CHAPTER 4

RESULT AND DISCUSSION

4.1 Cd concentration in sampling site

Seven soil and one water samples were collected from agricultural area and analyzed for their contamination. Cd concentration in water was lower than detection limit while in soil samples was ranging between 0.22 to 19 mg/kg. Density of microbial (Table 6).

Table 6 The concentration of Cd in soil and water samples and number of total viable bacteria.

Sample site	Cd in soil (mg/kg)	Cd in water (mg/L)	Density (CFU/ g. soil)
MT	19	NOT Detected	4×10^4
MTM	14		6×10^3
RF	8.6		1×10^4
FP 1	2.0		6×10^3
FP 2	1.0		3×10^5
FP 3	0.14		1×10^4
FP 4	0.22		3×10^5

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Confirming the report from Akkaji in 2015, Cd contamination in agricultural soil around the Pha De village, Mae Sot District, Tak Province were ranging from 0.63 to 30.4 mg/kg. This high Cd contamination water was ran through the canal along the Mae Taw creek (Sriprachote *et al.*, 2012). Moreover, Cd concentration was found in soil more than in water. As reported by Ruangsomboon in 2004, 96.59 % of Cd in water was decreased after exposed to the soil within 70 hours.

4.2 Relationship between quantity of viable bacteria and Cd concentration in soil

Microorganism in environment was change by heavy metals (Sobolev and Begonia, 2008) Total viable bacteria were ranging between 6 x 10^3 to 3 x 10^5 CFU/g soil. High total number of bacteria were found in low contamination soil (Table 5). Correlation between quantity of viable bacteria and Cd concentration in soil were calculated. The result shown none significantly different (r^2 = 0.2218) in negative correlations (figure 20). As reported by Chen *et al.* in 2014 high contamination of heavy metals significantly decreased microbial biomass carbon (MBC), activity, abundance, and diversity of microbes.

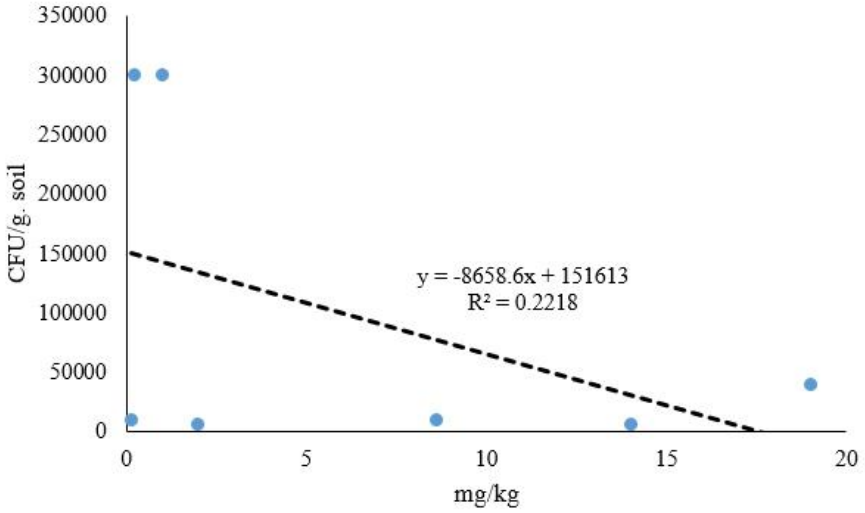

Figure 20 Correlation graph between Cd concentration in soil and viable bacteria quantity

The result indicated that the concentration of Cd not highly effected on the number of bacteria. It might be got the effected by others environmental condition such as soil moisture and soil properties.

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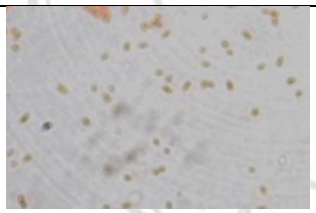
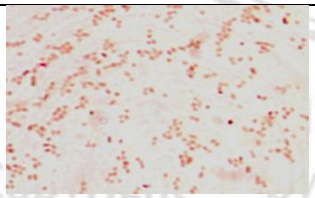
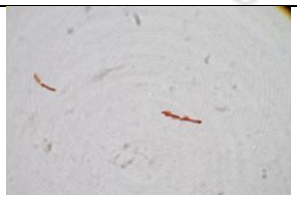
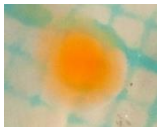
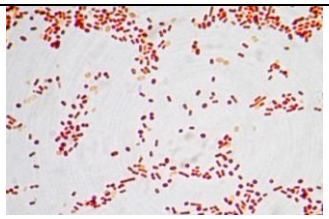
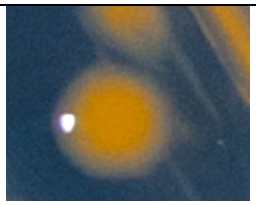
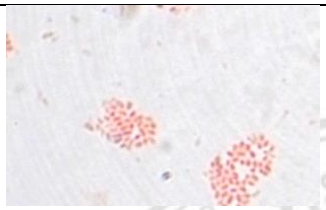
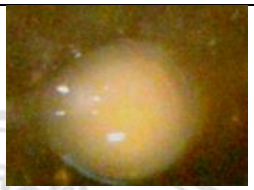
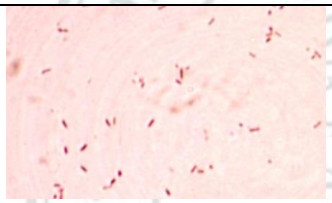
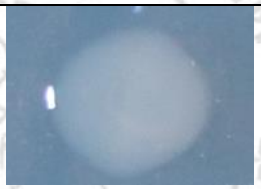
4.3 Selecting of Cd resistant bacteria

After suspended soil samples in a 0.85% of NaCl, soil solution was spread in Nutrients agar with 0.5 mM of $CdCl_2$•2.5 H_2O and incubated at 25 ºC, 48 hours. Bacterial strain was separated by morphology and shape. Gram strain was observed under microscope.

Morphology differentiation was used to isolated bacteria in to 7 strains. 6 strains were in Gram negative rod shape (Table 7). As a reported by Hoorman in 2011 that Gram Positive bacteria was tend to resist water stress. On another hand Gram negative bacteria be more sensitive to water stress. In this study, all of soil sample were collected in high moisture environment such as riparian zone, fish pond and irrigation canal. Therefore, Gram positive were found in this study.

Table 7 Colony, Morphology and Gram stain of Cd resistant bacteria.

Strain No.	Cell Shape and Gram reaction	Colony Morphology
1	 Rod Shape Gram Negative	 Circular, Smooth margin, Smooth and shine surface, Moist texture, Opaque White-green 0.9 cm. of diameter
2	 Rod Shape Gram Negative	 Circular, Smooth margin, Smooth and shine surface, Moist texture, Cloudy White-yellow-green, 1.1 cm. of diameter
3	 Rod Shape Gram Negative	 Circular, Wrinkled margin and surface, Moist texture, Cloudy White, 0.31 cm. of diameter
4	 Rod Shape Gram Negative	 Circular, Smooth margin, Smooth and shine surface, Moist texture, Translucent yellow-orange, 1.07 cm. of diameter

5	 Rod Shape Gram Positive	 Circular, Smooth margin, Smooth and shine surface, Moist texture, Cloudy yellow, 2.44 cm. of diameter
6	 Rod to coccoid Gram Negative	 Circular, Smooth margin, Smooth and shine surface, Moist texture, Opaque white-yellow, 0.67 cm. of diameter
7	 Rod shaped Gram Negative	 Circular, Smooth margin, Smooth and shine surface, Flat, Moist texture, Cloudy white, 3.27 cm. of diameter

4.4 Identification of Cd resistant bacteria

Species of resistant bacteria were identification by using 16S rDNA sequencing analysis. DNA extraction and PCR amplification was conducted in Microbiology program, Biology Department, Faculty of Science, Chiang Mai University.

Most bacteria (6 species) were classified as phylum Proteobacteria, but one species was classified as Phylum Firmicutes. Seven Cd-resistant bacterial strains were identified by 16S rDNA sequencing analysis (Figure 21)

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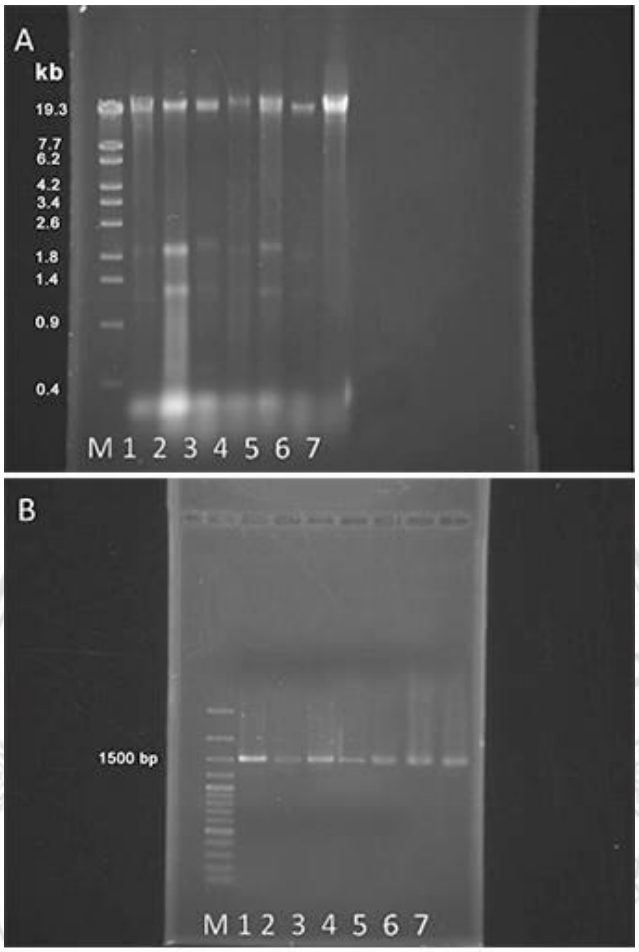

Figure 21 A: Gel 0.8% agarose gel electrophoresis of DNA extraction. M refer as Marker λDNA/Eco130I (StyI), 1-7 Refer as DNA extraction products from bacterial isolates. B: Gel 1% agarose gel electrophoresis of amplified 16S rDNA M refer as Marker 100 bp DNA Ladder pluse 1-7 line refer as PCR products from bacterial isolates

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*. The result of species identification were shown in table 8 (Data were shown in appendix)

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Table 8 Cd-resistant bacterial isolates

Isolated strain	Species	Base pair of sequencing	Percentage of Homology
1	*Pseudomonas lundensis*	1042	95%
2	*Pseudomonas monteilii*	1168	97%
3	*Alcaligenes faecalis*	1072	96%
4	*Brevundimonas vesicularis*	1246	97%
5	*Exiguobacterium acetylicum*	805	99%
6	*Aeromonas allosaccharophila*	1197	95%
7	*Aeromonas hydrophila*	1081	97%

All of Cd resistant bacteria species were commonly found in soil and in fish pond environment. For *Pseudomonas* spp. was the common bacteria that found in tropical aquatic animals. Ulcer disease in fish farm may cause by *Pseudomonas* spp. (Khatun *et al*., 2011). Many research were reported that *Pseudomonas* spp was used for cadmium removal in environment (Table 9). *Al. faecalis* AG3 was reported by Gupta *et al*. in 2015. Soil of Chambal region was used for isolated bacteria. Cadmium chloride, lead acetate, nickel chloride, mercury and cesium chloride were used for estimated tolerant of isolated bacteria. *Al. faecalis* AG3 was growth in range 10-50µg/ml. Non-living biomass of *B. vesicularis* was studied about Zn and Cd absorption. 100 mg/L was the suitable concentration for removal both metals (Singh and Gadi, 2012). *Exiguobacterium* sp. was study draft genome sequence by using The RAST (Rapid Annotations using Subsystems Technology). Protein in stress tolerance, include cold, cadmium, drug from sequence of BMC-KP was found in this experiment (Hyson *et al*., 2015). Cd absorbtion by *Ae. caviae* was investigated. Well-stirred batch reactor in equilibrium and kinetic experiments (initian concentration, temperature, biomass and ionic back ground) were performed (Loukidou *et al*, 2007).

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Table 9 The previous study of *Pseudomonas* spp. in Cd removal experiment.

2001	Lee *et al*.	*P. putida*	Study *P. putida cadA* and *cadR* gene Chromosomal Locus for Cadmium Resistant gene. Cd (II), Pb (II), and Zn(II) were induced *cadA* promoter, while Cd(II) were only induced *cadR* promoter.
2007	Pagès *et al*.	*P. brassicacearum*	*P. brassicacearum* from plant was study by microarray technique. In different phases of *P. brassicacearum* were study gene expression and cd resist mechanism.
	Vullo	*P. veronii* 2E	Cd, Zn and Cu removal efficiency of *P. veronii* 2E was studied. Absorption efficiency Free and immobilised cells in teflon membranes silicone rubber and polyurethane foam were compared. For the result, *P. veronii* 2E was successful for adsorb metals in all 3 immobilized materials.
2009	Sinha and Mukherjee	*P. aeruginosa* KUCD1	Remove from Industrial waste water result shown that membrane and periplasm to be the major accumulating site in this strain
2014	Nath *et al*.	*P. aeruginosa* SN1 and SN3	*P. aeruginosa* in Seed Germination of Rice (*Oryza sativa*) shown increasing shoot growth more than without bacteria.
2016	Halder and Basu	*P. stutzeri* MTCC101	SEM-EDX was used for observed *P. stutzeri* MTCC101 in log phase. Extracellular polymeric substance (EPS) was produced in highly stress from high Cd condition.

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4.5 Selected high Cd resistant bacteria and Growth Curve determination

4.5.1 Selected high Cd resistant bacteria and Minimal Inhibitory Concentration (MIC)

Selection of high efficiency bacteria by using Kirby-Bauer disc diffusion method (Bauer *et. al*, 1959). 6 concentration of filter paper disc (0, 0.5, 3, 5,10 mM and 1M) subsequence placed into the lawn of cell. After incubated in 25 ºC in 48 hr. The result were shown in figure 22 and Table 10. (Data were shown in appendix)

Form the statistical analysis, bacterial can classified in to 6 group. The most tolerant species was *P. lundensis* and *P. monteilii*. Abbas *et al*. (2014) found *Pseudomonas* sp. from Cd contaminant industrial wastewater. Yamina *et al.* (2014) isolated *Klebsiella neumonia* and *P. aeruginosa* and estimated resistant ability of heavy metals from hospital waste water. The ability of *P. aeruginosa* had been previously described by Chakraborty and Das (2014) that extracellular polymeric substances (EPS) secreted from cell were the major role of Cd attachment. Sulhydryl group (-SH) from EPS could bind with Cd ion. Shamim *et al*. (2013) added the ATPase inhibitor into *P. putida* culture medium Cd containing and was found that Cd was decreased. Additionally, Cd accumulation was found in killed cells as well

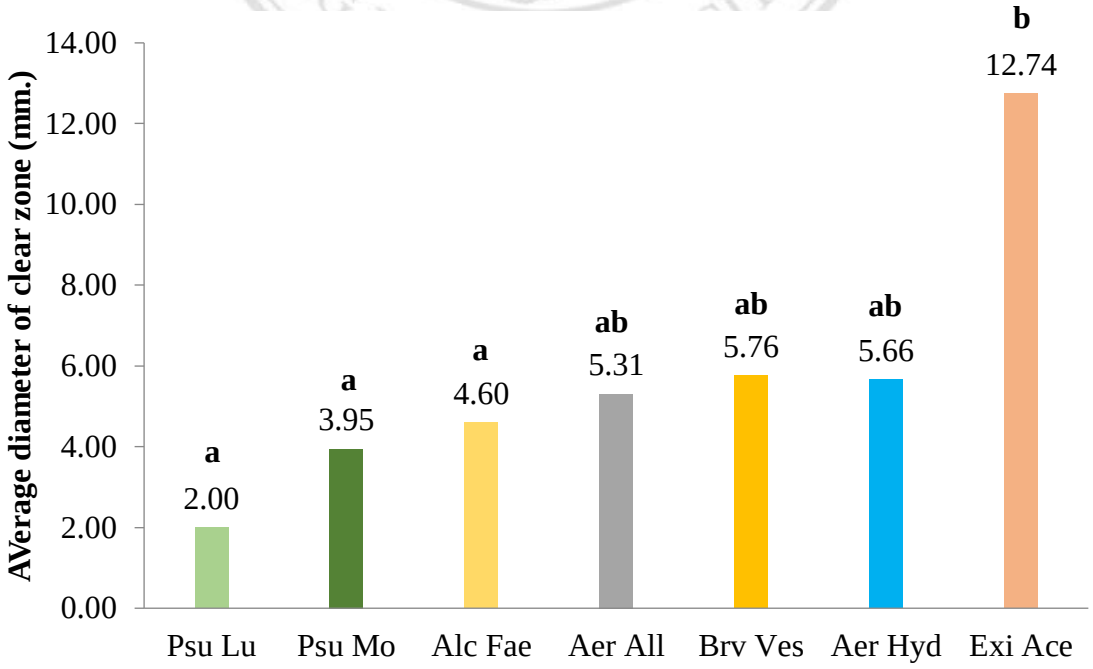


Figure 22 Average diameter of clear zone of cd resistance bacteria strain

P. lundensis, *P. monteilii* and *Al. faecalis* were used for Minimal Inhibitory Concentration (MIC) by using Kirby-Bauer disc diffusion method (Bauer *et. al*, 1959). 7 concentration of filter paper disc (0, 0.05, 0.125, 0.25, 0.5, 0.75 and 1M) subsequence placed into the lawn of cell. After incubated in 25 ºC in 48 hrs. The result were shown in figure 23. (Data were shown in appendix).

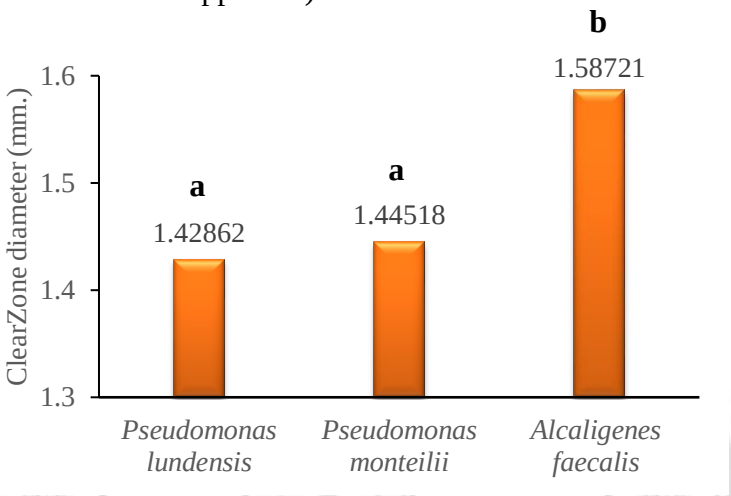
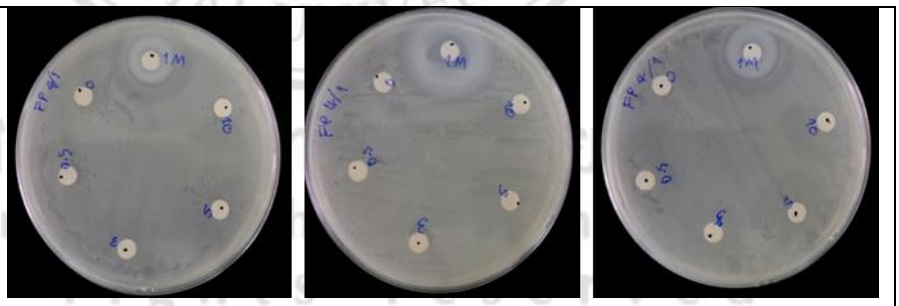
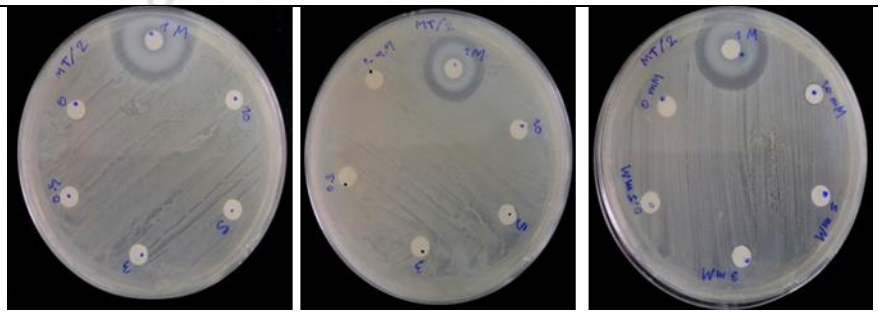
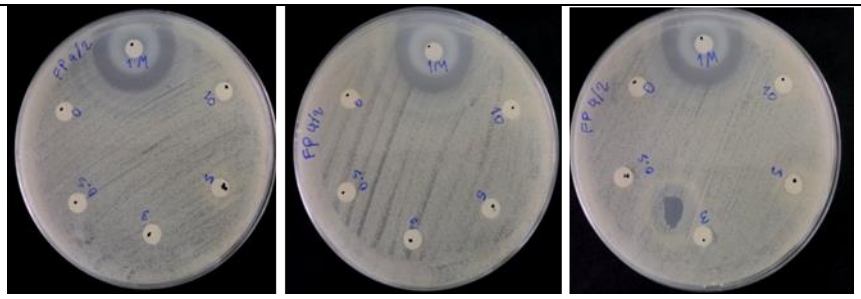
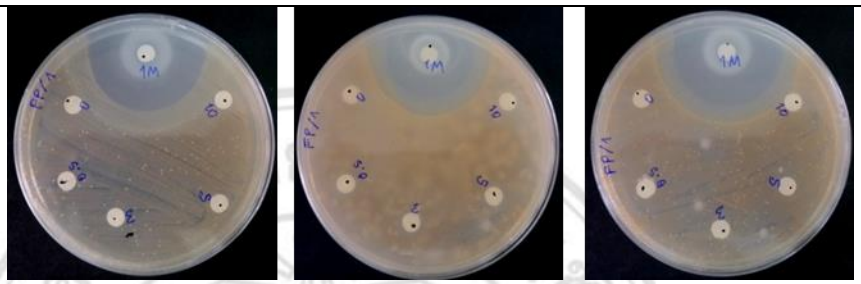
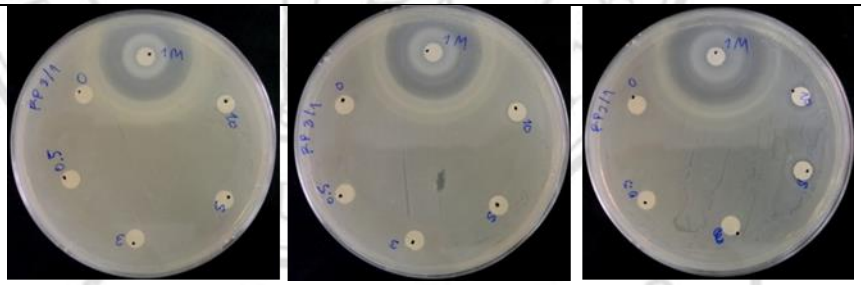
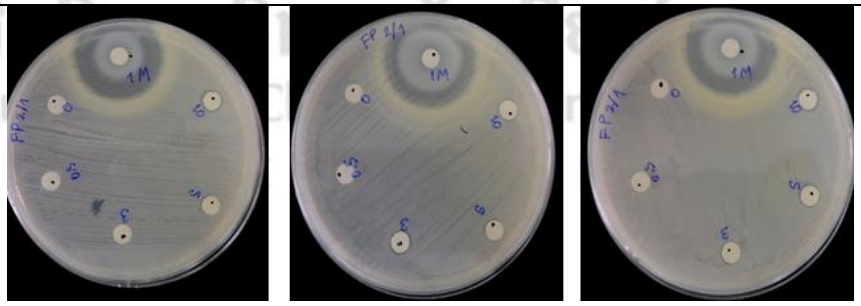


Figure 23 Average diameter of clear zone of Cd resistant bacteria strain

P.lundensis and P. monteilii were selected for high Cd resistant bacteria in this experiment.

Table 10 the picture clear zone in 7 species in different concentration of $CdCl_2$•2.5 H_2O

P. lundensis	
P. monteilii	

Al. faecalis	
B. vesicularis	
E. acetylicum	
Ae. allosaccharophila	
Ae. hydrophila	

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4.5.2 Growth Curve determination

Bacterial growth of each pure culture was monitored in NB by measuring the optical density OD600. Cell density was collected by spectrophotometer with 2 hours interval. Growth curve was plotted between cell density and Cultivation (figure 24). (Data were shown in appendix)

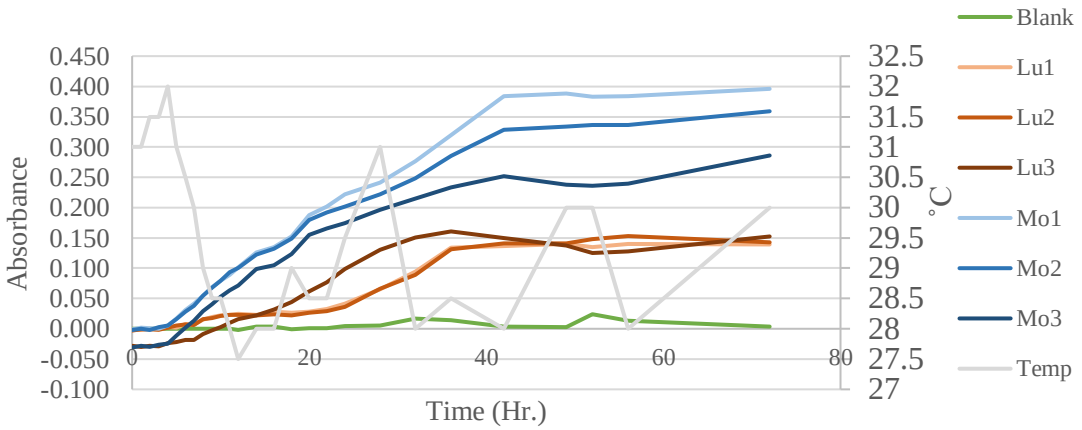

Figure 24 Growth curve determination by using absorbance in 600OD of *P. lundensis* and *P. monteilii*.

Growth pattern of the Cd resistant bacteria 2 strain (*P. lundensis* and *P. monteilii*). Found that lag phase of 2 species was 3 hr. and reaching to exponential log phase in 4 hr. after inoculation. After 45 hrs. optical density slightly constant. Result shown that growth were slightly constant in 45 hrs. Stationary phase time were applied to use for bacteria efficiency determination.

4.6 Remediation Efficiency of Cd resistant bacteria

4.6.1 Immobilized ability

Comparison with 2 species of cd resistance Bacteria (*P. lundensis* and *P. monteilii*). *P. lundensis* (73.37%) was higher immobilized ability value more than *P. monteilii* (49.79%). Result were showed in table 11. (Data were showed in appendix)

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Table 11 immobilized ability data of *P. lundensis* and *P. monteilii*

Species	Average value of Immobilized ability (%)	SD
P. lundensis	73.37	14.34
P. monteilii	49.79	17.45

As a result of Biofilm Formation Characteristics of *P. lundensis* Isolated from Meat research by Yong-Ji Liu *et al*. in 2015. The result of this study were reported that *P. lundensis* was the most prominent species of *Pseudomonas* spp. that cause the spoilage of chilled meat during aerobic storage. That's means *P. lundensis* may formed the biofilm easier that *P. monteilii*.

In 2000, According to the study of *Pseudomonas* Strain GCH1 in ceramic support as biocatalysts in an immobilized cell system by Martin *et al.* 0.5 mM Propachlor (2-chloro-N-isopropylacetanilide) elimination reached into 98%, in 3 hr. The viability of immobilized cells increased to 34-fold after 120 days.

As a report by Wasi S. *et al.* in 2011 *P. fluorescens* SM1 Strain for Remediation of phenols, heavy Metals (Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} and Cr(VI)), and pesticides from Water in immobilized cell treatment. Calcium alginate beads was used for immobilized ability. The efficiency of free cell removal heavy metals was 75% and 7–9% better efficiency under the immobilized conditions.

4.6.2 Cadmium removal

Initial and final concentrations of Cd was analyzed in the different condition of free cell and immobilized cell culture. *P. lundensis* and *P. monteilii* were used for determined and Cd removal efficiency (Table 12). (Data were shown in appendix)

Table 12 Cd removal efficiency in different condition and different species.

Row Labels	**Average of Cd removal (%)**	**StdDev of Cd removal (%)**
LU	29.40	1.25
LUS	39.55	6.44

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Table 12 (Continued) Cd removal efficiency in different condition and different species.

Row Labels	Average of Cd removal (%)	StdDev of Cd removal (%)
MO	39.47	4.29
MOS	38.62	2.68

From the result were show not significantly different between % of removal in free call and immobilized cell and not significantly between % removal between free cell and immobilized cell of *P. monteilii* and *P. lundensis*. On another hand, when compare with free cell of *P. monteilii* and *P. lundensis*, the higher significantly between 2 species were show ($p<0.05$).

Psudomonas spp. species was widely used in many research Vullo *et al*. in 2008 were study about Cadmium, zinc and copper biosorption mediated by *Pseudomonas veronii* 2E. The result show the biosoption was more over 40% in every metal with teflon membranes immobilized materials. Abbas *et al*. in 2014 were isolated the *Pseudomonas* sp. from the wastewater collected from industrial area of Penang, Malaysia. *Psudomonas* spp. strain RZCd1 was high effective 70% of the cadmium removal in log phase in 35°C and pH 7.0.

4.7 Biological Toxicity test

10 Mosquito larvae (instae3) /L will be cultured in different Cd contaminated water and Cd treated water for calculation of LD_{50} in 48 hrs.

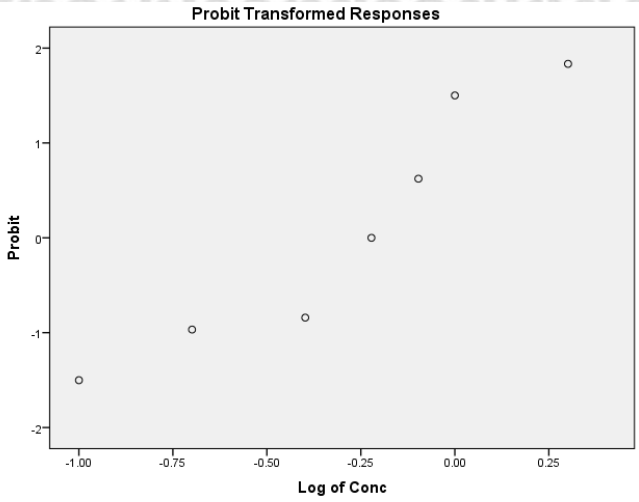

Figure 25 LC_{50} Log concentration of Cadmium and lethal of mosquito larva

From the data analysis were show Probit Analysis y=0.879 + 2.913x, $LC50_{48hrs.}$ = 0.499 ppm., 95% confidence 0.420-0.588, Std. Error = 0.322 (Figure 25). The result were compare with the Cd removal efficiency and resistant concentration. Concentrations that bacteria can resist were very higher than others species.

Mosquito larva were used for study about toxicity. Kitvatanachai *et al*., (2005) used the *Culex quinquefasciatus* mosquito for studies about the led toxicity and led uptake. Mosquito larvae were cultured in led different concentrations of 0.05, 0.1, and 0.2 mg/l. The result show the significantly reduced hatching, egg-production, and emergence rates, compared with the unexposed group ($p<0.05$). In 24 hours, The LC_{50} of lead was 0.18 mg/l. *Aedes aegypti* egg was report that tolerant in dry condition. Perez *et al*. in 2012 were study *Aedes aegypti* pharate 1^{st} instar in different of quiescence affects. The result shown that old larva (egg quiescence more than 2 months) significantly higher mortality than new larvae (egg quiescence less than 1 week) (85% vs. 53% respectively).

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