltd., Japan) for their body shape, size, color, part of the head, thorax, and abdomen. The life stage and species of the cassava mealybug were identified as described by Williams (2004) and Parsa *et al.* (2012).

Investigation of the Mealybug Structure Using Light Microscope

A specimen of the dominant cassava mealybug species was selected for its structure investigation to look into the locations that could be the invasion sites of the bacteria by sectioning and observing under the light microscope (Olympus Optical Co., Ltd., Japan). The fresh mealybug specimen was prepared for paraffin section using the methods as described by Xie *et al.* (2014), by fixing in 2.5% glutaraldehyde (v/v) in a 0.2 M phosphate buffer at pH 7.2 and at 4°C for 24 h, then dehydrating 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]), then immersing in an equal volume mixture of xylene and paraffin for 48 h at 56°C, and only paraffin for 48 h at 56°C before embedding in paraffin. The embedded specimen was serially sectioned at a thickness of 0.6 µm and immersed in 100% xylene, then in 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). The prepared specimens were then stained with carbol fuchsin and methylene blue, intermediately rinsed in distilled water (10 min), and then observed under a light microscope (Olympus Optical Co., Ltd., Japan).

Study of Cassava Mealybug Morphology Using Scanning Electron Microscope

The cassava mealybug specimens were prepared for observation so that their bodies could be examined for bacterial cell invasion under a JSM-6010 scanning electron microscope (SEM) (JEOL, Ltd., Tokyo, Japan) using the method as described by Xie *et al.* (2014). The fresh insect specimens were fixed in 2.5% glutaraldehyde (v/v) in a 0.2 M phosphate buffer (pH 7.2) at 4°C for 24 h, rinsed 3 times in a 0.2 M phosphate buffer, then fixed in 1% OsO₄. The specimens were further dehydrated through ethyl alcohol at concentrations of 70, 80, 90, 100% (5 min in each concentration), and 3 changes in 100% ethyl alcohol. The alcohol was then displaced by liquid carbon dioxide and the samples were dried using a critical point dryer (Leica Microsystems GmbH, Wetzlar, Germany), mounted on copper stubs, then coated with gold in the sputter coater (Coater model JFC-1100, JEOL, Ltd., Japan). The prepared insect specimens were finally scanned at different angles using the SEM at an operating voltage of 15 kv.

Proximate Composition Analysis of Cassava Mealybugs

Crude fat was determined by using Soxhlet apparatus for AOAC methods 960.39 and 948.22 (AOAC International, 2006).

Crude protein was done by the Kjeldahl method for AOAC methods 968.06 and 992.15 (AOAC International, 2006).

Crude chitin was analyzed by demineralization of the cassava mealybugs using 1 M hydrochloric acid at room temperature for 30 min. The resulting solids were washed with distilled water until neutral. Then, demineralized samples were dried in the oven at 60°C to constant weights. Deproteinization of chitin was carried out using 1.0 M sodium hydroxide (20 mL/g) in a beaker at 70°C for 24 h. The crude chitin was then dried, and the chitin

content was determined from the weight differences of the raw materials (Al Sagheer *et al.*, 2009).

Crude wax collected from the adult female of the cassava mealybugs was dissolved in chloroform. The chloroform was then evaporated and the waxy particles extracted with n-hexane to remove short-chain compounds which were more soluble in hexane than were the long-chain compounds (Soroker *et al.*, 2003).

Carbohydrate content was calculated by $\text{carbohydrate} = 100 - (\text{moisture} + \text{ash} + \text{crude fat} + \text{crude protein} + \text{chitin}) \times 100\%$, as described by Yang *et al.* (2014).

The ash content of the cassava mealybugs was determined by a dry-ashing procedure at 550°C according to the methods as described by the Codex-adopted AOAC method 942.05 (AOAC International, 2006).

Selection of Bacteria by Testing their Growth in the Modified TSB Medium Containing Cassava Mealybugs

Bacterial isolates were tested for their growth in the cassava mealybug broth prepared by boiling 1 part (dry weight) of ground pink cassava mealybugs (*Phenacoccus manihoti* Matile-Ferrero) with 2 parts of distilled water for 20 min, which was modified from the method of Yongsawatdigul *et al.* (2007). The mealybug slurry was filtered through cheesecloth and supernatant was collected and the pH adjusted to 7.0. The broth was sterilized by autoclaving at 115°C for 10 min. The selected bacterial isolates were prepared as the inoculum by culturing in 5 mL of trypticase soy broth (TSB) incubated at 35°C for 18 h. The inoculum size of 1% (approximate 10^6 CFU/mL) of each isolate was added to 10 mL of cassava mealybug broth and incubated at 35°C in a Gyromax™ 737R orbital incubator shaker (Amerex Instruments, Inc., Concord, CA, USA) at 150 rpm for 48 h. Growth was detected at 0, 24, and 48 h incubation using the standard spread plate method on TSA and incubated at 35°C for 24 h. The pH of the broth culture was measured at 0, 24, and 48 h using a pH meter (MP220, Mettler Toledo Schwerzenbach, Switzerland).

Preliminary Test for the Possibility to Invade the Live Cassava Mealybug body by a Selected Bacterial Strain

Two milliliters of a selected bacterial suspension (approximately 10^8 CFU/mL at the late log phase of growth) were sprayed on 40 mealybug bodies inhabiting cassava leaves which contained both nymphs and adults; they were placed into a plastic box (10×10 cm) containing a moist sterile tissue paper. The control set was performed by spraying with the sterile 0.85% normal saline solution. All treatments, in triplicate, were incubated in a moist chamber at room temperature (32°C) and with a 12:12 h light:dark photoperiod as described by Sattayawong *et al.* (2016) for 5 days. The dead cassava mealybugs were counted and observed daily during incubation. The dead insect specimens were counted and prepared for observation under the stereo-microscope and SEM as in the method described by Xie *et al.* (2014) mentioned above.

Results and Discussion

Soil Sample and Insect Specimen

Fourteen soil samples were collected in triplicate from the soil surface and below the surface at a depth of about 10 to 15 cm, from 14 cassava plantation locations in Nakhon Ratchasima Province. The average pH of these soil samples was 6.61 and the average moisture was 48.93%. The other 2 soil samples were collected from Buriram Province and contained an average moisture content of 47.49% and an average pH of 6.21. A further 2 soil samples with an average pH of 6.52 and an average moisture content of 44.06% were collected from Chaiphaphum Province. The soil samples collected from the 3 provinces were rather acidic (pH 6.02-7.07) and the average moisture contents of these soil samples ranged from 43.00-54.00% (Table 1). These soil samples were used for enumeration and isolation of soil bacteria capable of producing extracellular enzymes with the emphasis on chitinase, lipase, and proteinase.

The live mealybug insects were also collected from the same cassava plantation areas as the collected soil samples. The mealybugs were commonly found on the lower side of the cassava leaves, stem tips, and young stems, as well as in the soil. These samples were collected rapidly and carefully kept in the moisture chamber for further identification, classification, and testing against the potential selected soil bacteria.

Enumeration, Screening, and Selection of Extracellular Enzyme-Producing Bacteria from Soil

Bacteria able to produce the extracellular enzymes, chitinase, lipase, and proteinase were enumerated and isolated from 14 soil samples using 3 standard media, colloidal chitin agar (Nurderbyandaru *et al.*, 2010), tributyrin agar (Atlas, 2010), and skim milk agar (Atlas, 2010). Four hundred and sixteen non-mucoid colonies with clear zones, were

selected (Table 1). The soil samples from Nakhon Ratchasima Province were found to contain bacteria having chitinase, proteinase, and lipase, ranging from 2.90×10^4 - 2.72×10^5 , 1.30×10^5 - 6.85×10^5 , and 1.05×10^5 - 1.86×10^6 CFU/g, respectively. Then 173, 96, and 33 different colonies were selected from the enumerating CCA, SMA, and TBA media, respectively. Another 2 soil samples collected from Buriram Province, were found to contain a number of bacteria grown on the 3 media around 2.05×10^5 - 1.23×10^5 , 2.45×10^5 - 2.33×10^6 , and 1.38×10^5 - 1.61×10^5 CFU/g, respectively, and then 22, 22, and 5 different colonies, respectively, were kept for further study. For the soil samples collected from Chaiyaphum Province, numbers of bacteria growing on CCA, SMA, and TBA, were detected around 1.40×10^5 - 2.40×10^5 , 2.70×10^5 - 6.65×10^5 , and 1.24×10^5 - 2.85×10^5 CFU/g, respectively; then 29, 28, and 8 isolates, were selected from the enumeration media, CCA, SMA, and TBA, respectively. In this

Table 1. Enumeration results of bacteria from soil collected from 14 locations of cassava plantation areas in North-Eastern Thailand, using colloidal chitin agar (CCA), skim milk agar (SMA), and tributyrin agar (TBA), and for detecting chitinase-, lipase-, and proteinase-producing bacteria, respectively

No. and location of soil sample collection	Soil sample		Viable cell counts on the medium (CFU/g)			No. of selected isolate from the medium		
	pH	Moisture (%)	CCA	SMA	TBA	CCA	SMA	TBA
Nakhon Ratchasima Province								
1	6.84	54.43	9.79×10^4	1.34×10^5	1.86×10^6	24	24	6
2	6.63	51.56	2.72×10^5	2.41×10^5	1.42×10^5	24	8	3
3	6.82	55.25	3.25×10^4	7.55×10^5	1.07×10^5	32	14	2
4	6.58	46.64	1.58×10^5	1.36×10^5	1.05×10^5	16	8	3
5	6.53	46.35	2.41×10^5	6.85×10^5	2.09×10^5	12	8	2
6	6.91	42.44	2.47×10^5	4.30×10^5	7.60×10^5	11	6	2
7	6.02	45.11	1.35×10^5	2.11×10^5	2.01×10^5	25	8	2
8	6.59	54.68	2.90×10^4	1.30×10^5	1.13×10^5	12	8	4
9	6.11	47.71	6.50×10^4	1.71×10^5	1.91×10^5	9	5	4
10	7.07	45.08	1.72×10^5	1.37×10^5	1.33×10^5	8	7	5
Buriram Province								
1	6.15	48.89	2.05×10^5	2.33×10^6	1.61×10^5	7	8	2
2	6.27	46.09	1.23×10^5	2.45×10^5	1.38×10^5	15	14	3
Chaiyaphum Province								
1	6.52	43.50	2.40×10^5	6.65×10^5	2.85×10^5	16	16	5
2	6.51	44.62	1.40×10^5	2.70×10^5	1.24×10^5	13	12	3

step, the total of 416 isolates (224, 146, and 46 isolates were selected from the enumeration media, CCA, SMA and TBA, respectively) were selected for further screening for their cell morphology and confirmed for their extracellular enzyme production by point inoculation on the same agar media as mentioned above.

Screening and Selection of Gram-Negative, Non-Spore Forming Soil Bacteria

This step aimed to obtain dominant soil bacteria that had their small cells suitable for invading some pores on the cassava mealybug

body wall. The target bacteria should not produce spores that could contaminate the cassava tubers and cause problems to the quality of cassava starch in the starch production industry. The Gram-negative, non-spore forming bacteria, thus, became the potential candidates.

Forty-four out of 416 isolates were selected since they were Gram-negative, non-spore forming bacteria having small cell sizes between 0.2-1.0×1.0-3.5 µm, which occurred singly or in pairs. These characteristics supported the ability to penetrate into the mealybug's body as mentioned in the study of cassava mealybug morphology using the SEM as stated below.

Table 2. Morphological and biochemical characteristics of seven isolates; SUTT 15, SUTT 16, SUTT 26, SUTS 80, SUTS 53, SUTS 65, and SUTS 59, of soil bacteria able to produce chitinase, lipase, and proteinase, compared to closest relatives

Characteristics	Selected bacterial isolates compared to closely related reference strains										
	SUTT 15	SUTT 16	1	SUTT 26	SUTS 80	2	SUTS 53	3	SUTS 65	4	SUTS 59
Colony on TSA (24 h) and colony pigment color	Circular, raised, entire, smooth, opaque, purple	Circular, raised, entire, smooth, opaque, purple	Circular, raised, entire, smooth, violacein	Circular, raised, entire, smooth, opaque, yellow	Circular, raised, entire, smooth, opaque, yellow	Circular, raised, entire, smooth, opaque, yellow	Circular, raised, entire, smooth, opaque, white	Circular, raised, entire, smooth, opaque, white	Circular, raised, entire, smooth, opaque, white	Circular, raised, entire, smooth, opaque, white	Circular, raised, entire, smooth, opaque, white
Cell shape	Rods	Rods	Rods or coccoids	Rods	Rods	Rods	Rods	Straight rods	Rods	Straight rods	Rods
Cell size (µm)	0.2-0.6 ×1.0-1.5	0.2-0.5 ×1.0-1.5	0.6-0.9 ×1.5-3.0	0.2-0.5 ×1.2-2.5	0.2-0.5 ×1.0-3.0	0.2-0.5 ×1.0-3.0	0.5-1.0 ×1.5-0.5	0.5-1.0 ×1.5-0.5	0.2-0.6 ×0.7-1.5	0.5-1.0 ×1.5-0.5	0.3-0.5 ×2.0-2.4
Gram	-	-	-	-	-	-	-	-	-	-	-
Spore forming	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	nd	+	nd	+	nd	+
Oxidase	+	+	+	+	+	+	+	+	+	+	-
Motility	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of:											
Casein	+	+	+	+	+	nd	+	+	+	+	+
Tween 80	+	+	+	+	+	nd	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+
Chitin	+	+	+	-	-	nd	+	nd	+	nd	+
Indole	+	-	+	-	+	-	+	+	-	+	-
Citrate utilization	+	+	+	-	-	+	+	+	+	+	+
Nitrate reduction	+	+	+	-	-	d	+	+	+	+	-
Arginine dihydrolase	+	+	+	-	-	nd	+	nd	+	nd	-
H ₂ S production	+	-	+	-	-	nd	-	-	-	-	-
Urea hydrolysis	-	-	-	+	+	nd	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-	nd	-	-	-	-	+
Utilization of:											
D-sucrose	-	-	-	-	-	-	-	-	-	-	+
L-arabinose	-	-	-	-	-	-	-	-	+	+	-
D-glucose	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	-	-	-	-	-	-	+	+	+	+	-
D-sorbitol	-	-	-	-	-	-	-	-	+	+	+
D-melibiose	-	-	-	-	-	-	-	-	-	-	+
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+
Growth at 4	-	-	-	-	-	-	-	-	+	+	-
35	+	+	+	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 7.0	+	+	+	+	+	+	+	+	+	+	+
Growth on MacConkey	+	+	nd	+	+	d	+	nd	+	nd	+

+, Positive test or presence; -, negative test or absence; d, 11-89% of the strain was positive; nd, not detected; ¹, *Chromobacterium violaceum* NCTC 13278 (Garrity, 2006); ², *Chryseobacterium gleum* DSM 16776 (Vandamme et al., 1994); ³, *Pseudomonas aeruginosa* ATCC 10145 (Garrity, 2006); ⁴, *Pseudomonas fluorescens* DSM 50090 (Garrity, 2006); ⁵, *Serratia marcescens* NCTC 8900 (Garrity, 2006)

After testing their chitinase, lipase, and proteinase production by point inoculation on CCA, SMA, and TBA, and incubation at 35°C for 24 h, 44 isolates were found to exhibit clear zones surrounding their colonies growing on the 3 media, which indicated the production of the extracellular enzymes, chitinase, proteinase, and lipase, respectively. Then, only 7 out of 44 isolates showing clear zones surrounding colonies around 3.0-4.0 mm for CCA, 15.0-22.0 mm in diameter for SMA, and 4.0-6.0 mm in diameter for TBA, were selected for further investigation.

Characterization and Identification of the Selected Bacteria

The 7 selected bacterial isolates, designated as SUTT 15, SUTT 16, SUTT 26, SUTS 80, SUTS 53, SUTS 65, and SUTS 59, were characterized and identified by their morphological, cultural, and biochemical characteristics as well as by 16S rRNA gene sequence analysis. From the morphological, cultural, and biochemical characterization results, all selected isolates were Gram-negative, non-spore forming rods, with an average cell size of 0.2-1.0×1.0-3.0 µm (Table 2), and it was found that SUTT 15 and SUTT 16 were similar to the closest relatives *Chromobacterium violaceum* NCTC 13278 (Garrity *et al.*, 2005). SUTT 26 and SUTS 80 were similar to *Chryseobacterium gleum* DSM 16776 (Vandamme *et al.*, 1994). SUTS 53, SUTS 65, and SUTS 59 were similar to *Pseudomonas aeruginosa* ATCC 10145 (Garrity, 2006), *Pseudomonas fluorescens* DSM 50090 (Garrity, 2006), and *Serratia marcescens* NCTC 8900 (Garrity, 2006), respectively (Table 2). The 16S rRNA gene sequences of SUTT 15, SUTT 16, SUTT 26, SUTS 80, SUTS 53, SUTS 65, and SUTS 59 were deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank>) with the accession numbers NR113595, KR780470, JN544159, KF017580, HQ018742, KF770987, and KJ721215, respectively. These DNA sequences were compared to sequences from other bacteria from the nucleotide database provided by NCBI using BLAST to ascertain their closest relatives.

BLAST analysis of the generated 16S rRNA gene sequences for these bacteria showed similarities of 97.74, 98.18, 98.19, 98.23, 96.19, 98.60, and 93.19%, respectively, compared to *Chromobacterium violaceum* NBRC 12614 (NR113595), *Chromobacterium violaceum* CRh33 (KR780470), *Chryseobacterium gleum* PRR1 (JN544159), *Chryseobacterium indologenes* ZYF120413-7 (KF017580), *Pseudomonas aeruginosa* ASFP45 (HQ018742), *Pseudomonas fluorescens* CPB pse (KF770987), and *Serratia marcescens* IHB B7064 (KJ721215), respectively (Figure 1). These similarities were less than 99% implying that these selected isolates could be the same genera but different species to the closest relatives. The selected soil bacteria were, therefore, identified as *Chromobacterium* sp. SUTT 15, *Chromobacterium* sp. SUTT 16, *Chryseobacterium* sp. SUTT 26, *Chryseobacterium* sp. SUTS 80, *Pseudomonas* sp. SUTS 53, *Pseudomonas* sp. SUTS 65, and *Serratia* sp. SUTS 59.

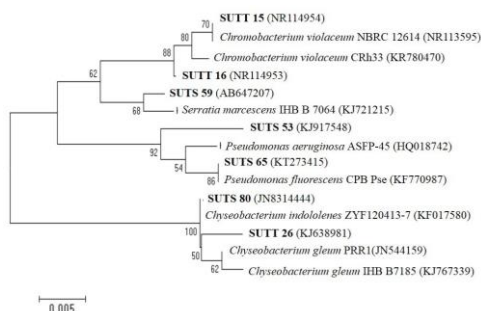


Figure 1. Phylogenetic tree of 7 selected bacterial isolates, SUTT 15, SUTT 16, SUTS 59, SUTS 53, SUTS 65, SUTS 80, and SUTT 26, based on 16S rRNA gene sequences using the neighbor-joining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 5 programs. Branch lengths are scaled 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

Classification and Identification of Cassava Mealybugs Specimens

Cassava mealybugs were collected from 14 different plantation areas in Northeastern Thailand. From their morphological characteristics, the insect specimens were classified and identified according to Williams (2004) and Parsa *et al.* (2012), as belonging to 4 species (Table 3). which were the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero) having a pink oval shape with short wax filaments, the green mealybug (*Phenacoccus maderiensis* Green) having a yellowish green, oval shape with short wax filaments, Jack Beardsley mealybug (*Pseudococcus jackbeardsleyi* Gimpel & Miller) having a long and oval body and wax filaments around the periphery, and the striped mealybug (*Ferrisia virgata* Cockerell) also having a long and oval body with wax removed and 2 stripes on back, with a few filamentous glassy strands of wax protruding from the body (Table 3). The dominant cassava mealybug specimens were

the pink mealybugs (*Phenacoccus manihoti* Matile-Ferrero). The stages of the life cycle found were nymph, adult, and eggs with ovisac (Figure 2). The minor populations found belonged to the striped mealybug (*Ferrisia virgata* Cockerell), and Jack Beardsley mealybug (*Pseudococcus jackbeardsleyi* Gimpel & Miller) (Figure 3). Soroush *et al.* (2012) reported that 5 species of mealybugs were found to attack cassava plants in Thailand. These species were the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero), Madeira mealybug (*Phenacoccus madeirensis* Green), Jack Beardsley mealybug (*Pseudococcus jackbeardsleyi* Gimpel & Miller), striped mealybug (*Ferrisia virgata* Cockerell), and solenopsis mealybug (*Phenacoccus solenopsis* Tinsley). This finding was the same as reported by Sattayawong *et al.* (2016). Of the 5 species of cassava mealybugs reported to be attacking cassava in Thailand, the solenopsis mealybug, *Phenacoccus solenopsis* Tinsley and the pink mealybug, *Phenacoccus manihoti* Matile-

Table 3. Cassava mealybug specimens collected from cassava fields in North-eastern Thailand, identified according to Williams (2004) and Parsa *et al.* (2012)

Cassava mealybug specimen	Color Body shape		Morphological characteristics under stereo microscope				Identification result
			Size (lengthxwidth; nm)	Dorsal	Lateral	Ventral	
1	Gray	Oval; slightly rounded in lateral view	2.80-3.10x1.8-2.0	The covered with powdery white wax, without stripes on back, and antenna 8 segments	Thin filaments around body, with 17 pairs of lateral filaments	Caudal pair about one-half length of body or more	<i>Pseudococcus jackbeardsleyi</i> Gimpel & Miller
2	Dark gray	Elongate oval	2.20-4.10x0.90-2.50	With wax removed with 2 stripes on back, with a few filamentous glassy strands of wax protruding from the body, and antenna 8 segments	Non have waxy filaments on the lateral	Only 1 pair of conspicuous anal wax filaments present, hind filament one-half length of body	<i>Ferrisia virgata</i> Cockerell
3	Pale green	Oval	0.88-2.30x0.74-1.38	Covered with mealy white wax, which is not thick enough to hide the yellow body, no markings on back, and antenna 9 segments	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	<i>Phenacoccus maderiensis</i> Green
4	Pink	Oval	0.60-2.50x1.00-1.50	Dusted with white mealy powder, without stripes on back, and antenna 9 segments	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	<i>Phenacoccus manihoti</i> Matile-Ferrero

Ferrero were recorded to be rapidly increasing in numbers and dispersed across the cassava-growing regions, causing yield losses in large areas of cassava fields. (Papadopoulou and Chryssohoides, 2012).

Investigation of Cassava Mealybug Structure Using Light Microscope

From the collected cassava mealybug specimens, the adult female of the pink mealybug, (*Phaenococcus manihoti* Matile-Ferrero), was found to be the dominant species in the plantation areas. The adult female of the insect species was selected for its structure

investigation to evaluate the target site for invasion by the bacteria. The longitudinal section of the insect's body showed a thick cuticle layer (3–4 μm) covering the body, and wax glands secreting wax filaments around the body. For the cross section of the abdominal terminal of the adult female, a series of wax glands were observed closely arranged in the epidermis. There were more wax glands on the dorsal than the ventral surface (Figure 4). Papadopoulou and Chryssohoides (2012) mentioned that the wax was synthesized by the glandular cells. The wax reservoir was found to be located at the central cells. The tube duct

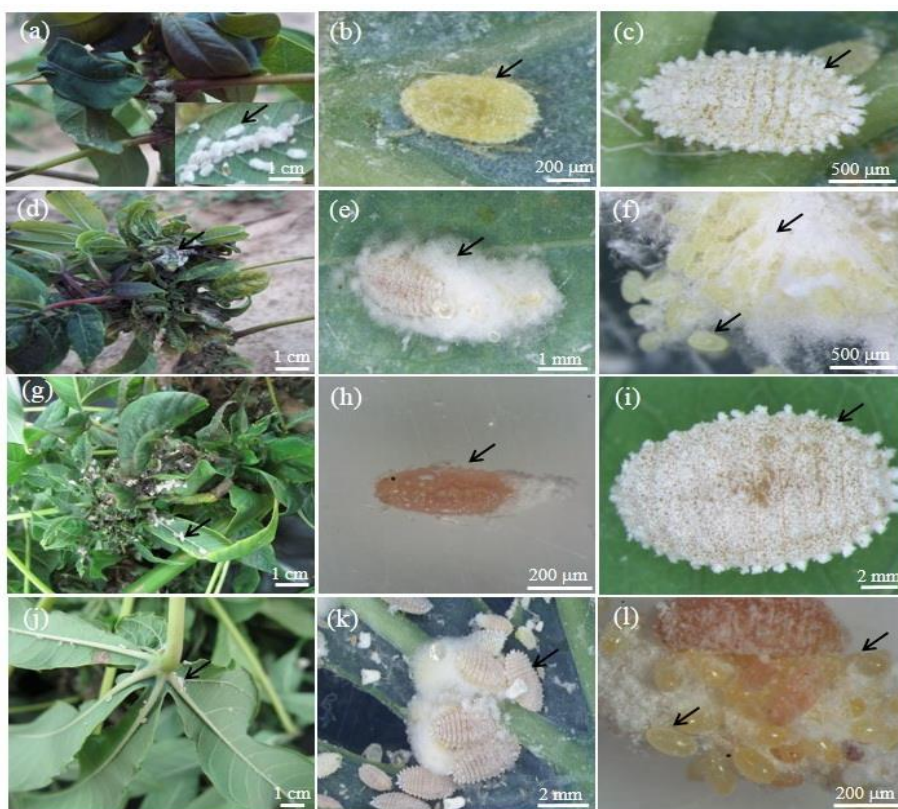


Figure 2. Green and pink cassava mealybugs found in their natural habitats in North-eastern Thailand, and their morphology observed under stereo microscope. Green mealybug (*Phenacoccus maderiensis* Green): (a) and (d) its habitat under cassava leaves; (b) nymph; (c) adult female; (e) adult female with eggs, and (f) ovisac of eggs. Pink mealybug (*Phenacoccus manihoti* Matile-Ferrero): (g) and (j) its habitat under cassava leaves; (h) nymph, (i) adult female, (k) adults with eggs, and (l) ovisac of eggs. Scale bars as indicated in the Figure

had its bottom end connected to the center cell, through which the excreted wax substances were led to the outer cuticle surface. Sirisena *et al.* (2015) described the integument of the mealybug as consisting of 3 main layers: the procuticle, epidermis, and basement membrane. The procuticle was the outermost integument layer, containing a further 4 sub-layers: the epicuticle, exocuticle, endocuticle, and formation zone. *Phenacoccus madeirensis* Green has a grey oval body covered by thin, white, mealy wax, with red legs and dark dorso-submedial bare spots on the intersegmental areas of the

thorax and abdomen. These areas form a pair of dark longitudinal lines on the dorsum. The ovisac covered the entire dorsum, with 18 pairs of lateral wax filaments, the posterior pairs being the longest. They were approximately the length of the body or a little shorter. Similar results were reported by Papadopoulou and Chryssohoides (2012). From the results of this study, the open pores around the body could be the target of invading bacteria such as spiracles, ostioles, triocular, multiocular, quinquelocular, discoidal, and circulus pores.

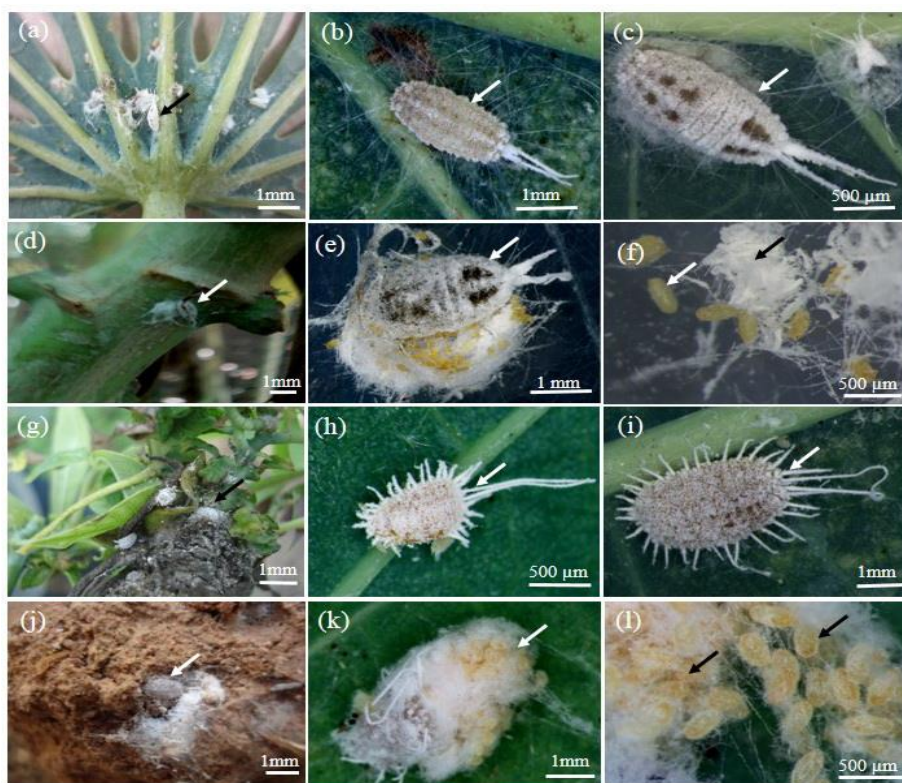


Figure 3. Striped and Jack beard cassava mealybugs found in their natural habitats in North-eastern Thailand, and their morphology under stereo microscope. Striped mealybug (*Ferrisia virgata* Cockerel): (a) and (d) its habitat under cassava leaf, and stem, respectively; (b) nymph, (c) adult female, (e) adult female with eggs, and (f) ovisac of eggs. Jack beard mealybug (*Pseudococcus jackbeardsleyi* Gimpel & Miller): (g) and (j) its habitat under cassava leaf, and the insect found in soil, respectively; (h) nymph, (i) adult female, (k) adult female with eggs, and (l) ovisac of eggs. Scale bars as indicated in the Figure

Study of Cassava Mealybug Morphology Using Scanning Electron Microscope

The adult specimen of the female pink cassava mealybug *Phenacoccus manihoti* Matile-Ferrero was prepared and its body morphology observed using SEM. The adult female was used because of its main role in reproduction: on the dorsal surface of the adult female, there were ostiole, antenna, cerarius, and wax-secreting pores, and on the ventral surfaces which included the mouth, spiracle, vulva, legs, and circulus (Figures 5 and 6). The nymph morphology was also observed (Figure 7). The following structures were found from the observation under SEM.

Ostioles: each ostiole was a lip-like cuticular structure on the dorsal body surface near the eyes (Figures 5(a-i)). An outer pair of lips of the epicuticle, surrounding an inner pair of membranous lips was shown. Sirisena *et al.* (2015) described that the inner lips form a muscularly controlled valve at the outer end of

an internal canal. When it was disturbed, the inner lips may be relaxed to release a globule of liquid that congeals quickly on contact with the air; this is believed to be a defensive mechanism, fouling the mouthparts of a predator. The outer lips bear trilocular pores and sometimes flagellate setae probably to prevent the exudates fouling the mealybug's surface. Paired ostioles are a diagnostic feature of the family Pseudococcidae (Sirisena *et al.*, 2015).

Antenna: 9 antennal segments were present in this insect. The apical and sub-apical segments are clearly separated. The apical segment was the longest, followed by the third and second segments (Figures 5(j), 5(k), and 5(l)). Early workers used the number of antennal setae as primary characteristics to separate species (Sirisena *et al.*, 2015).

Discoidal pores: the second type of the wax-secreting pores, these had a dense distribution on the dorsal abdominal segments,

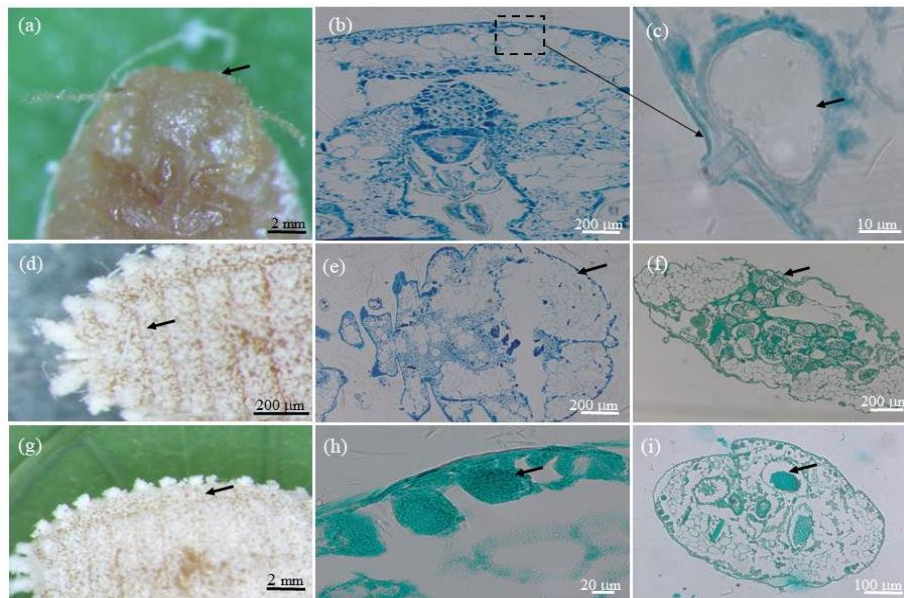


Figure 4. Morphology and structures of adult female, the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero), observed under stereo (a, d, and g) and light (b, c, e, f, h, and i) microscopes: (a) and (b) the wax glands in the anterior part (head) (arrows); (c) wax secreted by the wax gland (arrow); (d) and (e) position of the wax gland in the posterior part (anus) (arrows); (f) ovisac and eggs of the insect (longitudinal section); (g) position of the wax gland, (h) and (i) sections of the wax glands. Scale bars as indicated in the Figure

the thoracic margin, and submargin, but were absent in the middle area of the head (Figure 5(m)).

Quinquelocular pores: the third type of wax secreting pores, they were generally restricted to the stigmatic furrow on the ventral body surface. Each quinquelocular pore consisted of 5 circular loculi, arranged in a circle or pentagon with each loculus further secreting 1 wax filament (Figure 5(m)).

Multilocular disc pores: the fourth type of wax pores are usually present on the venter, at least around the vulva (Figure 5(n)). Each pore contains a circle of more than 5 openings

(loculi). In this insect, 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. These filaments were broken into short and curved fragments after being secreted from the pore and conglutinated on the surface of the eggs, both to prevent them from sticking and to protect them from immersion and desiccation (Sirisena *et al.*, 2015).

Trilocular pores: the first type of wax pore produced spiral wax filaments that fragmented to form the fine, mealybug body covering (Sirisena *et al.*, 2015). In this study the

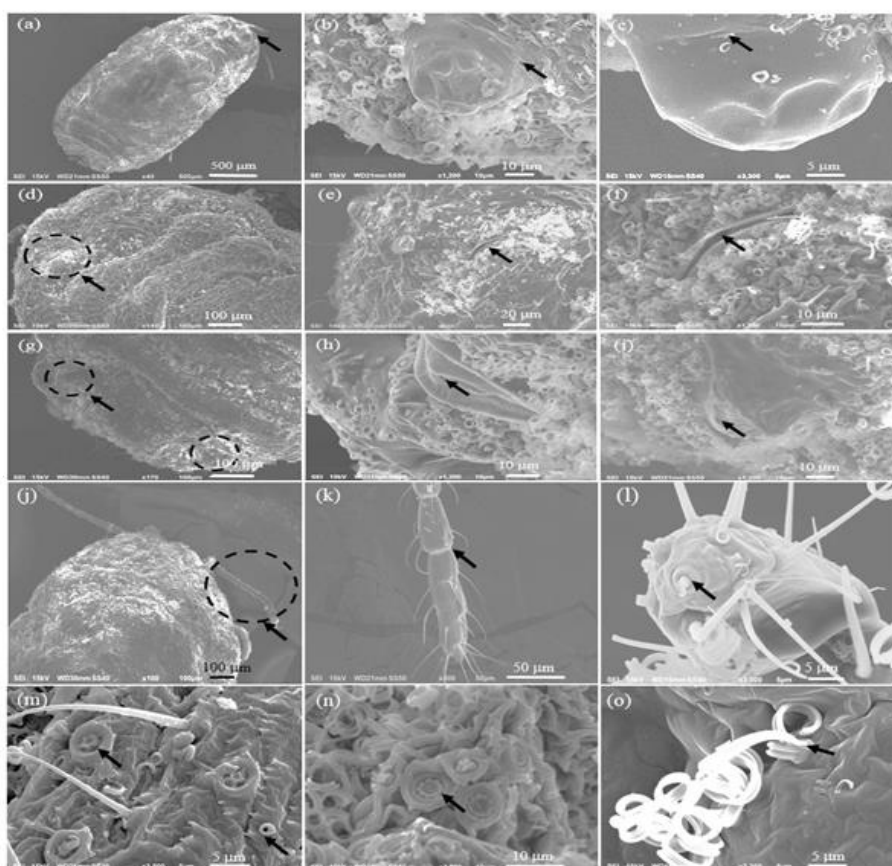


Figure 5. SEM micrographs of the dorsal body of the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero) adult female: (a), (b), and (c) eye (arrows; (d), (e), and (f) the anterior ostiole (arrows); (g), (h) and (i) the posterior ostioles (arrows); (j), (k), and (l) the antenna (arrows), and (m), (n), and (o) discoildal and quinquelocular pores, multilocular pore, and trilocular pore (arrows). Scale bars as indicated in the Figure

wax pores were abundant and spaced evenly over both the dorsal and ventral body surfaces but were absent in intersegmental areas. Each the trilobular pores had a diameter of about 3-4 μm . Each opening secreted a flat shaped wax filament, 2 μm in width. These wax filaments linked on the surface of the body (Figure 5(o)).

Circulus: the circulus is a large, membranous structure found on the midline of the abdominal venter in the mealybug species (Figures 6a, 6b, and 6c). It helps the mealybug adhere to the substrate (Sirisena *et al.*, 2015).

Spiracles: the respiration in this insect was mediated by the tracheal system, which

has cuticular openings called spiracles located in part of the thorax and abdomen (Figures 6(d-f)).

Legs: the cassava mealybugs had only 1 tarsal segment and a single claw on each leg. At the expanded base of the claw, there was usually a pair of seta-like digitules (claw digitules) (Figures 6(g-k)).

Cerarii: these paired, dorsal structures on the body margins are present only in the mealybug (Sirisena *et al.*, 2015). They secrete and support the lateral wax filaments during the mealybug's life. Each cerarius consists of an aggregation of setae of 1 or 2 types, trilobular pores (Figures 7(h) and 7(i)), and sometimes discoidal pores.

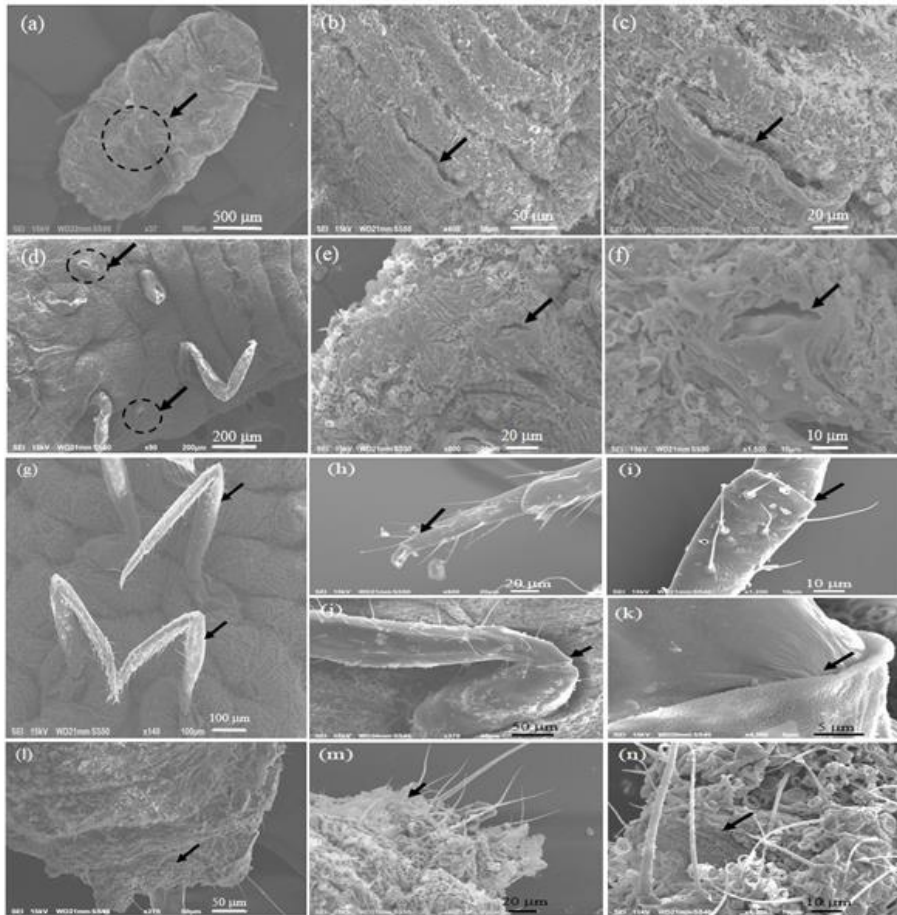


Figure 6. SEM micrographs of the ventral body of the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero) adult female: (a), (b), and (c) circulus (arrows); (d), (e), and (f) spiracles (arrows); (g) legs, (h) claw, (i), (j), and (k) thigh (arrows); (l), (m), and (n) anus (valve) (arrows). Scale bars as indicated in the Figure

In this study, trilocular pores and discoidal pores were the major types of the wax-secreting pores in the integument of the pink cassava mealybug. Based on the observation, the trilocular pores were fairly numerous with the wax filaments that were secreted to form the wax covering on the mealybug surface in all instars of the nymph and adult. A similar wax was present on the lateral filaments formed from the cerarii. The trilocular and discoidal pores were also reported in other scale insects, such as *Ceroplastes japonicus* Green, a species of the family Coccidae (Zhang *et al.*, 2012). However, the trilocular pores only occurred on the dorsal surface in nymphs and adults. In this finding, the mealybug structures that could be invaded by some specific bacterial strains were the

anterior and posterior ostioles, wax-producing filaments, abdomen (circulus), and respiration (spiracles), with 2-59, 2-7, 17-20, and 9-30 μm diameters, respectively. Eggs and adults were detected in the pink mealybug collected from its natural habitat (Figure 8).

Proximate Composition of Cassava Mealybugs

The chemical compositions of the adult female of the pink cassava mealybug *Phenacoccus manihoti* Matile-Ferrero were analyzed in order to be tested as bacterial nutrients. The fresh insect was found to contain 22.70% moisture content, and composed of crude chitin, crude protein, and carbohydrate of 15.06, 3.09, and 6.15%, respectively (Table 4). Because of its high wax content, the adult

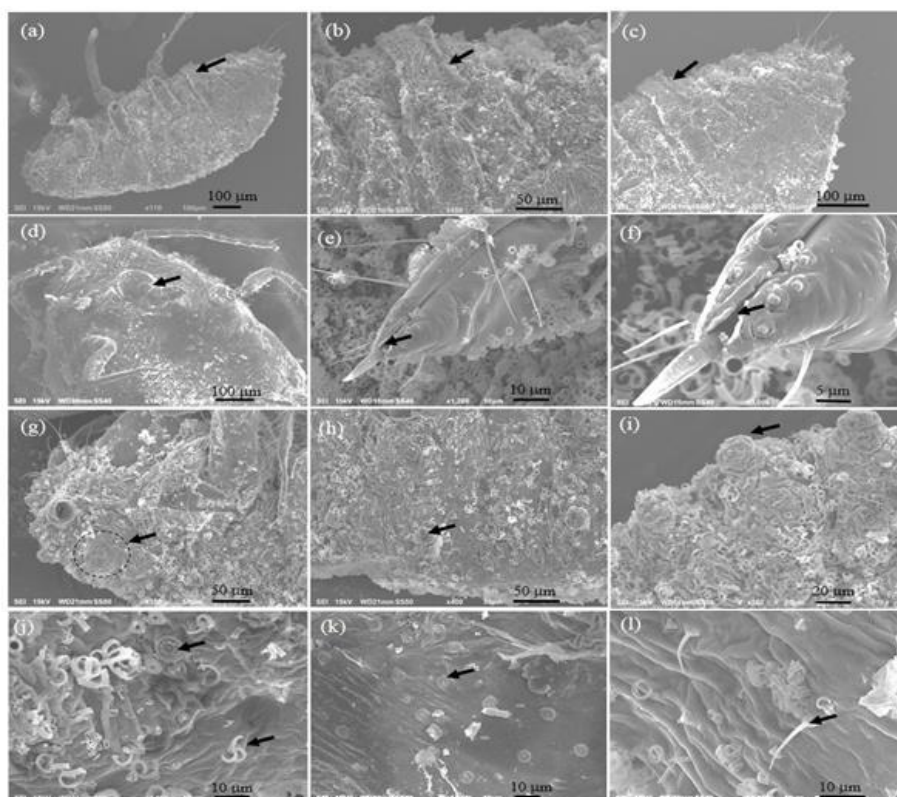


Figure 7. SEM micrographs of nymph of the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero): (a), (b), and (c) the position of circulus (abdomen) (arrows; (d), (e), and (f) the mouth (arrows; (g) the position of eye (arrow); (h) and (i) the cerarii (the position of lateral filament) (arrows); (j), (k), and (l) multilocular and trilocular pores, quinquelocular pore, and discoidal pore (arrows). Scale bars as indicated in the Figure

Phenacoccus manihoti Matile-Ferrero had a high wax to protein ratio. The liquid inside the fresh body of the pink mealybug was also analyzed. High-performed liquid chromatography of the liquid of the adult female pink cassava mealybug body was carried out, and the liquid was found to contain glucose, fructose, maltose, and sucrose. The major preparation was glucose (about 82.73%). The insect cuticle was reported to be

a very complex structure composed of protein, chitin, and fatty acid. The protein constituent was reported to be nearly 50-80% of the total mass (Gupta *et al.*, 1992). However, the structure varies considerably depending upon the insect source. The adult *Phenacoccus manihoti* Matile-Ferrero had a low protein content. The protein content of insects varies widely among species (Yang *et al.*, 2014).

Table 4. Proximate compositions of adult pink cassava mealybugs (g/100g dry weight)

Parameter	Concentration (g/100 g dry weight)
Moisture (%) ^a	22.70±0.72
Wax particle ^b	31.37±1.02
Crude fat ^c	15.40±2.28
Crude protein ^d	3.09±0.04
Crude chitin ^e	15.06±0.14
Ash ^f	6.23±0.39
Carbohydrate ^g	6.15±0.89

Values are expressed as means ± standard deviations of triplicate analyses. ^a, AOAC International Methods 934.01 (2006); ^b, Soroker *et al.* (2003); ^c, AOAC International Methods 960.39 and 948.22 (2006); ^d, AOAC International, Methods 968.06 and 992.15 (2006); ^e, Al Sagheer *et al.* (2009); ^f, AOAC International Method 942.05 (2006); ^g, Yang *et al.* (2014)

Growth of the Selected Bacteria in the Medium Containing Cassava Mealybugs

Seven bacterial isolates that were selected based on the dominant isolates found in the soil, the small size of the cells, and the capability to produce enzymes for hydrolyzing the fresh mealybug, were tested for growth in the cassava mealybug medium. The isolate *Serratia* sp. SUTS 59 showed the highest growth (from approximately 10^6 cells/mL to 6.20×10^8 CFU/mL within 24 h, and to 3.89×10^9 CFU/mL, within 48 h) followed by *Pseudomonas* sp. SUTS 53 (from approximately 10^6 cells/mL to 4.68×10^8 CFU/mL within 24 h, and to 3.31×10^9 CFU/mL within 48 h) (Figure 9). *Serratia* sp. SUTS 59 was also a facultative anaerobe that could grow inside the mealybug body. The strain was then selected to test for the ability to invade cassava mealybugs.

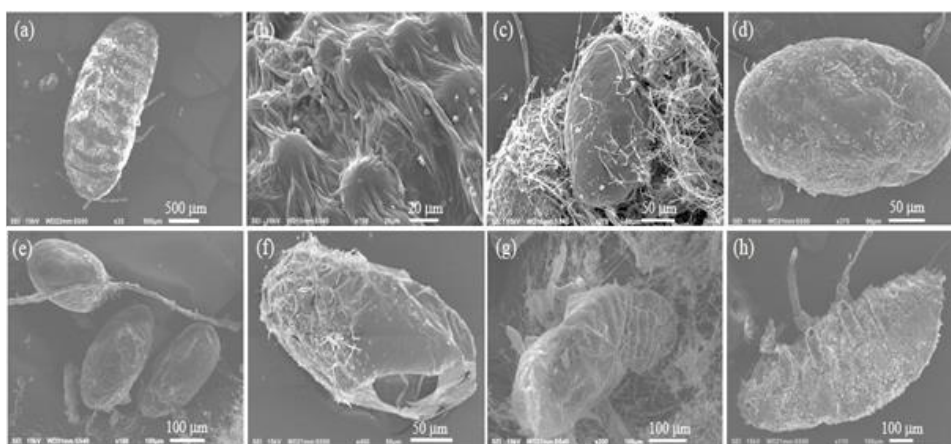


Figure 8. SEM micrographs of the pink mealybug (*Phenacoccus manihoti* Matile-Ferrero): (a) adult female; (b) eggs inside adult female, scale bar = 20 µm; (c) egg outside adult female; (d) egg shape outside; (e) hatched egg; (f) egg after hatching; (g) molt stage; and (h) the nymph. Scale bars as indicated in the Figure

Preliminary Test for the Possibility to Invade the Live Cassava Mealybug by a Selected Bacterial Strain

Serratia sp. SUTS 59 was selected to perform the preliminary test for the ability to invade the live cassava mealybug. After spraying the bacterial cell suspension (0.85% sodium chloride solution containing approximately 10^8 CFU/mL) on the pink mealybug, the effect of the tested isolate was monitored by counting the number of living and dead mealybugs after observing the insect response to stimulus and change in color of the insect body. It was found that the isolate *Serratia* sp. SUTS 59 could kill the pink mealybug. The mortality rate of live cassava mealybugs of 15.83, 37.50, 52.50, and 70.00% was detected at 2, 3, 4 and 5 days after spraying. The results were better than the use of fungi (*Metarhizium anisopliae* and *Beauveria bassiana* (10^7 spores/mL) in which 50% mortality was found at day 6 (Amutha and Banu, 2011). The dead insect bodies showing a color change from the normal color to reddish brown to black, were then observed under stereo microscope and SEM. Bacterial cells were found to occupy the insect body (Figure 10). Salunkhe *et al.* (2013) also reported that *Serratia marcescens* isolated from dead green mealybugs, *Maconellicoccus hirsutus* (Green), could have the potential to be used as a biocontrol.

Conclusions

Fourteen soil samples were collected from 10, 2, and 2 cassava plantation locations in Nakhon Ratchasima, Buriram, and Chaiyaphum Provinces, respectively, in Northeastern Thailand; the dead bodies of cassava mealybugs were found for isolation and screening of potential bacteria for controlling the mealybugs. All target bacteria selected from the isolation did not produce spores that could contaminate cassava tubers and starch products. Seven isolates of the dominant soil bacteria were finally selected from their potential to produce hydrolytic extracellular enzymes, chitinase, proteinase, and lipase, to hydrolyze cassava mealybug structures; their small cell sizes ($0.2\text{-}1.0 \times 1.0\text{-}3.0$ μm occurring singly or in pairs) supported their ability to penetrate into the mealybug body via some pores on the cassava mealybug body wall, and their perfect growth in the medium containing cassava mealybug. The 7 selected bacterial isolates were Gram-negative, designated and identified as *Chromobacterium* sp. SUTT 15, *Chromobacterium* sp. SUTT 16, *Chryseobacterium* sp. SUTT 26, *Chryseobacterium* sp. SUTS 80, *Pseudomonas* sp. SUTS 53, *Pseudomonas* sp. SUTS 65, and *Serratia* sp. SUTS 59, according to their physiological

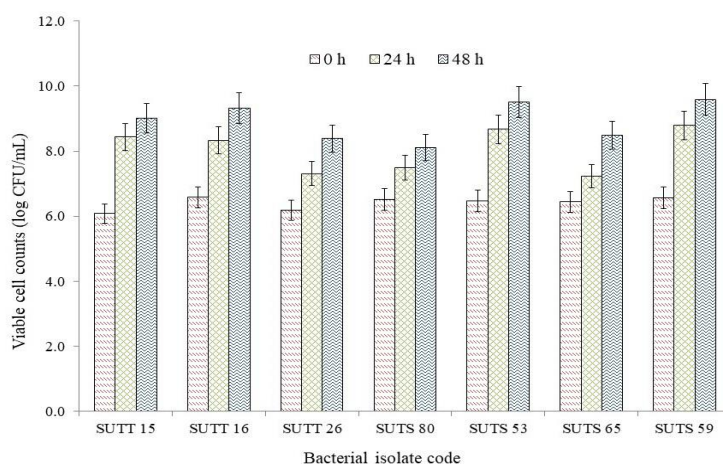


Figure 9. Growth of seven selected bacterial strains isolated from soil, in the cassava mealybug broth with shaking at 150 rpm, 35°C for 48 h

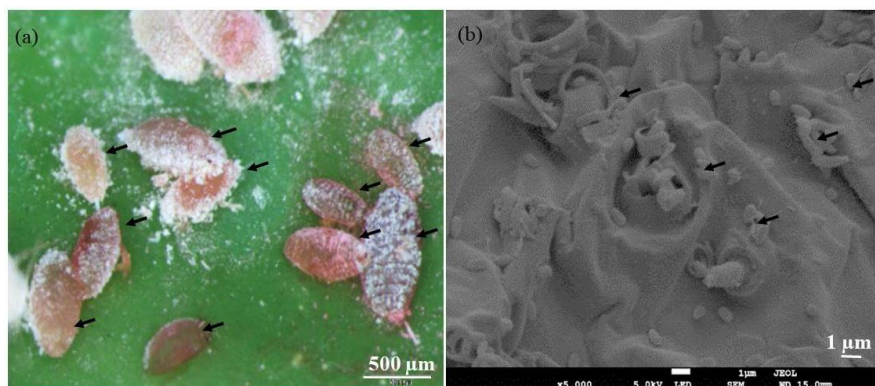


Figure 10. The pink mealybug on fresh cassava leaf after spraying with cell suspension of the selected bacterium, *Serratia* sp. SUTS 59 and incubating in a moist chamber at room temperature (32°C) for 5 days: (a) the dead cassava mealybugs (arrows) under stereo microscope, (b) SEM micrograph of the dead insect with invading bacterial cells (arrows). Scale bars as indicated in the Figure

characteristics and 16S ribosomal RNA sequences similarity compared to their closest reference relatives.

The cassava mealybugs were also collected from the same locations as the collected soil samples, to be investigated as the bacterium-invading targets. The insects were found to live on the cassava plant, and accumulated in the soil under the plant and to be nutrients for soil bacteria. The collected insect specimens were classified and identified as belonging to the green mealybugs (*Phenacoccus maderiensis* Green), pink mealybugs (*Phenacoccus manihoti* Matile-Ferrero), striped mealybug (*Ferrisia virgata* Cockerel), and Jack Beardsley mealybug (*Pseudococcus jackbeardsleyi* Gimpel & Miller). The pink mealybug (*Phenacoccus manihoti* Matile-Ferrero) was mainly found. Its body is covered with a 3-4 µm thick cuticle layer having several pores, particularly ostioles, cerarii, and spiracles with 2-59, 2-7, 17-20, and 9-30 µm diameters, respectively, which could be invaded by the selected soil bacteria. Then, the facultative anaerobic bacterial isolate, *Serratia* sp. SUTS 59, exhibiting very good growth in cassava mealybug broth, was selected for testing by spraying its cell suspension (approximately 10^8 cells/mL) onto the live adult females of the pink cassava mealybug. The insect mortality of 52 and 70% was detected after treating for 4 and 5 days,

respectively, which was better than the use of reported well-known fungi (*Beauveria bassinia* and *Metarhizium anisopliae*). The bacterium could be a good candidate for further investigation as a possible biocontrol agent for cassava mealybug population.

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References

- Aggarwal, C., Paul, S., Tripathi, V., Paul, B., and Khan, M.A. (2015). Chitinolytic activity in *Serratia marcescens* (strain SEN) and potency against different larval instars of *Spodoptera litura* with effect of sublethal doses on insect development. *BioControl*, 60(5): 631-640.
- Al Sagheer, F.A., Al-Sughayer, M.A., Muslim, S., and Elsabee, M.Z. (2009). Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohydr. Polym.*, 77:410-419.
- Amutha, M. and Banu, J.G. (2011). Susceptibility of cotton mealy bugs, *Phenacoccus solenopsis* and *Paracoccus marginatus* at different developmental stages to entomopathogenic fungi. *Indian J. Plant Prot.*, 39(3):242-246.

- AOAC International. (2006). Official Methods of Analysis. 18th ed. AOAC International, Washington, DC, USA.
- Atlas, R.M. (2010) Handbook of Microbiology Media. CRC Press, Boca Raton, FL, USA, 2,040p.
- Beishier, L. (1991). Microbiology in Practice. 5th ed. Harper Collins, New York, NY, USA, 528p.
- Calatayud, P.A. and Le Rü, B. (2006). Cassava-Mealybug Interactions. IRD Editions, Paris, France, 110p.
- Ciesla, J., Kopycinska, M., Lukowska, M., Bieganski, A., and Janczarek, M. (2016). Surface properties of wild-type *Rhizobium leguminosarum* bv. *trifolii* strain 24. 2 and its derivatives with different extracellular polysaccharide content. PLoS ONE, 11(10):e0165080.
- Dorsch, M., Ashbolt, N.J., Cox, P.T., and Goodman, A.E. (1994). Rapid identification of *Aeromonas* species using 16S rDNA targeted oligonucleotide primers: a molecular approach based on screening of environmental isolates. J. Appl. Bacteriol., 77(6):722-726.
- Garrity G.M., Brenner D.J., Krieg N.R., and Staley J.T. (2005). Bergey's Manual of Systematic Bacteriology Vol. 2, The Proteobacteria (Part C). Springer-Verlag, New York, NY, USA, 1,256p.
- Greenfield, M., Gómez-Jiménez, M.I., Ortiz, V., Vega, F.E., Kramer, M., and Parsa, S. (2016). *Beauveria bassiana* and *Metarhizium anisopliae* endophytically colonize cassava roots following soil drench inoculation. Biol. Control, 95:40-48.
- Gupta, S.C., Leathers, T.D., El-Sayed, G.N., and Ignoffo, C.M. (1992). Production of degradative enzymes by *Metarhizium anisopliae* during growth on defined media and insect cuticle. Exp. Mycol., 15:310-315.
- Kaiser, K., Wemheuer, B., Korolkow, V., Wemheuer, F., Nacke, H., Schöning, I., and Daniel, R. (2016). Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests. Scientific Reports, 6:33696.
- Kumar, R. and Vats, R. (2010). Protease production by *Bacillus subtilis* immobilized on different matrices. New York Science Journal, 3(7):20-24.
- Neuenschwander, P. (2003). Biological control of cassava and mango mealybugs in Africa. In: Biological Control in IPM Systems in Africa. Neuenschwander, P., Borgemeister, C., and Langewald, J., (eds). CABI Publishing, Oxford, UK, p. 55-59.
- Nurderbyandaru, N., Mubarik, N.R., and Prawasti, T.S. (2010). Chitinolytic bacteria isolated from chili rhizosphere: Chitinase characterization and application as biocontrol for *Aphis gossypii*. Microbiol. Indones., 4(3):103-107.
- Otieno, N., Lally, R.D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K.J., and Dowling, D.N. (2015). Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. Front. Microbiol., 6:745-753.
- Papadopoulos, S. and Chrysosoides, C. (2012). *Phenacoccus madeirensis* Green, 1923 (Homoptera: Pseudococcidae) on *Ocimum basilicum*: a new geographical record for Greece. Bull. OEPP, 42(1):146-147.
- Parsa, S., Kondo, T., and Winotai, A. (2012). The cassava mealybug (*Phenacoccus manihoti*) in Asia: first records, potential distribution, and an identification key. PLoS ONE, 7(10):1-11.
- Salunkhe, R.B., Patil, C.D., Salunke, B.K., Rosas-García, N.M., and Patil, S.V. (2013). Effect of wax degrading bacteria on life cycle of the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae). BioControl, 58(4): 535-542.
- Sambrook, J. and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY, USA, 2,368p.
- Sattayawong, C., Uraichuen, S., and Suasa-ard, W. (2016). Larval preference and performance of the green lacewing, *Plesiochrysa ramburi* (Schneider) (Neuroptera: Chrysopidae) on three species of cassava mealybugs (Hemiptera: Pseudococcidae). Agr. Nat. Resour., 50(6):460-464.
- Sharma, A., Bruce, K.L., Chen, B., Gyoneva, S., Behrens, S.H., Bommaris, A.S., and Chernooff, Y.O. (2016). Contributions of the prion protein sequence, strain, and environment to the species barrier. J. Biol. Chem., 291(3):1,277-1,288.
- Sirisena, U.G.A., Watson, G.W., Hemachandra, K.S.O., and Wijayagunasekara, H.N.P. (2015). Scanning electron microscopy of six selected mealybug (Hemiptera: Pseudococcidae) species of Sri Lanka. Trop. Agric. Res., 26(2):237-247.
- Sirisha, E., Rajasekar, N., and Narasu, M.L. (2010). Isolation and optimization of lipase producing bacteria from oil contaminated soils. Adv. Biol. Res., 4(5):249-252.
- Soroker, V., Nelson, D.R., Bahar, O., Renah, S., Yablonski, S., and Palevsky, E. (2003). Whitefly wax as a cue for phoresy in the broad mite, *Polyphagotarsonemus latus* (Acari: Tarsonemidae). Chemoecology, 13:163-168.
- Soroush, P., Kondo, T., and Winotai, A. (2012). The cassava mealybug (*Phenacoccus manihoti*) in Asia: first records, potential, distribution, and an identification key. PLoS ONE, 7(10):e47675.
- Taluengphol, K., and Thaochan, N. (2015). Horizontal transmission of *Metarhizium anisopliae* PSUM04 infected mustard aphid, *Lipaphis erysimi* (Kalt.) (Hemiptera: Aphididae), in healthy population. Khon Kaen Agr., 43(1):764-768.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., 28:2,731-2,739.
- Vandamme, P., Bernardet, J.F., Segers, P., Kersters, K., and Holmes, B. (1994). New perspectives in the classification of the flavobacteria: Description of *Chryseobacterium* gen. nov., *Bergeyella* gen. nov., and *Empedobacter* nom. rev. Int. J. Syst. Evol. Microbiol., 44(4): 827-831.
- Veliz, E.A., Martínez-Hidalgo, P., and Hirsch, A.M. (2017). Chitinase-producing bacteria and their role in biocontrol. AIMS Microbiol., 3(3):689-705.

- Wang, Y.N., Tsang, Y.F., Wang, L., Fu, X., Hu, J., Li, H., and Le, Y. (2018). Inhibitory effect of self-generated extracellular dissolved organic carbon on carbon dioxide fixation in sulfur-oxidizing bacteria during a chemoautotrophic cultivation process and its elimination. *Bioresource Technol.*, 252:44-51.
- Williams, D.J. (2004). *Mealybugs of Southern Asia*. Southdene Sdn. Bhd., Kuala Lumpur, Malaysia, 896 p.
- Xie, Y., Tian, F., Liu, W., Zhang, Y., Xue, J., Zhao, Y., and Wu, J. (2014). The wax glands and wax secretion of *Matsucoccus matsumurae* at different development stages. *Arthropod Struct. Dev.*, 43:193-204.
- Yang, Q., Liu, S., Sun, J., Yu, L., Zhang, C., Bi, J., and Yang, Z. (2014). Nutritional composition and protein quality of the edible beetle *Holotrichia parallela*. *J. Insect Sci.*, 14(1):1-4.
- Yongsawatdigul, J., Rodtong, S., and Raksakulthai, N. (2007). Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter cultures. *J. Food Sci.*, 72(9):382-390.
- Zhang, Y., Xie, Y., Xue, J., Fu, X., and Liu, W. (2012). The structure of integument and wax glands of *Phenacoccus fraxinus* (Hemiptera: Coccoidea: Pseudococcidae). *Zoological Research*, 33(12):13-17.