CHAPTER TWO

MATERIALS AND METHODS

2.1 Soil sample collection

Agricultural soil (IMO4) sample was kindly provided from Serdang Agricultural Center, Selangor, Malaysia and kept at room temperature at Molecular Bacteriology and Toxicology Laboratory (MBT Lab), Institute of Science Biology (ISB), Faculty of Science, University of Malaya, Kuala Lumpur.

2.2 Bacterial cultures collection

All the bacteriological preparations based on to the guidelines of *Laboratory Manual in Molecular Bacteriology* (Salmah, et al 2008). All bacterial slant stocks used in this study were kept at 4^{o}C in the Molecular Bacteriology and Toxicology Laboratory (MBT Lab), Institute of Science Biology (ISB), Faculty of Science, University of Malaya, Kuala Lumpur. Plasmid-free *E. coli* ATCC 25922, with known sensitivity to chloramphenicol (MIC of 4 ug/ml) (Washington II and Shutter, 1980), and *S. aureus* ATCC 25923 were used as controls in all antibiotic sensitivity tests. A novel species *Paenibacillus haemolyticus* strain 139SI ATCC BAA-2268 (JF825470.1) (Appendix 1A & 1B) isolated from a Malaysian agricultural soil was used as positive control in haemolytic and emulsification activity, and as negative control in mice lethality test.

2.3 Experimental animals

Specific pathogen free ICR strain mice ages, 6 to 8 weeks (22 to 27grams) were obtained from the Animal House, Faculty of Medicine, University of Malaya. The animals were housed in individual cages and throughout the period of study the mice were maintains at a high sanitation with their health and well-being assessed daily; commercial feed and water were provided *ad libitium*.

2.4 Chemicals, reagents, solvents and antibiotics

All chemicals used were of Analar grade, or of the highest grade available commercially, obtained from BDH Chemical Ltd., England; Bethesda Research Laboratories (BRL), U.S.A.; Sigma Chemical Co., U.S.A.; BD. Co., Promega Co. and Oxoid Ltd. The chemical and reagents used were agarose powder, supercoiled DNA ladder (2-10 kb), bromophenol blue, ether, chloroform, absolute ethanol, isopropanol, phenol-CHISAM, ethidium bromide, glycerol, teepol solution, EDTA disodium salt, potassium acetate, sodium hydroxide, sodium chloride, glacial acetic acid, boric acid, sodium acetate, sodium deodecylsulphate (SDS), sucrose-EDTA, bacteriological agar, commercial antibiotic discs, Gram Stain Kits & Reagents (crystal violet, iodin, decolorizer/acetone, safranin), Aminopeptidase-strip test and Minipreps plasmid DNA kit. The media used for bacterial growth were Brain Heart Infusion (BHI) agar and broth powder. The media used for sensitivity susceptibility testing were Mueller-Hinton broth and agar.

Membrane filters (0.22 um and 0.45 um) were supplied by Millipore Corp., U.S.A. Tuberculin syringes (1ml) with needles with the size of (27G x 1/2 inches) were supplied by Terumo Corporation, Japan and parafilm was from American Can, U.S.A.

2.5 Media used

2.5.1 Brain Heart Infusion (BHI) broth

BHI broth powder	37.0 g
Distilled water to	1000.0 ml

This medium was used to culture soil bacterial.

2.5.2 Brain Heart Infusion (BHI) agar

BHI agar powder	52.0 g
Distilled water	1000.0 ml

The BHI agar was prepared, autoclaved then autoclaved at 121°C, 15 p.s.i for 20 minutes before and cooled at 45-50°C. 7% of the sterile sheep blood was then added aseptically to the BHI agar and was mixed thoroughly before plating.

2.5.3 Mueller-Hinton (MH) agar medium

MH broth powder	25.0 g
Distilled water to	1000.0 ml

This medium was used to culture bacterial strains for antibiotic sensitivity tests. 15 g of bacteriological agar was added to MH powder, autoclaved and was cooled to 40-45°C before preparing plate.

2.6 Solutions for Gram negative plasmid DNA extractions

2.6.1 Sucrose-EDTA-Tris (SET) buffer, pH 8.0

Sucrose 25% (w/v)	20.0 g
Disodium EDTA (50 mM)	1.86 g
Tris base (50 mM)	0.61 g

Distilled water to	100.0 ml

The pH was adjusted to 8.0 and autoclaved before stored at 4°C.

2.6.2 Alkaline SDS (lysing) solution (1%)

Solution 1 - 0.4 N NaOH

Sodium hydroxide pellets	1.6 g
Distilled water to	100.0 ml

Soution 2 - 2% SDS

SDS powder	2.0 g
Distilled water to	100.0 ml

Working solution 1X strength and pH was adjusted to pH 12.7. Both the solutions were mixed in equal amounts just prior to use.

2.6.3 Sodium acetate, 3 M, pH 4.8

Sodium acetate	24.6 g
Acetic acid glacial	few drops
Distilled water to	100.0 ml

The pH was adjusted to pH 4.8 with glacial acetic acid prior to final volume of 100.0 ml. The solution was stored at 4°C after autoclave.

2.6.4 Potassium acetate, 5 M (Sambrook *et al.*, 1989)

Potassium acetate	29.44 g
Acetic acid glacial	11.5 ml
Distilled water to	100.0 ml

The pH of autoclaved solution was adjusted to 4.8 and was stored at 0°C prior to use.

2.7 DNA electrophoresis

2.7.1 Agarose gel (0.7%)

Agarose powder	0.7g
1X of Tris-Borate-EDTA top up to	100ml

Agarose powder was dissolved in 1X TBE buffer by boiling in a microwave oven and cooled to 50^{o}C before ethidium bromide solution (0.5 ug/ml) was added. The molten gel was poured into a sealed gel casting tray with a comb placed at one end of the tray to form wells. The gel was then allowed to solidify. This agarose gel was used in the DNA electrophoresis to detect the plasmid presence in soil bacterial samples.

2.7.2 Tris-borate-EDTA (TBE) buffer (Peacock & Dingman, 1967)

To prepare 10X strength:

Tris-base	108.0 g
Boric acid	55.0 g
Disodium EDTA	9.3 g
Distilled water to	1000.0 ml

This buffer was used for agarose gel electrophoresis. A 10X strength stock solution was diluted to IX strength as a working solution. The pH of the 1X strength solution was adjusted to pH 8.3. Long storage of the 10X buffer was avoided and buffer was kept refrigerated when not in use.

Working solution, 1X strength, pH 8.3

10X Tris-borate-EDTA (TBE) buffer	100ml
Sterile distilled water	900ml

The solution was mixed well and kept at 4°C

2.7.3 Ethidium bromide (10 mg/ml)

Ethidium bromide powder	0.2 g
Sterile distilled water	20.0 ml

Ethidium bromide intercalates between nucleic acids groups and enables them to fluoresce under UV light. It is used for the detection or visualization of nucleic acids in agarose gel. This solution was stored at 4°C in dark (protected from light). Gloves were worn at all times when handling solutions and agarose slab gels containing ethidium bromide, as it is a powerful mutagen and carcinogen.

2.7.4 Gel loading buffer (tracking dye)

Glycerol	50.0 ml
SDS	0.1 g
Bromophenol blue	0.05 g
Xylene Cyanole	0.05 g
Sterile distilled water to	100.0 ml

This solution was added to the DNA samples prior to electrophoresis to weigh down and track the movement of the DNA.

2.7.5 Tris-EDTA (TE) buffer, pH 8.0

Tris-HCl, pH 8.0	10 mM
EDTA, pH 8.0	1 mM

This buffer was prepared as shown as above and was used for dissolving precipitated DNA.

B. METHODS

2.8 Washing procedures and sterilization

All items and glassware were soaked overnight in a diluted teapol solution. Then, they were scrubbed with suitable brushes and were rinsed thoroughly with tap water followed by distilled water before they were air or oven dried (50°C)

All plasticware, including microfuge tubes, blue, white and yellow tips, eppendorf comfortips, beakers and polypropylene/polycarbonate centrifuge tubes were steam-sterilised by autoclaving at 121°C (15 p.s.i) for 30 minutes. Glassware including beakers, measuring cylinders, conical flasks, pipettes, universal and bijou bottles were sterilised in a dry heat oven at 180°C for 2 hours. Media and solutions were steamed sterilised at 121°C (15 p.s.i) for 20 minutes in an autoclave. Small volumes were autoclaved using a pressure cooker.

2.9 Storage and maintenance of bacterial cultures

For long term storage, overnight broth cultures of the soil bacteria were mixed with equal volume of sterile 50% glycerol and stored at -70°C in sterile tubes. The frozen stock cultures were plated on BHI agar medium supplemented with 5-7% sheep blood and incubated at 37°C. Working stock cultures were sub-cultured every two weeks. All the colonies were maintained as a glycerol suspension (25 %, w/v) at – 80^{o}C and BHI-slant agar at room temperature.

2.10 Bacterial colony screening and isolation

Approximately, 1 gram of soil sample was mixed and thoroughly suspended with 5 ml of sterile distilled water in bijou bottle before the suspension was streaked on Brain-Heart-Infusion (BHI) agar plates supplemented with 5-7% sheep blood. The plates were incubated for 16-24 hours at 37^{o}C.

Each hemolytic colony picked was sub-cultured onto BHI-blood agar (Appendix 2A). The BHI-blood plates with single colonies obtained and was picked at random and re-streaked twice to ensure its purity. Once a bacterial colony was purified, it was transferred to a fresh bijou bottle BHI agar slant, labeled prior to Gram identification, colony morphology observation, antibiotic sensitivity testing, biosurfactant with emulsifying activity, mice lethality test and plasmid screening.

2.11 Colony morphology

At this point, one hundred and thirty nine (139) soil bacterial colonies were isolated and collected from the BHI blood plates. Initial colony observations were carried out and recorded of the shape, color and size.

2.11.1 Morphological Analysis

Morphological characterization of microbes was commonly performed to distinguish the microbes based on colony and cellular morphologies. A stereo microscope (Olympus, Japan) was used to examine and characterize the formation of bacterial colonies on solid media, using magnification of 40 X to 50 X.

2.12 Bacterial Gram identification and differentiation

The cellular morphology of the bacterial isolate was determined by standard procedures of Gram staining (Burke's method), in-house lysis method and commercial test strips.

2.12.1 Gram staining

A total of one hundred and thirty eight (138) soil bacterial isolates were subjected to standard Gram staining (Appendix 2B). The microorganisms were mounted on microscope slides that fixed stained and viewed under microscope. Gram-positive bacteria were stained purple whereas Gram-negative bacteria appeared pink.

2.12.2 Aminopeptidase-strip test

Aminopeptidase-strip test kit was purchased from Merck KgaA, Darmstadt, Germany detects aminopeptidase presence in the cell walls. This GRAM-TEST® commercial kit contains L-alanine substrate that is used to detect the presence of L-alanine aminopeptidase enzyme that localized in the bacterial cell wall of Gram-negative organisms.

A single bacterial colony was removed with an inoculation loop and suspended in 0.2 ml of sterile distilled water in a tube prior to incubation for 30 minutes at 37°C. If the bacterial suspension turns to yellow color, L-alanine aminopeptidase is present to indicate that the bacteria are Gram-negative. If there is no yellow colorization, L-alanine aminopeptidase is absent and the bacteria are considered Gram-positive.

2.12.3 Alkaline lysis solution

Alkaline Lysis solution was used to observe lysis ability.Alkaline lysis solution (0.5 ml) comprising of 1% SDS and 0.2 N NaOH) were mixed with an equal volume of an overnight bacterial culture grown and incubated at 4^{o}C. A clear, viscous lysate was observed within 2 minutes, for Gram-negative and no changes were observed for Gram-positive. The standard Gram positive bacteria used were *Staphylococcus aureus* ATCC 25923 and Gram-negative *E. coli* ATCC 25922.

2.13 Haemolytic activity of isolated soil bacteria

One hundred and fifty seven (157) isolated soil bacterial colonies were tested for their ability to produce haemolysis on BHI agar plate containing 5-7% (v/v) sheep blood after incubated at 37°C for 24 hours. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carrillo et al., 1996). A clear zone around a single colony was considered positive haemolysis. Result was recorded based on the type of clear zone observed i.e α-hemolysis when the colony was surrounded by greenish zone, β-hemolysis when the colony was surrounded by a clear white zone and Ɣ-hemolysis when there was no change in the medium surrounding the colony.

2.14 Antibiotic susceptibility testing of isolated soil bacteria

Antibiotic susceptibility test were conducted essentially by the disc diffusion method described by (Bauer et.al., 1966). Mueller Hinton plates were dried at 37°C in an incubator for 30 minutes prior to use. All the 138 isolated bacterial colonies were tested for their susceptibilities towards 11 different antibiotics. The antibiotic discs used were ampicillin (10 ug), chloramphenicol (30 ug), erythromycin (15 ug), gentamicin (10 ug), kanamycin (30 ug) , spectinomycin (10 ug), streptomycin (10 ug), tetracycline

(30ug) , triple sulphonamides (30 ug), trimethoprim + trimethoxazole (25 ug) and vancomycin (30 ug).

2.14.1 Standardization of soil bacterial broth culture

The inocula of soil bacteria for seeding the antibiotic susceptibility plates were prepared from overnight fresh grown pure cultures in BHI broth. The culture was then diluted with sterile saline (0.85% NaCl) until it visually matched the turbidity of the McFarland 0.5 turbidity standard. This was achieved by holding the culture suspension and McFarland standard in front of a light against a white background with contrasting black lines and comparing the turbidity. If the turbidity is too heavy, the suspension should be diluted. If the turbidity is too light additional cells should be added to the suspension.

2.14.2 Inoculation of the susceptibility test media

After proper turbidity was achieved, the test culture was streaked evenly over the prepared Mueller Hinton agar plates using a sterile cotton wool swab and allowed to dry for 5 to 10 minutes before the discs were placed on the plates. The discs were placed on the agar with sterile fine pointed forceps and tapped gently to ensure the adherence to the agar. The plates containing the disks were placed inverted and incubated at 37°C for 16 to 18 hours.

2.14.3 Interpretation of zone inhibition

After overnight incubation, the diameter of each zone of inhibition was measured to the nearest millimeter. In all measurements, the zones of inhibition were measured from the edges of the last visible colony-forming growth. The ruler should be

positioned across the center of the disc to make these measurements. The results were recorded in millimeters (mm) and interpretation of susceptibility was obtained by comparing the results to the standard zone sizes.

The diameter of the inhibition zones for individual antibiotics was translated into susceptible or resistant categories by referring to the interpretative chart (NCCLS, 1985) provided by the manufactures. *E. coli* strains K12 (ATCC 25922) and *S. aureus* (ATCC 25923) were used as a control as it is sensitive to all antibiotics.

2.15 Preparation of soil bacterial filtrates

A single colony from 138 soil bacteria was inoculated into 3 ml BHI broth and incubated for 48 hours incubation at 37°C. The broth culture was chilled on ice for 20 minutes before centrifugation at 13,000 rpm at room temperature for 15 minutes. The supernatant was filtered using syringe Minisart filter (pore size 0.20 µm) and collected into a fresh 1.5 ml centrifuge tube before stored at −70°C until used. 100 µl of filtrate was streaked onto the entire surface of blood agar plate and incubated at 37°C overnight. The plate was observed for any bacterial growth. The filtrate (supernatant) should be free from bacterial cells and were used in biosurfactant activity and mice study

2.16 Biosurfactant activity assays – Drop collapsing test with milky activity

The drop-collapse technique was performed on clean glass slide following the method described by Bodour and Miller-Maier (1998). 1 drop of oil immersion was added to the glass slide and 100 µl of each 138 bacterial filtrate was added to the surface of oil using 10 µl micropippetor by holding the pipet at an angle of 45º. The shape of the drop on the oil surface was inspected after 1 minute. If the drop collapsed,

(biosurfactant-producing cultures giving flat drops) the result was scored as positive '+'. At the same time, emulsification activity was observed as milky color indicating the oil and filtrate were mixed together. Those bacterial filtrates cultures that gave rounded drops or remained beaded, the result was scored as were scored as negative '-', indicative of the lack of biosurfactant production (Youssef et al., 2004) and their emulsification activity was observed as non-milky color indicating the oil and filtrate were not mixed together.

2.17 Animal study

The 138 soil bacterial cultures and filtrates were used to test the lethality and toxicity in mice.

2.17.1 Mice lethality test

Soil bacteria were grown overnight in BHI broth at 37°C and centrifuged for 5 minutes. The cells pellet was resuspend in normal saline (0.85% NaCl) and adjusted tubidometrically to contain 10^9 colony forming units (CFU) per ml. The mice were injected intraperitoneally (i.p) with 10^9 colony forming units (CFU) of the soil bacterial cultures and observed their lethality twice daily within 72 hours.

2.17.2 Mice toxicity test

Each of the138 soil bacterial filtrate prepared in Section 2.15 was tested with 0.5 and 1 ml in mice. Positive and negative controls were always used in mouse pathogenicity study. The control mice used were inoculated with 0.5 and 1 ml of normal saline (0.85% NaCl) and *P. haemolyticus* BAA 2268. The mice toxicity was observe in within 72 hours.

2.18 Detection of plasmid DNA from soil bacteria

2.18.1 DNA extraction using kit

Wizard® Plus SV Minipreps Kit from promega, USA was used and steps was followed as per manufacturer‘s instructions. A single isolated colony from a fresh bacterial culture plate was selected and inoculated into 10ml of BHI/ampicillin broth and incubated in a shaking incubator at 37^{o}C overnight. The culture was harvested by centrifugation at 14000 rpm for 5 minutes in a tabletop centrifuge. The supernatant was poured off and excess media was removed by blotting on a paper towel. The pellet was thoroughly resuspended in 250 ul of Cell Resuspension Solution by vortex in a 1.5 ml microfuge tube. 250 ul of Cell Lysis Solution was added and mixed by inverting the tube several times. The cell suspension was incubated at room temperature until the suspension cleared not longer than 5 minutes followed by addition of 10 ul of Alkaline Protease Solution. The solution was mixed gently by inverting the tube several times and incubated at room temperature for 5 minutes. The lysate was neutralized by adding 350 ul of Wizard® Plus SV Neutralization Solution and mixed immediately by several gentle inversions followed by centrifugation at 14000 rpm for 10 minutes at room temperature. The clear lysate was carefully transferred to a Spin Column inserted in a 2 ml Collection Tube. The solution was centrifuged 14000 rpm for 1 minute at room temperature. The flow-through was discarded from the Collection Tube and 750 ul of Column Wash Solution was added to the Spin Column and centrifuged at 14000 rpm for 1 minute, the flow-through was discarded and was repeated with 250 ul of Column Wash Solution and centrifuged 14000 rpm for 2 minutes. The Spin Column was transferred to a new sterile 1.5 ml microfuge tube. The plasmid DNA was eluted by adding 50 ul of Nuclease-Free Water to the Spin Column and centrifuged at 14000 rpm for 1 minute. The Spin Column was removed and the plasmid DNA was stored at -20^{o}C.

2.18.2 Small scale plasmid extraction

2.18.2.1 Alkaline lysis for Gram negative bacteria

Bacterial cell was grown overnight at 37ºC in 3 ml of BHI broth. The culture was transferred into a 1.5 ml microcentrifuge tube and spun down in a microcentrifuge at 13000 r.p.m for 3 minutes to pellet the cells. The supernatant was decanted and resuspend the cell pellet obtained in 200 µl of SET buffer. 400 µl of lysing solution was added to the cell suspension and the tube was inverted gently several times before kept at 4ºC for 5 minutes. When the lysate become clear and viscous, 300 µl of 5 M potassium acetate was added and mixed gently. The mixture was incubated further at 4ºC for 15 minutes and recentrifuged at 13000 r.p.m for 15 minutes at 4ºC. The supernatant obtained was transferred to a fresh tube and 0.6 volumes of isopropanol was added before emulsified by inverting a few times. The solution was cold at 0ºC for 20 minutes before centrifugation at room temperature for 5 minutes. The resulting plasmid DNA pellet was washed with 1 ml of 70% ethanol and air dried for 20 to 30 minutes before redissolved in 30 µl of sterile distilled water.

2.18.2.2 Plasmid isolation for Gram positive bacteria

The method of Klaenhammer (1984) with modification was adapted to extract plasmid DNA for gram positive soil bacteria. 1 ml of culture was centrifuged and the pellet resuspend in 200 ul of cold 25% sucrose, 0.05 M Tris, pH 7.5, 5 mM EDTA before kept on ice for 10 min. 5 ul of lysozyme (20mg/ml in 0.05 M Tris, pH 7.5, 5 mM EDTA) was added and incubated at 37°C for 1 hour. Cells were centrifuged and 500 ul of lysis solution (3% SDS in 0.05 M Tris, 5 mM EDTA, 0.05 M glucose) was added immediately before 10 ul of 10 N NaOH was added. The sample was heated at 65°C for

1 hr before allowed to cool slowly (approx.15 min) at room temperature. 50 µl of 2 M Tris, pH 7.0 was add and mixed gently before added 70 µl of 5 M NaCl. 500 µl of phenol which is saturated with 3% NaCl (mix gently until emulsification) was added and left at room temperature for 5 min. 300 µl of chloroform was added and mixed gently before centrifuged for 5 min. 600 ul of chloroform: isoamylalcohol (24:1) was added and left at room temperature for 5 min, centrifuged and the phase aqueous was harvested for ethanol precipitation.

2.19 Agarose gel electrophoresis

The presence of the soil bacterial plasmid was checked and visualized by agarose gel electrophoresis. The electrophoresis was run on 0.7% agarose slab gel which was submerged horizontally in the electrophoresis tank. The required amount of agarose powder to produce 0.7% agarose slab gel was dissolved in 1X TBE buffer by boiling. The dissolved molten agarose solution was cooled at 50^{o}C before addition of 3 ul ethidium bromide (0.5 ug/ml). The solution was mixed properly and was poured in a gel casting tray containing a comb inserted to form wells. The gel was then allowed to solidify at room temperature for about 45 minutes before slowly removing the inserted comb to prevent any damage to the wells. Then the gel casting tray was placed horizontally in the electrophoresis tank filled with 1X TBE buffer. 20 µl of the extracted bacterial DNA samples was well-mixed on a square of parafilm M (American Can Co., USA) with 5 µl of gel loading buffer. The sample mixture was then carefully loaded into the wells of the gel by using a 10 µl micropipette.

Electrophoresis was carried out at room temperature (20-25°C) from cathode (-) to anode (+) at a constant voltage ranging from 70-80 V for about 60-90 minutes. The power for electrophoresis was supplied by a power pack (Model EC 105 E-C Apparatus

Co. Florida). Once the loading dye reached about 1 cm from the end of the gel, the electrophoresis was stopped. The DNA-ethidium bromide complex in the gel was visualized using UV transilluminator (Model TFX, Vilber Lourmat, France). The gel photo was taken using the digital camera. Gloves were worn at all times when handling solutions and agarose slab gels containing ethidium bromide, as it is a powerful mutagen and carcinogen.