CHAPTER 3

Materials and Methods

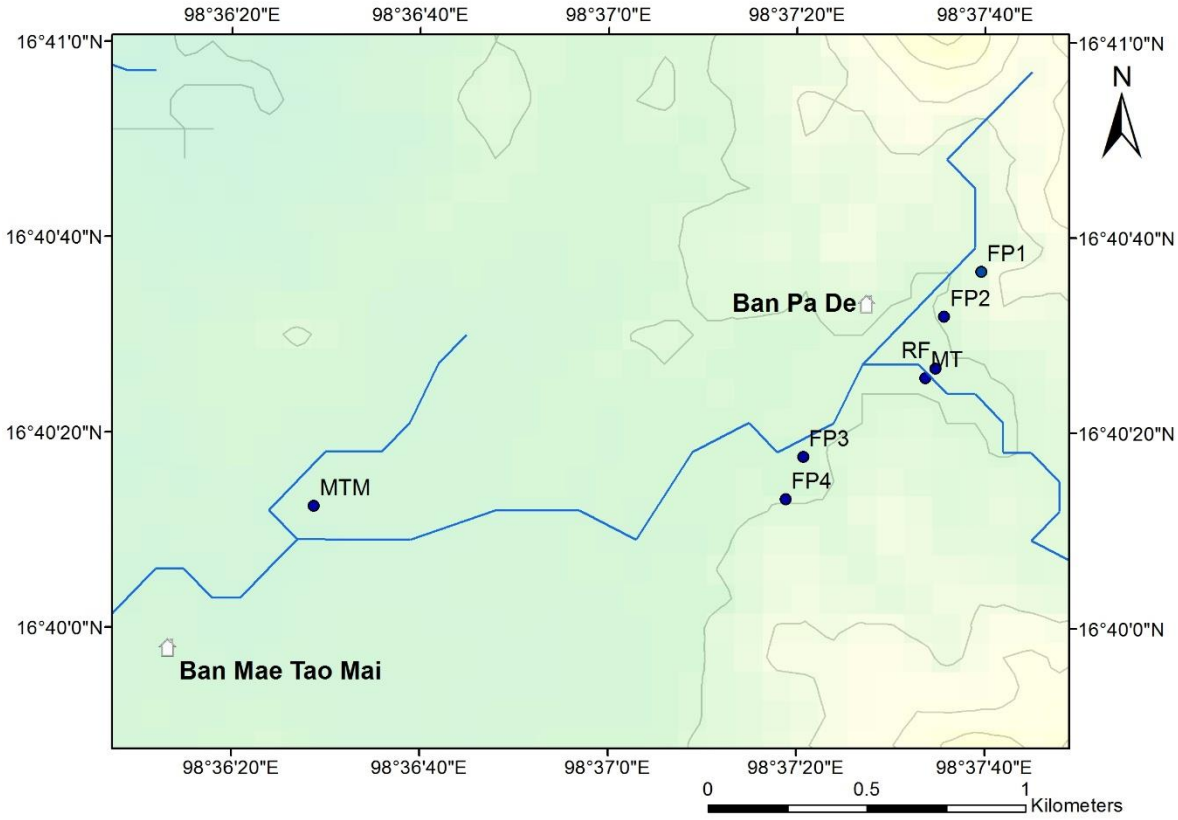
3.1 Research concept

This study were isolated and identified Cd resistant bacteria from contaminated soil. After selected the high resistant strain, removal and immobilized ability between second highest ranks were compared. Then mosquito larva were used for Cd biological toxicity test.

3.2 Study area

3.2.1 Site description

Cd resistant bacteria was isolated from the soil sample collected at Mae Sot district. Sampling sites was selected according by high Cd contamination area report by Simmon *et.al* (2004). Agricultural soil and riparian soil from 4 sites of fish pond, 1 site of rice field and 2 sites of riparian zone in Mae Sot district, Tak Province, Thailand were collected in September 2013. (Figure 6). The locations of study sites, in co-ordinates, were determined. The positions identified on site using GPS navigation (Table 5).



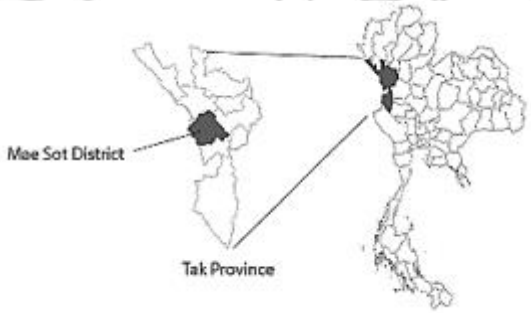


Figure 6 Sampling sites fish pond No.1 (FP1), No.2 (FP2), No.3 (FP3), No.4 (FP4), rice field irrigation canal (RF) ,Mae Tao creek (MT) riparian zone at Ban Pa De village and Mae Tao creek riparian zone (MTM) at Ban Mae Tao Mai village Mae Sot District, Tak Province.

Table 5 Latitude and longitude of collection location on the Mae Sot District

Site	Latitude and longitude
FP1	16º 40.547´ N, 98º 39.613´ E
FP2	16º 40.535´ N, 98º 37.595´ E
FP3	16º 40.292´ N, 98º 37.364´ E
FP4	16º 40.220´ N, 98º 37.315´ E
RF	16º 40.443´ N, 98º 37.580´ E
MT	16º 40.427´ N, 98º 37.562´ E
MTM	16º 40.207´ N, 98º 36.480´ E

I. **Sampling site 1** fish pond number 1 (Figure 6 and 7) was located on Ban Pa De village. This site was pond that cultivated cat fish (*Clarias macrocephalus*) and has the lotus (*Nymphaea lotus*) and some submerged plant under the water. Tap water from mountain irrigation upper to the Cd contaminated area was used for this site.

Figure 7 Sampling site 1 and sample site 2 (at the upper part of the picture)

II. **Sampling site 2** fish pond Number 2 (Figure 6 and 8) was located nearby site 1. Natural canal fish species were cultivated in this pond. But, the aquatic plant was not found and higher light intensity. Because of site 2 was located near paddy field and was not found

any big tree around this site. Moreover, duck were found in this site. Tap water from mountain irrigation upper to the Cd contaminated area was used for this site.

Figure 8 Sampling site 2 Fish pond number 2

III. **Sampling site 3** fish pond Number 3 (Figure 6 and 9) this site was the Nile tilapia (*Tilapia nilotica*) fish farm nearby paddy field. The aquatic plant was not found and higher light intensity. The riparian garden vegetation was found mainly banana and papaya. Tap water from mountain irrigation upper to the Cd contaminated area was used for this site.

Figure 9 Sampling site 3 Fish pond number 3

IV. **Sampling site 4** fish pond number four (figure 6 and 10) this site was the Nile tilapia (*Tilapia nilotica*) fish farm in the garden. The aquatic plant was not found and lower light intensity. The riparian garden vegetation was found many species. Tap water from mountain irrigation upper to the Cd contaminated area was used for this site.

Figure 10 Sampling site 4 Fish pond number 4

V. **Sampling site 5** Rice field irrigation canal (Figure 6 and 11) this site was the paddy field near Mae Taw creek. Rice was cultivated in the wet season and Corn was cultivated in dry season. Water in Mae Taw creek was used in this field.

Figure 11 Sampling site 5 Rice field irrigation canal

VI. **Sampling site** 6 Mae Tao creek riparian soil at Ban Pa De village (figure 6 and 12) this site was the riparian soil form the water that through the zinc mining zone. Bamboo (*Thyrsostachys* sp.) was the mail vegetation of this site.

Figure 12 Sample site 6 Mae Tao creek riparian soil at Ban Pa De village

VII. **Sampling site 7** Mae Tao creek riparian zone at Ban Mae Tao Mai village (figure 6 and 13) this site was the riparian soil form the water that through the zinc mining zone. The stream was larger than channel at Ban Pa De village.

Figure 13 Sampling site 7 Mae Tao creek riparian zone at Mae Tao Mai village

3.3 Soil sampling

Soil form the different types of land used was sampling. According with the recorded of high Cadmium concentration area. (AOAC, 1990) Follow by this step;

1. Agriculture area soil and riparian soil in 7 sampling sites (4 sites of fish pond soil, 1 site of rice field soil and 2 sites of riparian zone soil) was collected in 20-30 g at 15 cm depth by clean weeding hoes.
2. Soil samples were transferred into sterile plastic bag.
3. Soil samples were kept in ice box at 4 ºC and transported to laboratory.

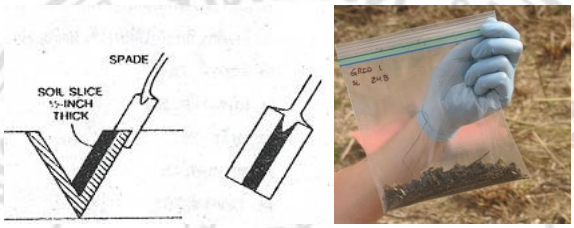


Figure 14 Soil sampling method and plastic bag with soil sample

3.4 Heavy metal content measurement

Atomic absorption spectrophotometry was used to quantify heavy metals in soil sample and water sample in the study site. After the removal experiment by bacteria, the water sample were analyzed.

3.4.1 Soli sample preparation

1. Soil samples were dried at a hot air oven at 70 ºC for 3 days.
2. After ground and filtered through a 0.025 mm. mesh sieve, soil samples (0.5 g.) were added by 5 ml of Nitric-Perchloric acid solution ($HClO_4$ and HNO_3 2:1) with high temperature incubation (AOAC, 1990).
3. After digestion, soil solution were filtrated by Whatman® filter paper No.1 and kept the solution in 4 ºC.

3.4.2 Water sample preparation

1. Water samples were collected in PP plastic container
2. Kept into 4 ºC ice box

3.4.3 Analyzed concentrations of Cd

Cd concentration was analyzed by atomic absorption spectrophotometer (PerkinElmer's PinAAcleTM 900 model).

3.5 Microbiology method

3.5.1 Screening of Cd resistant bacteria

1. Ten grams of soil samples were suspended in to 90 mg of sterile normal saline (0.85% NaCl).
2. Soil samples were serially ten-fold serial diluted. The suspension was spread at 10^{-4}, and 10^{-6} 0.1 ml on nutrient agar medium (NA) supplemented with $CdCl_2 \cdot 2.5\ H_2O$ 0.5 mM.
3. All plates were incubated at 25 ºC, 48 hours.
4. The colonies were counted and calculated to CFU per grams of soil sample (FAO-APHCA, 2002).

3.5.2 Isolation and selection of Cd resistant bacteria, and growth studies

1. Each single colony was streaked in the nutrient agar medium (NA) supplemented with $CdCl_2 \bullet 2.5\ H_2O$ 0.5 mM and separated based on the different morphology of colony.
2. Ensure the pure culture of isolation, the single colony of each isolate was re-streaked 3 times and keep in 4 ºC.
3. Selection of high efficiency bacteria by using Kirby-Bauer disc diffusion method (Bauer *et. al*, 1959). Bacterial isolate that has the smallest number of clear zone in the highest concentration was assumed to be the “high resistant bacteria”
4. Pure culture of high Cd resistant bacteria was cultured and amplified in nutrient broth medium (NB) with aeration by shaking in incubator for 48 hours.
5. Growth study was performed by measurement the turbidity of suspension after pure culture strain was inoculated by 500 µL of the same cell concentration (follow by OD 625 nm of McFarland Standard No. 0.5 by McFarland and Jama, 1907) in nutrient agar medium (NA) supplemented with different concentration of $CdCl_2 \bullet 2.5\ H_2O$ and incubated at 25 ºC in the dark until bacterial growth reach to stationary phase.

6. Bacterial growth of each pure culture was monitored in liquid medium in the presence of different concentration by measuring OD600 using spectrophotometer with 2 hours interval. Growth curve was plotted between cell density and Cultivation.
7. Minimal Inhibitory Concentration (MIC) was calculated from the minimal concentration of Cd that inhibits growth of each bacterial species.

3.5.3 Characterization of the bacterial isolates recovered

1. Bacteria colony morphology were observed the colony surface, margin and color after incubated at 25 ºC for 48 hours.
2. Bacterial cell morphology was determined with an optical microscope after incubated at 25 ºC for 48 hours.
3. Each isolate was tested Gram straining. Method was using the Hucker method as described by Doestch (1981). Pure colonies were fixed to a glass slide. Slides were then covered by crystal violet for one minute and gently rinsed with water. Gram iodine was then applied for one minute. Slides were then washed with Decolorize solution. Slides were then covered by safranin O for one minute and excess safranin O rinsed with water. Cell morphology was observed under compound light microscope.

3.6 Molecular biology

3.6.1 Microbial DNA extraction

The bacterial isolates was cultivate in the nutrient broth at 25 ºC for 48 hours for fresh cell. Then the cell was break down and Extraction the DNA followed by Siripornmongcolchai Method in 2002. 1 mL of each bacterial sample solution in nutrient broth was centrifuged and the cell pellet was washed and suspended in 560 µL of TE Buffer. Cell wall and protein was digested by 30 µL of 10% sodium dodecyl sulfate and 5 µL of 20 mg/mL Protenase K respectively when incubated for 1 hr. at 37 ºC. 100 µL of 5 mol/L sodium chloride were added into DNA mixed solution and incubated for 10 min. at 65 ºC. After incubation, 600 µL of Phenol: Chloroform: Isoamyl alchhol (25:24:1) were added and centrifuged. The upper aqueous phase containing the DNA was transferred to a new tube and 600 µL of Chloroform: Isoamyl alcohol (24:1) were added. After which

a centrifugation step was carried out at maximum speed. The supernatant was removed into new tube. The DNA was dissolved in 800 µL of 100% cold ethanol. The samples were then kept at -20 ºC overnight allowing the DNA to precipitate. After 24 hrs. supernatant was discarded. The remaining ethanol evaporated. 30 µL of TE buffer was added to dissolve the DNA that was then kept at -20 ºC. The DNA product was separated on 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining. The Marker λDNA/Eco130I (StyI) DNA ladders were used as DNA molecular weight standards.

3.6.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) were used for multiply specific DNA molecules in the test tube. The enzyme DNA polymerase were used to copies DNA molecules. Universal primers were bind to homologous regions of the DNA then PCR cycle consists of three steps:

1. Strand separation (denaturing) – the DNA double strand is separated by heating the solution.
2. Hybridization of primers (annealing) – the solution is cooled to allow each primer to hybridize to a DNA strand
3. DNA systhesis (extension) – the solution is then heated to 72 ºC, the optimal temperature for the Taq DNA polymerase. The polymerase elongates both primers in the direction of the target sequence because DNA synthesis is in the 5‟ to 3‟ direction.

Rainey *et al*. (1996) was described 16sRNA PCR. Amplification reactions were performed in a total volume of 50 µL containing: 5 µl of 1x PCR buffer with $MgCl_2$, 1 µL of 0.2 mM deoxynucleoside triphosphate (dNTP), 0.5 µl of 1U Taq polymerase, 0.5 µM primer 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') and 2 µl of DNA extraction. Samples were subjected to 30 cycles of amplification, as follows (94 ºC 5 min.)(94 ºC 1 min., 55 ºC 1 min.) X 30 (72 ºC 5 min.) The amplification product was separated on 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The 100bp DNA ladders were used as DNA molecular weight standards.

3.6.3 Sequencing and identification

Sequencing of the purified PCR products was carried out by First Base Inc. – Singapore. Homology of 16S rDNA sequence from the selected bacteria were analyzed by BLAST program from *GenBank Database*.

3.7 Immobilized cell and free cell experiment

Immobilized cell and free cell were incubated in NB with $CdCl_2$•2.5 H_2O 1 mM. and shake in rotary shaker at 150 rpm for 48 hrs. Immobilized ability, Cd removal and Uptake capacity were analyzed. The immobilized experiment followed by;

1. Immobilized materials (Scoring web) were prepared as size 2x1x1 cm^3 and dry into hot air oven at 100 ºC and wrapped with aluminum foil and autoclaved before bacterial cultivation.
2. Immobilized material were submerged into culture media and shake in rotary shaker at 150 rpm for 48 hrs.
3. Supernatant were sampled for measure the Cd removal (APHA, 1992) by atomic absorption spectrophotometer (PerkinElmer's PinAAcleTM 900 model).
4. Immobilized ability was measured by dry weight of immobilized cell and non-immobilized cell.

3.8 Biological toxicity test

1. Mosquito egg (*Anopheles minimus*) were got from the Malaria Research Unit 2, Chiang Mai province
2. Embryonated eggs (30-50 eggs) were cultured in a free Cd environment. On the fifth day after hatching, mosquito larva were used in experiments.
3. 10 Mosquito larvae (instar 3) /L were cultured in different Cd contaminated water and Cd treated water for calculation of LD_{50} in 48 hrs.

3.8.1 Protocol for Mosquito Rearing (Das S., *et al*. 2007 applied by Malaria Research Unit 2, Chiang Mai province)

This protocol describes mosquito rearing in the insectary. The insectary rooms are maintained at 28°C and ~80% humidity, with a 12 hrs. day/night cycle.

Day 1: The 3-5 day old adult female mosquitoes are fed on blood to lay eggs. Guinea pig were used for mosquito blood-feeding (figure 15).

Figure 15 Guinea pig in the cage for mosquito feeding

Day 3: The females will lay eggs two days after they feed on blood. A small filter paper wrapped in a conical shape is put in a small Aluminum dish with distilled water, making sure that filter paper gets moist. The dish is kept inside the cage overnight for the mosquitoes to lay eggs (figure 16).

Figure 16 Mosquito cage with Lay egg dish

Day 4: The filter paper containing the mosquito eggs is placed in a plastic tray (Figure 17) with ~300 mL distilled water. A pinch of grounded fish food was added to the tray and eggs are allowed to hatch to larvae during the next days.

Figure 17 Plastic tray for rearing *Anopheles minimus* larvae.

Days 5 - 8: Growing larvae was fed every day with two tablets of grounded fish food, and monitored for density and population. On the eighth day (5 day old larvae), the larvae population is diluted from 1 tray to ~10 trays.

Days 9 - 12: The larvae was fed every day with grounded fish food. On the 12th day (9 day old larvae), the water is changed with fresh water, and food is added. The pupae starts developing at this stage. The trays are covered with nets to avoid escape of adults. (~ 8-10 mins)

Days 13 - 15: The pupae was allowed to emerge to adults for the next 2 - 3 days. Food was given every day to the larvae/pupae by carefully removing the net to avoid escaping of adults. Then put the pupa bow in the cage.

Day 16: The pupa was become adult in the cage (Figure 18). The cage consists of a small 100 ml bottle with a cotton wick that was soaked with 10% sucrose (autoclaved) (Figure 19).

Figure 18 Mosquito rearing cage

Figure 19 Cotton wick that soaked with 10% sucrose

Days 17 - 21: The adults (both males and females) are then kept in the insectary room for 4-5 days, fed on 10% sucrose before they are again blood-fed to begin the next cycle. The same mosquitoes can be used to lay eggs more than once.

The mosquitoes that are not needed for experiments or rearing can be killed by placing the cage in a freezer. The used trays and cages need to be cleaned and dried before they can be used again.

3.8.2 LD50 calculation by Reed Muench method

$$\text{Proportionate distance} = \frac{(\%\ \text{mortality at dilution next above } 50\%) - 50\%}{\left(\begin{matrix}\%\ \text{mortality at next}\\ \text{dilution above } 50\%\end{matrix}\right) - \left(\begin{matrix}\%\ \text{mortality at next}\\ \text{dilution below } 50\%\end{matrix}\right)}$$

LD_{50} = Log (negative logarithm of the next dilution above 50% mortality + Proportionate distance) x dilution factor

3.9 Calculation and Statistical Analysis

For Statistical Analysis, all data were subjected to analysis of variance followed by Duncan's multiple range tests. Differences will be considered significant at $P<0.05$.

1. Immobilized ability

$$\text{Immobilized ability (\%)} = \frac{(W_f - W_i) \times 100}{W_{ni} + (W_f - W_i)}$$

Where:

W_i is the initial dry weight of immobilized material (g)

W_f is the final dry weight of immobilized material (g)

W_{ni} is the final dry weight of non immobilized cell in solution (g)

2. Cadmium removal

$$\text{Cadmium removal (\%)} = \frac{(C_i - C_f) \times 100}{C_i}$$

Where:

C_i is the initial cadmium concentration (mg/L)

C_f is the final cadmium concentration (mg/L)