CHAPTER III

MATERIALS AND METHODS

3.1 SOURCES OF MICROORGANISMS

3.1.1 Samples

Copper-resistant bacteria were isolated from fifty samples collected from different sites, i.e., metal processing plants, laboratory, plating industry, dump and natural source. Those samples were collected in various forms, i.e., soil, mud, sediment, industrial wastewater, municipal wastewater, natural water and sludge. The samplings were conducted sequentially in 11 months, from February to December, 1997 (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

Bacterial references strains used in this study were provided by MIRCEN*, Thailand, namely, ***Zoogloea ramigera*** (TISTR 1329) and ***Bacillus licheniformis*** (TISTR 1010). They were served as copper-sensitive strains for resistance tests and also for confirmation of identification of selected bacterial strains.

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* MIRCEN = Microbiological Resources Center

3.2 CHEMICALS, REAGENTS AND INSTRUMENTS

3.2.1 Chemicals and Reagents

Chemicals used in this study were all analytical grade: $CuSO_4.5H_2O$ (Merck, Darmsatadt, Germany); $ZnSO_4.7H_2O$ (Merck); $CdCl_2.H_2O$ (Merck); $AgNO_3$ (Merck); $MnSO_4.H_2O$ (May and Baker, Dagenham, England); $NiSO_4.H_2O$ (May and Baker) and K_2CrO_4 (Seelze-Hannover, Hannover, Germany).

Reagents used in this study were 0.1N HCl (Merck); 0.1N NaOH (Merck); and 0.85% Normal Saline (Merck); Heavy metal standard solution (Merck); Gram Stain, namely, crystal violet (Merck); 95% ethyl alcohol (Merck); safanin O (BDH Chemical, Poole, England) and iodine (May and Baker).

3.2.2 Instruments

Many instruments were used, i.e., High-Speed Centrifugation (ALC Model 4239 R, Italy); Spectrophotometer (Spectronic® 20 Genesys™ , U.S.A.); pH meter (Model pH scan 1, Singapore); Rotary Shaker (Lab-Line Instrument, Melrose Park, Illinois, U.S.A.); Atomic Absorption Spectrophotometer (Perkin-Elmer, model 403, Norwalk, Connecticut, U.S.A.); Autoclave (Labo Autoclave, Sanyo Electric, Japan, grateful thanks to Viriya Insurance, Co., Thailand, for donation of two sets to the Department of General Science, Faculty of Science, Chulalongkorn University); Microwave (Sanyo Electric, Japan, grateful thanks to Thanes, Co., Thailand for donation to the Department of General Science); Refrigerator (Samsung, Korea, grateful thanks to Becthai, Co., Thailand for donation to the Departmentof General Science); Incubator (Memmert GmbH, Model 700, Schwabach,

Germany); and Oven (Memmert GmbH, Model 700, Schwabach, Germany).

3.3 CULTURE MEDIA

3.3.1 General Media

The formula and preparation of each culture medium were found in **APPENDIX B**. Those were Tryptic Soy Broth (TSB; Difco laboratories, Detroit, Michigan, U.S.A.); Tryptic Soy Agar (TSA; Difco). They were used as liquid medium and semisolid medium, respectively, for general media.

3.3.2 Selective Media

Identification of selected bacterial isolates, some selective media were used, i.e., MacConkey-Inositol-Potassium Tellurite (MCIK, Difco) Agar, Pseudomonas Selective Isolation Agar (PSIA, Difco), Shigella-Salmonella Agar (SSA; Difco) and MacConkey Agar (MA; Difco).

3.3.3 Medium for Metal Resistant Test

Metal resistance of those selected bacterial strains was determined in either 1/3 strength TSA or 1/3 strength TSB or both (Chao and Chen, 1991) containing various concentrations of Tested metals, i.e., Cu, Zn, Mn, Cd, Ag, Ni and CrO_4^{2-} in the forms of $CuSO_4.5H_2O$; $ZnSO_4.7H_2O$; $MnSO_4.H_2O$; $CdCl_2.H_2O$; $AgNO_3$; $NiSO_4.H_2O$ and K_2CrO_4, respectively.

3.4 STAINS AND BIOCHEMICAL TESTS FOR IDENTIFICATION

Formula, preparation and procedure of each stain and some biochemical tests were found in **APPENDIX C**. Gram's Stain and Indian Ink were used. Indian Ink examined capsule and endospore forming in bacterial.

Some biochemical tests were done, i.e., Citrate Utilization; Triple Sugar Iron (TSI) Agar; Motility Test; Oxidase Test; Catalase Test; Oxidation-Fermentation Test; Acetate Utilization; MacConkey Agar; Salmonella-Shigella (SS) Agar; Capsule Stain.

3.5 ANALYSES OF SOME HEAVY METALS

Procedures of determination of heavy metal concentration were shown in **APPENDIX D**. By using of Atomic Absorption Spectrophotometer equipped with an air-acetylene flame and appropriate single-element lamps in all experiments, the amounts of each metal in whole cell or biomass, EPS, cells without EPS or cells, and medium or experiment, were performed by standard methods, i.e., Cu ($CuSO_4.5H_2O$); Zn ($ZnSO_4.7H_2O$); Mn ($MnSO_4.H_2O$); Cd ($CdCl_2.H_2O$); Ag ($AgNO_3$); Ni ($NiSO_4.H_2O$); and CrO_4^{2-} (K_2CrO_4).

3.6 SAMPLING AND CULTIVATION PROCEDURES

3.6.1 Sampling Procedures

Samples of soils, waters, mud and sediments were collected from certain sites that have been contaminated by heavy metal(s) and also from natural sources. At each sampling site, three replicates (1 kg and/or 1 L for one replicate) were conducted. Those samples were preserved at

low temperature (4-6°C) in the icebox for transport and then stored in the refrigerator. Isolations of copper-resistant bacteria were performed either in the same or after day of collection.

3.6.2 Isolation of Copper-Resistant Bacteria

Each sample of soils or mud or sediments was serially diluted in normal saline (0.85 mg/L NaCl); and then spread on 1/3 strength TSA plates containing 400 μg/ml Cu^{2+} (Kitti-anong, 1995). After 24-hr. incubation at 37°C, isolated colonies were individually picked and purified at least 2 times and then stabbed in the stock culture tubes or pure culture. Those bacterial isolates or strains were further proceeded.

Certain amount of each water sample was filtered through Millipore filter devices. The membrane containing bacteria from each sample was washed out and serially diluted and the dilutions were spread on 1/3 strength TSA plates containing 400 μg/ml Cu^{2+}. Thereafter isolates were proceeded as mention above.

3.6.3 Stability Test of Copper Resistance in The Selected Bacterial Isolates

Each time of subculturing of the seven selected bacterial isolates, i.e., CuR-4, CuR-14, CuR-24, CuR-25, CuR-32, CuR-38 and CuR-40, on TSA containing small amount of Cu (400 μg/ml), resistance to the highest copper concentration (700 μg/ml) of those isolates were tested for at least 20 times of subculturing.

3.6.4 Copper Resistance Test

Dilution (1:400) of 24-hr. culture (grow in 1/3 strength TSB in 37°C) of the test organisms were placed in the well of a multipoint inoculator device. The inoculator was used to transfer the culture to the control plates (1/3 strength TSA and 1/3 strength TSA containing 400 μg/ml Cu) and to the copper plates (1/3 strength TSA plus 500, 600, 700, 800, 900 and 1,000 μg/ml of Cu^{2+}, respectively). The inoculum was about 10^3-10^4 organisms. The results were read after 24-hr. incubation at 37°C. Growth on both control and test plates was taken as resistance to the test concentration of copper. At least 3 tested sets of replicate were performed in all experiments.

3.6.5 Identification of Selected Bacterial Strains

Seven of 350 bacterial strains were found to be resistant to 700 μg/ml Cu, the highest concentration of copper. After primary examining, i.e., sticking appearance in liquid or semisolid media, exopolysaccharide production and spore formation by Indian ink staining, it was found that only 2 of 7 highest resistant strains should be further processed. Each of them was 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. Thereafter, the expected genus or genera of selected isolates were confirmed by biochemical tests, comparing with bacterial reference strains.

3.6.6 Effect of Copper Concentration on pH Values of Culture Medium

The actual amounts of copper, i.e., 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1,000 μg/ml, were individually added into TSB, TSA and distilled water. The pH values of each copper concentration were determined. The results were shown in **APPENDIX D**. In TSB, TSA and distilled water, no copper ion added, the pH values of all were neutral (7.1). When 100 μg/ml Cu was added in each test solution, the significant reduction of pH values immediately occurred (from neutral to 5). The pH of TSB, TSA and distilled water was about 4, when copper ions were added more (200-300 μ/ml), and the pH was 4 in all test solutions containing 400 μg/ml Cu individually. Thereafter, adding more Cu ions (500-1,000 μg/ml), the pH values of TSB, TSA and distilled water were about 4, no pH reduction, e.g., at 1,000 μg/ml Cu, the pH values were 3.9, 3.9 and 4, respectively. No pH adjustment is needed because the higher in the amount of copper ions added, the lower in the pH values of the culture media and distilled water occurred, spontaneously, if neutral pH was adjusted in any solution, the precipitation of copper was detected. It may imply that any kind of bacteria being able to grow in the medium containing at least 400 μg/ml seems to be either tolerant or acidophilic.

3.7 EFFECTS OF SOME GROWTH FACTORS ON COPPER-RESISTANT BACTERIAL ISOLATES

3.7.1 Effect of pH

Two selected bacterial strains were cultivated separately in 5-ml TSB culture tubes. The pH values of the culture medium were

adjusted to be 4, 5, and 6 by adding of 0.1 N HCl and 8, 9 and 10 by adding 0.1 N NaOH. Certain amounts of the test organisms were inoculated into those sterile TSB in culture tubes, and then incubated at 37°C for 24 hours. The number of each test organism was determined by viable count method. The highest amount of the test 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 TSB was adjusted to 7. Certain amounts of the test organisms were inoculated into those sterile TSB in culture tubes and then incubated for 24 hr. at different temperature, i.e., 30°, 37° and 40°C. 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.

3.8 EFFECT OF COPPER ON GROWTH RATE OF THE SELECTED BACTERIAL STRAINS

Determination of the effect of Cu on growth rate and EPS production of the selected bacterial strains was performed. Certain amounts of each of the test organisms, i.e., the first and the second condition, the pH values of the culture medium were adjusted to be 4 and 7, respectively, and the third condition, 700 μg/ml Cu was added into the culture medium (pH of medium was 4, spontaneously). After inoculation of the test organisms in sterile 250-ml culture flask containing 125-ml sterile culture medium, they were incubated at 37°C for 24 hr. The growth of bacteria or turbidity increase was determined by

spectrophotometer (measured the absorbance at wavelength 500 nanometer, nm).

3.9 EXTRACTION OF EXOPOLYSACCHARIDE

After incubation at 37°C, for 48 hr., the test organisms or selected isolates in TSB (pH 7) were harvested by centrifugation at 8,000xg for 10 minutes, and washed three times by normal saline. The test organisms were suspended in phosphate buffer saline containing 0.5% formaldehyde. Bacterial cells were removed by centrifugation again at 10,000xg for 10 minutes. Ice-cold acetone was added to the supernatant to precipitate the dissolved exopolysaccharide. Frequently, the precipitation was complete in 18 hr at 4°C. The precipitation was recovered by low-speed centrifugation, and washed several times by acetone, dissolved in water and dried in the oven at 105°C for 4 hr. (Hancock and Poxton, 1988).

3.10 EFFECT OF SOME ENVIRONMENTAL FACTOR ON EXOPOLYSACCHARIDE PRODUCTION

3.10.1 Effect of pH, Temperature and Incubation Period

The procedures of those effects were done similarly to the previous procedures (**3.7 in this chapter**), i.e., the effect of pH and temperature. The effect of the incubation period was conducted by observing the growth of the test organisms or the selected bacteria strains in sterile TSB (pH 7) and incubated in various duration, i.e., 24, 48 and 72 hr. at 37°C. The quantities of exopolysaccharide were determined. The detail of EPS extraction was shown (3.11 in this chapter).

3.10.2 EFFECT OF COPPER ON EXOPOLYSACCHARIDE PRODUCTION

The selected bacterial isolates were individually inoculated into sterile 250-ml culture flask containing 125-ml sterile TSB. Various amounts of copper, i.e., 0, 10 and 20 μg/ml were added into the culture medium. They were incubated at 37°C for 48 hr. (the best incubation period). After incubation, the quantities of EPS were investigated. And also, the detail of EPS extraction was shown (3.11 in this chapter).

3.11 ACCUMULATION OF COPPER AND OTHER HEAVY METALS

3.11.1 Contact Time of Copper Accumulation by EPS

The highest Cu accumulation on exopolysaccharides at the shortest time of the interaction was performed. Copper (500 μg/ml) and EPS 1.0 g dry weight/L were mixed and stirred continuously. Contact time or exposure times are 15, 30, 45 and 60 min. Centrifugation (10,000xg for 10 min.) was used to separate EPS from the metal solution. The amount of Cu accumulated by EPS was determined by measuring the residual copper concentrations in the supernatant, that the analyses were performed by AAS.

3.11.2 Effect of Copper Concentrations on Copper Accumulation by Exopolysaccharide

The effect of them was studied by adding equal amounts of EPS (1.0 g dry weight/L) to solution with different copper concentration with continuous stirring, various from 100-700 μg/ml. At the end of exposure time (15 min.), centrifugation 10,000xg for 10 min. was the

separation method. The supernatant was decanted and used for analysis of remaining copper by AAS.

3.11.3 Comparison the Copper Accumulation by Exopolysaccharide and Whole Cells

The strains Cu R-38 and Cu R-40 were inoculated in 250-ml flasks which containing 125-ml of TSB medium at 37°C for 48 hr. A sample was pipettes out of the solution. In one experiment whole cell were centrifuged at 10,000xg for 10 min. to separate from TSB medium and wash three times with distilled water. In the second case EPS were isolation from cell by the method mentioned above. Quantification of EPS and whole cells was by means of wet weight determination in this experiment.

The biosorbent (EPS and whole cell) was added to the metal solution at room temperature. The mixture was equilibrated for 15 min. with continuous stirring. The accumulation phase was terminated by centrifugation at 10,000xg for 10 min., which thoroughly separated the biosorbent from the metal solution. The amount of copper accumulated by the biosorbent was determined by measuring the residual copper concentration in the supernatant. The analyses were performed by AAS.

3.11.3 Comparison the Copper Accumulation by Whole Cells, Exopolysaccharide and Cells without Exopolysaccharide

The strains Cu R-38 and Cu R-40 were inoculated in 250-ml flasks which containing 125-ml of TSB medium at 37°C for 48 hr. A sample was pipettes out of the solution. In one experiment whole cells were harvested from TSB medium by centrifuged at 10,000 x g for 10

min. In second case EPS were isolation from cell by the method mentioned above, for cell were collected after EPS isolation process. All of samples were dry at 105°C for 4 hr. Quantification of sample was by means of dry weight determination in this experiment.

The whole cells, EPS and cells without EPS (same equal amount) were added to the metal solution. After 15 min., The mixture was separate by centrifugation (10,000 x g for 10 min. The remained coppers in the supernatant were investigated by AAS.

3.11.5 Accumulation of Copper and Other Heavy Metals by Exopolysaccharide

In this study, the accumulation of each metals (Cu, Zn, Mn and Cd). The concentration of EPS was 1.0 g (dry wt/L) added to each metals solution at room temperature. After 15 min., the mixture was separate by centrifugation at 10,000xg for 10 min. The residual metals were quantified using AAS.

The concentrations of EPS was 1.0 g (dry wt/L) added to mixed metals solution, which containing Cu, Zn, Mn and Cd (112, 114, 109 and 111 μg/ml, respectively, of each individual metal). Exposure time was 15 min. The residual metals were quantified as mention above.

3.11.6 Recovery of Metals

EPS (final concentration of 1.0 g dry wt/L) was added to a metal solution (the concentration of each metal 100 μg/ml) at room temperature. A withdrawn sample was centrifuged and the supernatant was analyzed with respect to remaining metals. The mixture was then

adjusted to pH 3.0 by adding 0.1 M HCl. After exposure (30, 60 and 120 min.) a withdrawn sample was treated as described above.

3.11.7 Efficiency of Exopolysaccharide after Regeneration

Initial copper concentration was 100 μg/ml. Mixing was achieved by continuous stirring. EPS was adding to a final concentration 1.0g. After equilibrium (15 min.), 40 ml of slurry was centrifuged (10,000 x g for 10 min.). The supernatant was discarded. Distilled water was added to sediment to restore the initial volume. The HCl (0.1M) add to the slurry (final pH 3.0). After 120 min, the sample was analyzed with respect copper. The residual pellet from the experiment described above was added in the solution containing copper (100 μg/ml). This was done in order to determine whether this EPS could be used for reaccumulation of copper. Exposure, separation and analysis of copper concentration were performed as described previously.