CHAPTER 3

MATERIAL AND METHODS

3.1 SOURCE OF MICROORGANISMS

3.1.1 SAMPLE

Mercury-resistant bacteria were isolated from 61 samples collected from different sites, i.e., laboratory, painting industry, municipal waste and natural water. Those samples were collected in various form, i.e., soil, mud, sediment, industrial waste water and sludge. The samplings were conducted sequentially in 7 months from December, 1998 to June, 1999 (grateful thanks to everyone who kindly collected those samples are cordially performed here). The detail of samples was shown in Appendix A.

3.1.2 BACTERIAL REFERENCE STRAINS

The mercury-volatilizing bacterium ***Escherichia coli*** KP 245 which has a mercury-volatilizing plasmid (pRR 130) was used as mercury resistance strain for volatilization test. The author grateful thanks to Dr. Nakamura Kunihiko (National Institute for Minamata Disease, Minamata, Japan) for this bacterium.

Escherichia coli and ***Serratia macescens*** were used as mercury and heavy metal sensitive reference strain were provided by Microbiology, Faculty of Science, Chulalongkorn University.

3.2 CHEMICAL, REAGENT, STAINING DYE AND INSTRUMENT

3.2.1 CHEMICALS

All chemical and reagents used in this study were of analytical grade that are;

Ammonium sulfate $(NH_4)_2 SO_4$ Merck, Darmstadt.

Calsium sulfate $CaSO_4.2H_2O$ Merck,

Diphynylthiocarbazone $C_6H_5NH.NH.CS.N:N.C_6H_5$ Ajex chemicals, Aubern, Australia;

Di-potassium hydrogen orthophosphate K_2HPO_4 A-jax chemicals;

Di-sodium hydrogen phosphate dodecahydrate anhydrous Na_2HPO_4 , Merck;

Ethylenediamine tetraacetic acid disodium salt dihydrate EDTA May and Baker, Ltd.,England;

Hydroxylamine hydrochloride $NH_2OH.HCl$ Ajex chemicals;

Iodine resublimed May&Baker Ltd., Dagenham, England

Iron (II) sulfate heptahydrate $FeSO_4.7H_2O$ Merck

Magnesium acetate $(CH_3COO)_2Mg.4H_2O$ BDH

Manganese(II) sulfate monohydrate pure $MnSO_4.H_2O$ Merck

Magnesium sulfate heptahydrate $MgSO_4.7H_2O$ Merck

Mercury chloride $HgCl_2$ 99.5% Carlo

Potassium dihydrogen phosphate KH_2PO_4 Merck

Potassium hydroxide Pellets KOH May&Baker Ltd., Dagenham, England.

Potassium nitrate KNO_3 Merck

Potassium permanganate $KMnO_4$ Merck;

Sodium dihydrogen phosphate $NaH_2PO_4.2H_2O$, Merck;

Sodium nitrite $NaNO_2$, Merck;

Sodium thioglycolate $HSCH_2COONa$ Fluka,Switzerland; and

Urea $CO(NH_2)_2$, Merck.

3.2.2 REAGENT

Reagent used in this study are;

Acetic acid CH_3COOH 4 N, Merck;

Chloroform $CHCl_3$, Merck;

Hydrochloric acid solution 0.1 N for adjust pH

Nitric acid solution 0.1 M for adjust pH

Normal saline 0.85% NaCl

Phosphate buffer 0.07 M pH 7.0

Potassium hydroxide solution 0.1 M for adjust pH

Sodium hydroxide solution 1.0 N for adjust pH

Stock mercury 13.5 mg/ml

Sulfuric acid 9 N, Merck and

Volatilization mixture compose with; phosphate buffer 0.07M pH 7.0, 0.5 mM EDTA, 0.2 mM magnesium acetate, 5 mM sodium thioglycolate, and 50 μg/ml of $HgCl_2$.

3.2.3 STAINING DYE

Staining dye use in this study are;

Crytal violet 85% Merck, Darmstadt;

Methylene blue DAB Fluka and

Safranine-O BDH chemic Ltd., England

3.2.4 INSTRUMENTS

Autoclave Sanyo, Japan

High-Speed centrifugation (ALC Model 4239R, Italy)

Incubator Memmert, Germany

Kodak x-ray film

Microdilution plate or multiwell plate, Nunc, USA.

Microwave Sanyo, Japan.

pH meter Scant, Singapore

Spectrophotometer Spectronic 20 genesys, USA

3.3 CULTURE MEDIA

3.3.1 GENERAL MEDIA

1) Enrichment media: nutrient broth NB Difco, Detroit MI, USA.

2) Isolation media: basal salt agar (Nakamura, Fujisaki and Tamashiro, 1986) component and preparation was shown in Appendix B.

3) Stock media: nutrient agar NA. plus 20 μg/ml of $HgCl_2$.

3.3.2 SELECTIVE MEDIA

Identification of selected bacterial isolates, some selective media were used, i.e.,

Eosin Methylene blue Agar (E.M.B. Agar), Scharlau Microbiology, Sentmenat, Spain;

MacCONKEY agar, Difco Labolatories, Detroit MI, USA;

Nutrient Gelatin, Difco Labolatories ;

Simmons Citrate Agar, Difco Labolatories;

Salmonella-Shigella agar (SS), Difco Laboratory and

Triple sugar iron agar (TSI) , Difco Labolatory.

Preparation and component of these media were shown in Appendix B.

3.3.3 MEDIUM FOR RESISTANT TEST

Tryptic soy agar at 1/10 normal strength (Farrell, Germida and Huang, 1990) plus different concentration of mercury and different concentration of other heavy metals.

3.4 STAINING FOR IDENTIFICATION

Staining were used in this study, namely, Gram's stain, to determine activity of bacteria as gram positive or gram negative and to observe the dimension, the physiological characteristics and the cellular morrphology by microscope.

3.5 BIOCHEMICAL TEST FOR IDENTIFICATION

Biochemical test were use to confirm the determination of bacterial isolates after the test by selective media. In this study was test as follows.

Catalase test

Citrate Utilization test

Gelatin liquefaction Test

Indole test

Limus milk

Mac

MR.VP

Motility test

Nitrate test

Oxidase- test

Oxidation-fermentation test

Potassium cyanide test

SS agar

Triple sugar iron agar

Urease test

3.6 SAMPLING OF CULTIVATION PROCEDURE*

3.6.1 SAMPLING

Samples of sediment and sludge were collected from Industrial area, Labolatory area. The top 3 cm of sediments was collected by plastic bag.

3.6.2 ISOLATION OF MERCURY RESISTANT BACTERIA

1) 0.5 g of sediment was added to 4.5 ml nutrient broth containing 50 μg/ml of $HgCl_2$.

2) mixed with a vortex mixer, incubate at 37 °C for 4 hr.

3) 0.1 ml suspension were spread on Basal salt medium containing 50 μg/ml of $HgCl_2$. see Appendix B.

4) incubate at 37 °C for 18-24 h.

5) picked the single colony to 2 ml normal saline.

6) one loopful streak on the same medium 2-3 times to purification.

7) stock culture were prepare by streaking the isolation onto NA slants containing 20 μg/ml of $HgCl_2$.

8) incubate for 6 h at 37°C and stored at 4°C.

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*All chemicals, reagents, cultivation media, glassware and certain devices were sterilized by autoclaving at 121°C, for 15 mimute and at least three replicates were conducted in each test.

3.6.3 MERCURY-RESISTANCE TEST

Susceptibility was determined by an agar dilution method (Lederberg and Lederberg, 1953) with a multiple inoculator system. (Nieto, Ventosa and Berraquero, 1987; Nieto et al., 1989) as follows.

1) A loopful amount of bacterial was inoculated into 1/10 normal strength TSB 4 ml, incubate for 24 h at 37^0C.

2) Dilute (1:500) of 24 hr. culture from (1) in mormal saline (0.85 mg/l NaCl). The inoculum size was about 10^4-10^5 cells/ml.

3) Transfer the culture from (2) to the wel! of a multipoint inoculator device.

4) The inoculator was used to transfer the culture to the control plated (1/10 strength TSA and 1/10 strength TSA containing 50 μg/ml Hg^{2+}) and to the mercury plates (1/10 strength TSA plus 100, 150, 200 and 250 μg/ml of Hg^{2+}, respectively).

The results were read after 24 h incubation at 37^0C. Growth on both control and test plates was taken as resistance to the test concentration of mercury. At least 3 tested sets of replicate were performed in all experiments.

The lowest concentration of metal that completely prevented growth was termed the MIC.

3.6.4 SCREENING OF ISOLATES FOR VOLATILIZATION OF MERCURY CHLORIDE.

All strains isolated were screened for the ability to volatilize Hg^{2+} by using the X-ray film method (Nakamura and Nakahara, 1988., Nakamura, Sakamoto, Uchiyama and Yagi, 1990.,Tsai and Olson, 1990., Nakamura, Naruse and Takizawa, 1999).

The mercury-volatilizing bacterium, ***Escherichia coli*** KP 245, the bacterium has a mercury-volatilizing plasmid (pRR130), obtained from Dr. Nakamura Kunihiko,National Institute for MinamataDisease were used as controls of the method.

The simplified X-ray film method for determining bacterial volatilization of Hg^{2+} is as follows.

1) Bacterial strains were incubated on nutrient agar plates containing 1 μg/ml of $HgCl_2$, incubate for 24 hr at 37° C.

2) The strains were washed with 0.07 M Na-P buffer (pH 7.0) and suspended into volatilizing mixture (see materail and method in **3.2.2 reagents**) in a microplate.

3) The plate was covered with the X-ray film and set between two boards of acrylic plastic, securing the ends with clips in the darkroom (see Figure 3.1)

4) Then put into a dark box.

The X-ray film was developed after reacting for 60 min at 35 ° C. The foggy areas on the film were the results of the reduction of Ag^{+} emultion by mercury vapour.

3.6.5 IDENTIFICATION OF SELECTED BACTERIAL STRAINS.

There of 272 bacterial strains were found to be resistance to 250 μg/ml Hg. The highest concentration of mercury. After screening for ability of mercury volatilization, it was found that only 2 of 3 highest resistant strains should be further processed. Each of them were tested for some fundamental characteristics, e.g. size, color and appearance of colony, gram-staining and morphology of the cells (size, shape and spore

formation). The expected genus was primary performed by growing of the selected isolates on certain selective media. There after the expected genus of genera of selected isolates were confirmed by biochemical tests.

3.6.6 STABILITY TEST OF MERCURY RESISTANCE IN THE SELECTED BACTERIAL ISOLATED.

Each time of the two selectd bacterial isolates, i.e., Hg-R11 and Hg-R14 on 1/10 strength TSA with and without mercury resistance to the highest mercury concentration (250 μg/ml) of those isolates were tested for at least 20 times of subculturing.

3.7 EFFECT OF SOME GROWTH FACTOR AT 5 ppm of Hg ON MERCURY RESISTANT BACTERIA

3.7.1 EFFECT OF pH

1) Inoculating loopful of the working culture (Hg-R11, and Hg-R14) into 5 ml of 1/10 strength TSB plus 5 μg/ml of Hg^{2+}, incubate for 24 h at 37^0C.

2) 0.1 ml of the inoculum was transferred to 50 ml of 1/10 strength TSB plus 5 μg/ml of Hg^{2+}. The pH values of the culture medium were adjusted to be 4 5 and 6 by adding of 0.1 NHCl and 8, 9 and 10 by adding 0.1 NaOH.

3) Incubate for 24 hr at 37^0C.

The number of each test organism was determined by viable count method, The highest amount of the twat bacterial strain was found in medium adjusted to certain pH value.

3.7.2 EFFECT OF TEMPERATURE

The procedure for study the effect of temperature was performed similarly to the effect of pH, but the pH value of the medium was adjusted to 8. Certain amounts of the test organism were inoculated into 50 ml of 1/10 strength TSB plus 5 μg/ml of Hg^{2+} and then incubated for 24 hr. at different temperature i.e, 25, 30, 35,40, and 45^0C. The number of each test organism was determined by viable count method, The highest amount of the test bacterial strain was found in medium incubated at certain temperature of incubation.

3.7.3 EFFECT OF MERCURY CONCENTRATION

1) Inoculating a loopful of the working culture of Hg-R strains into 5 ml of 1/10 strength TSB plus. 5 μg/ml of $Hg^{2+,}$ incubate for 24 h at 37°C.

2) 0.1 ml of 24 hr. culture were transfer to 20 ml of 1/10 strength TSB at optimum pH from 3.7.1 plus difference concentration of mercury i.e., 0,4, 8, 50 and 150 μg/ml.

3) Incubate for 24 h at optimum temperature from 3.7.2.

The number of each test organism was determined by viable count method at 0, 2, 4, 6, 8, 12 and 24 hours.

3.8 EFFECT OF pH AND TEMPERATURE ON MERCURY VOLATILIZATION IN THE SELSECTED BACTERIAL ISOLATES

3.8.1 EFFECT OF PH

1) Inoculating loopful of the working culture (Hg-R11, and Hg-R14) into 5 ml of 1/10 strength TSB plus 5 μg/ml of Hg^{2+}, incubate for 24 h at 37^0C.

2) 1 ml of the inoculum was transferred to 50 ml in 1/10 strength TSB plus 5 μg/ml of Hg^{2+} at pH 6, 7, 8 and 9

3) Incubate for 24 hr at 35°C.

The amount of Hg volatilization was determined by measuring the residual mercury concentrations in the supernatant that the analyses were performed by calorimetric method with dithizone (see detection in Appendix C) and minus with initial mercury concentration compared with control (no cells add).

3.8.2 EFFECT OF TEMPERATURE

The procedure for study the effect of temperature was performed similarly to the effect of pH, but the pH value of the medium was adjusted to 8. And incubated for 24 hr at different temperature i.e., 25, 30, 35 and 40 and the amount of Hg volatilization was determined as 3.8.2.

3.9 REDUCTION OF MERCURY

1) Inoculating a loopful of the working culture (Hg-R11 and Hg R14) into 5 ml of 1/10 strength TSB plus 5 μg/ml of Hg^{2+}, incubate for 24 hr at 37°C.

2) Transfer 2 ml of 24 hr culture to sterile 100 ml of 1/10 strength TSB plus 50 μg/ml of Hg^{2+} at pH 8.

3) Incubated at 35°C for 24 hr.

The remaining of mercury in the medium were determined by colorilnitric method with dithizone at 1hr, 2hr, 3hr, 4hr, 6hr, 8hr, and 24hr.

3.10 RECOVERY OF METALS

1) Inoculating a loopful of the working culture (Hg-R11 and Hg R14) into 5 ml of 1/10 strength TSB plus 5 μg/ml of Hg^{2+}, incubate for 24 hr at 37°C.

2) Transfer 20 ml of 24 hr culture to sterile 1,000 ml of 1/10 strength TSB plus 150 μg/ml of Hg^{2+} at pH 8 in the 2.5 l bottom.

3) The bottom were sealed with plugs and connected to an $KMnO_4$-H_2SO_4 trap containing 100 ml of trapping solution (sec 3.2.2 reagent in this study) as shown in Figure 3.2

4) The bottom were incubated for 5cl at ambient temperature. Head gases were flushed to traps with a stream of vapor-saturated.

The remaing of mercury in the medium and trapping solution wer determined by colorimetric method with dithizone.

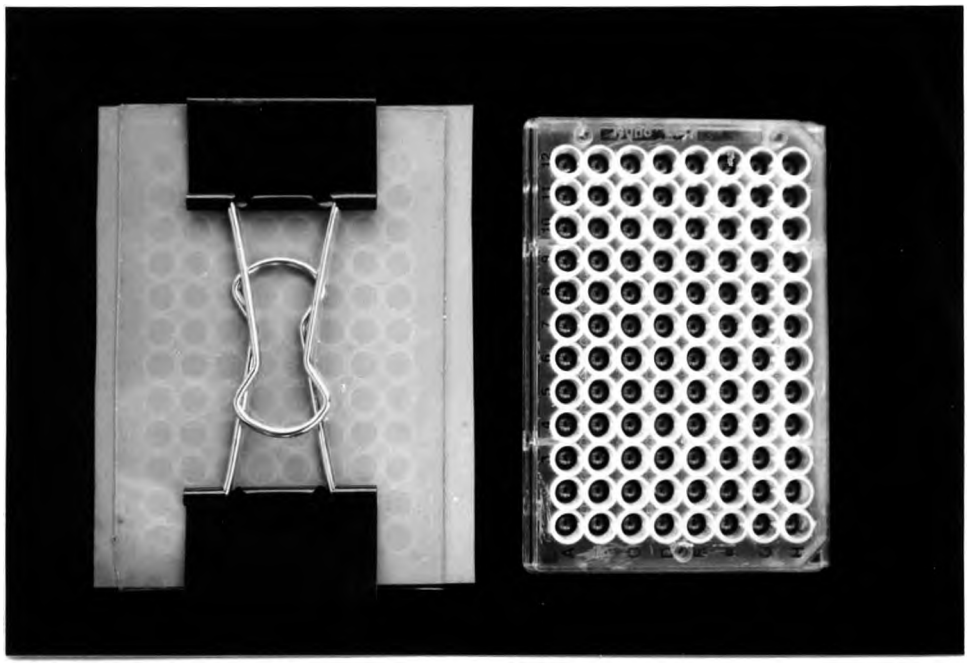

Figure 3.1 Normal multiwell plate (left) and multiwell plate set for Mercury volatilization test (right)

Figure 3.2 Bacterial culture and trap solution for determined volatilization Of mercury