CHAPTER 3

MATERIALS AND METHODS

3.1 SOURCES OF MICROORGANISMS

3.1.1 SAMPLES

Chromium-resistant bacteria, phenol-resistant bacteria and chromium/phenol-resistant bacteria were isolated from fifty samples collected from various sites, i. e., plating industry, painting, wood preserving, oil refining, chemical production, metal processing plant, laboratory, dump and natural sources, were collected in various forms, i. e., soils, mud, sediments, industrial wastewater, municipal wastewater, natural water and sludges. The samplings were conducted sequentially in 12 months during December 1997 to November 1998 (Grateful thanks to everyone who kindly collected those samples is cordially performed here). The detail of samples was shown in **Appendix A.**

3.1.2 BACTERIAL REFERENCES STRAINS

Bacterial reference strains used in this study were provided by MIRCEN*, Thailand, namely, ***Enterobacter cloacae***, ***Escherichia coli***, ***Klebsiella aerogenes***, ***Proteus vulgaris*** and ***Pseudomonas putida***. They were served as chromium - sensitive strains, phenol - sensitive strains and chromium/phenol - 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, STAINING DYES AND INSTRUMENTS

3.2.1 CHEMICALS

All chemicals used in this study were all analytical grade:

Amino - antipyrine ($C_{11}H_{13}ON_3$, Fluka, Messerschmittstr, Switzerland);

Arsenic oxide ($Na_2HAsO_4.7H_2O$, Hopkin & Williams, England);

Cadmium chloride ($CdCl_2.H_2O$, Merck, Darmstadt, Germany);

Copper sulfate ($CuSO_4.5H_2O$, Merck);

Diphenylcarbazide (1,5-diphenylcarbazide, Merck);

Dipotassium hydrogen phosphate (K_2HPO_4, Merck);

Iodine (May and Baker) and

Manganese sulfate ($MnSO_4.H_2O$, May and Baker, Dagenham, England);

Nickel sulfate ($NiSO_4.H_2O$, May and Baker);

p-Chlorophenol (C_6H_5ClO, Fluka);

p-Cresol (C_7H_8O, Sigma Chemical Co., St. Louis, U.S.A.);

Phenol (C_6H_5OH, Merck);

p-Nitrophenol ($NO_2C_6H_4OH$, Fluka);

Potassium chromate (K_2CrO_4, Seelze-Hannover, Hannover, Germany);

Potassium dichromate (K_2CrO_7, Merck);

Potassium dihydrogen phosphate (KH_2PO_4, Merck);

Potassium permanganate ($KMnO_4$, Merck);

Silver nitrate ($AgNO_3$, Merck);

Sodium azide (NaN_3, Merck);

Sodium chloride (NaCl, Merck);

Sodium hydroxide pellet (NaOH, Merck);

Tri - potassium hexa - cyanoferrate (III) ($K_3Fe(CN)_6$, Merck) and

Zinc sulfate ($ZnSO_4.7H_2O$, Merck).

3.2.2 REAGENTS

All reagents used in this study were also analytical grade or prepared from analytical grade chemicals as mentioned above, i. e.,

ammonium hydroxide (NH_4OH, Merck);

95% ethyl alcohol (Merck);

heavy metal standard solution (Merck);

hydrochloric acid (HCl, Merck);

normal saline (0.85% NaCl);

phosphate buffer (104.5 g K_2HPO_4 and 72.3 g KH_2PO_4 in 1000 cm^3 of distilled water, pH 6.8);

phosphoric acid (H_3PO_4, Merck);

0.1N sodium hydroxide solution (4 g NaOH in 1000 cm^3 of distilled water) and

sulfuric acid (H_2SO_4, Merck).

3.2.3 STAINING DYES

Staining dyes used in this study were also analytical grade, namely, Gram Stain, i. e.,

crystal violet (Merck);

safanin O (BDH Chemical, Poole, England).

3.2.4 INSTRUMENTS

Many instruments were used, i. e.,

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);

high - speed centrifugation (ALC Model 4239 R, Italy);

incubators (Memmert GmbH, Model 700, Schwabach, Germany);

microwave (Sanyo Electric, grateful thanks to Thanes, Co., Thailand for donation to the Department of General Science);

oven (Memmert GmbH, Model 700);

pH meter (Model pH scan 1, Singapore);

refrigerator (Samsung, Korea, grateful thanks to Becthai, Co., Thailand for donation to the Department of General Science);

rotary shaker (Lab-Line Instrument, Melrose Park, Illinois, U.S.A.) and

spectrophotometer (Spectronic® 20 Genesys™, U.S.A.).

3.3 CULTURE MEDIA

3.3.1 GENERAL MEDIA

The formula and preparation of each culture medium were found in **Appendix B**. Those were:

Nutrient agar (NA; Difco Laboratories, Detroit, Michigan, U.S.A.) and

Nutrient broth (NB; Difco).

They were used as liquid medium and semisolid medium, respectively, for general media, and also used for stock medium.

3.3.2 SELECTIVE MEDIA

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

Brain Heart Infusion agar (BHI; Scharlau Chemie, S.A., La Jota, Barcelona, Spain);

Eosin Methylene Blue agar (EMB; Scharlau Chemie);

Escherichia Coli broth (EC broth; Scharlau Chemie);

King A agar (Pseudomonas agar P; Scharlau Chemie);

Lactose broth (LB broth; Difco);

MacConkey agar (M; Difco);

MacConkey-Inositol-Potassium Tellurite agar (MCIK; Difco);

Pseudomonas Selective Isolation agar (PSIA; Difco);

Shigella-Salmonella agar (SS; Difco);

Simmons Citrate Medium (Scharlau Chemie);

Sulfate Reducing broth (Scharlau Chemie) and

Three Sugar Iron agar (TSI; Scharlau Chemie).

3.3.3 MEDIUM FOR RESISTANT TEST

Resistance of those selected bacterial strains was determined in either ½ strength NA or ½ strength NB (Bird, et.al., 1985) containing various concentrations of tested metals, i.e., CrO_4^{2-}, Cu, Zn, Cd, Ag, Mn, Ni and As in the forms of K_2CrO_4; $CuSO_4.5H_2O$; $ZnSO_4.7H_2O$; $CdCl_2.H_2O$; $AgNO_3$; $MnSO_4.H_2O$; $NiSO_4.H_2O$; $Na_2HAsO_4.7H_2O$ and C_6H_5OH, respectively.

3.3.4 MEDIUM FOR EFFICIENCY TEST

Basal Mineral Medium (g in 1 L): 1.023 NH_4Cl, 0.235 NaH_2PO_4, 1.250 K_2HPO_4, 0.070 $MgSO_4.7H_2O$, 0.014 $CaCl_2$, 0.001

$FeCl_3.6H_2O$; pH 7 and add phenol 300 μg/ml as carbon and energy source (Shen and Wang, 1995a).

3.4 STAININGS 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 morphology by microscope.

3.5 BIOCHEMICAL TESTS FOR IDENTIFICATION

Some biochemical tests were done, i. e., citrate, gelatin, indole, KCN, motility, MR-VP, nitrate, oxidase, phenylalanine deamination and urease, for identification, ***Enterobacter cloacae***; ***Escherichia coli***; ***Klebsiella aerogenes***; ***Proteus vulgaris*** and ***Pseudomonas putida***. Formula, preparation and procedure of some biochemical tests were found in **Appendix C**.

3.6 BACTERIOLOGICAL PRECEDURES

3.6.1 SAMPLING AND CULTIVATION PROCEDURES

3.6.1.1 Sampling Procedures

Samples of soils, water (natural water and wastewater), sludges and sediments were collected from certain sites that were expected to be contaminated by heavy metal(s) and/or chemical(s) and also from natural sources. At each sampling site, three replicates (½ kg and/or ½ L for one replicate) were conducted. Those samples were preserved at low temperature (4-6°C) in the icebox, then transport to the laboratory and stored in the refrigerator. Isolation of chromium-resistant bacteria, phenol-resistant bacteria and chromium/phenol-resistant bacteria were performed either in the same or after day of collection.

3.6.1.2 Isolation of Resistant Bacterial Strains

Each sample of soils or sludges or sediments was serially diluted in normal saline and then spreaded on 3 sets of ½ strength NA plates; i. e., that containing 600 μg/ml Cr(VI), containing 600 μg/ml C_6H_5OH and containing 300 μg/ml Cr(VI) plus 300 μg/ml C_6H_5OH, respectively (Shen and Wang, 1995a). After 24-hr. incubation at 37°C, isolated colonies were individually picked and purified for at least twice and then stabbed in the stock culture tubes, incubated in the refrigerator. Those bacterial isolates or strains were further proceeded.

3.6.1.3 Resistance Test of Resistant Bacterial Isolates

Dilution (1:400) of 24-hr. culture (grown in ½ strength NB 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 (½ strength NA and ½ strength NA containing 600 μg/ml) and to the plates (½ strength NA plus 800, 1200, 1600, 2000 and 2400 μg/ml, respectively). The inoculum was about 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. At least 3 tested sets of replicate were performed in all experiments.

3.6.1.4 Identification of The Selected Bacterial Strains

Three of 495 bacterial isolates were found to be resistant to 2400 μg/ml Cr, three strains resistant to 2000 μg/ml phenol and three strains resistant to 1200 μg/ml Cr/phenol, the highest concentration, and then examining i. e., appearance in liquid or semisolid media. Each was tested for some fundamental characteristics, e. g., size, color and appearance of colony; gram staining and morphology of the cells (size

and shape). 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.

3.6.1.5 Stability of the Resistance to Chromium or Phenol or Chromium/Phenol of The Selected Bacterial Strains after Repeated Culturing

Each time of subculturing of the nine selected bacterial isolates, i. e., CrR-2, CrR-14, CrR-15; PhR-26, PhR-33, PhR-64 and CPR-4, CPR-16, CPR-17, on NA containing small amount of concentration (600 μg/ml), resistance to the highest concentration of those isolates were tested for at least 20 times of subculturing.

3.6.1.6 Resistance of The Selected Bacterial Strains to Other Heavy Metals

The selected bacterial isolates were studied resistance to other heavy metals, i. e., $CuSO_4.5H_2O$; $ZnSO_4.7H_2O$; $CdCl_2.H_2O$; $AgNO_3$; $MnSO_4.H_2O$; $NiSO_4.H_2O$; $Na_2HAsO_4.7H_2O$, compared with K_2CrO_4 and C_6H_5OH.

3.6.2 EFFECTS OF SOME GROWTH FACTORS ON THE SELECTED BACTRIAL STRAINS

3.6.2.1 Effect of pH

Nine selected bacterial strains, i. e., CrR-2, CrR-14, CrR-15; PhR-26, PhR-33, PhR-64 and CPR-4, CPR-16, CPR-17 were cultivated separately in 5-ml NB culture tubes. The pH values of the culture medium were adjusted to be 4, 5 and 6 by adding of 0.1N HCl and 8, 9 and 10 by adding 0.1N NaOH. Certain amounts of the test organisms

were inoculated into these sterile NB in culture tubes and then incubated at 37°C for 24-hr. 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.

3.6.2 .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 NB was adjusted to 7. Certain amounts of the test organisms were inoculated into those sterile NB 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.

3.6.3 EFFECTS OF PHENOL ON GROWTH RATE OF THE SELECTED BACTERIAL STRAINS

The selected bacterial isolates, i. e., CrR-15, PhR-26, PhR-33, PhR-64 and CPR-16, were inoculated in 50-ml 0.85% normal saline and phosphate buffer, for control, and 50-ml 0.85% normal saline and phosphate buffer, adding phenol 300 μg/ml, for testing. And then compared between amounts of bacterial cells with concentration of toxic substance on normal saline solution and phosphate buffer solution with normal saline solution and phosphate buffer solution, adding phenol. If bacteria could grow in normal saline solution and phosphate buffer solution, adding phenol showed bacteria could use phenol as carbon and energy source.

3.7 CHEMICAL PROCEDURES

3.7.1 EFFICIENCY OF CHROMIUM DETOXIFICATION AND PHENOL DEGRADATION BY THE SELECTED BACTERIAL STRAINS

3.7.1.1 Incubation Periods and Contact Time

The highest resistant bacterial isolates at the shortest time of the interaction were performed. CPR-resistant bacterial isolate, CPR-16 were mixed into Basal Mineral medium; BM medium, with concentration (300 μg/ml), incubation periods: 6, 12, 24 and 48-hr. Contact time or exposure time was 15, 30, 45 and 60 min, individually. Centrifugation (5,000xg for 5 min.) was used to separate cells from cells-free (supernatant). The amount of chromium detoxification and phenol degradation was determined by measuring the residual concentration in the supernatant, that analyses were performed by spectrophotometer.

The analytical method:

a. Total chromium:
   1. Pipet 50-ml sample into two 250-ml beaker, the first beaker made volume to 100 ml with distilled water;
   2. added conc. NH_4OH, neutral pH (5-7);
   3. added H_2SO_4, pH ~ 4 and excess 1 ml;
   4. boiled on the hot plate;
   5. 2 drops $KMnO_4$, occured the dark red (added until occur the color for 2 minutes);
   6. added 1-ml NaN_3, the dark red disappeared (if the color appeared, will add 1-ml NaN_3);
   7. boiled again about 1 minutes, so cooled;
   8. added 0.25-ml H_3PO_4 and
   9. the sample were further proceeded.

b. Cr (VI)

1. The both beaker were adjusted pH 1.0 ± 0.3 with 0.2N H_2SO_4;
2. dilluted to 100 ml with distilled water;
3. add 2-ml diphenylcarbazide solution, mixed;
4. for 5-10 minutes, detected %Transmittance at 540 nm with spectrophotometer (used distilled water as blank) and
5. compared with the Standard Curve.

c. Standard Curve

1. Prepared the standard chromium solution, i. e., 10, 20, 30, 40, 50 and 60 μg by pipet the stock chromium solution 1 ml = 5 μg Cr; 2, 4, 6, 8, 10 and 12 ml, respectively;
2. added distilled water to 100 ml into the beaker;
3. followed above: a and b;
4. plotted graph between the concentration of chromium (μg) and %T (graph semilog), as shown in **Appendix D** and
5. Calculation

$$\text{Cr, mg/L} = \frac{\text{μg Cr, read from the curve}}{\text{amount of sample (ml)}}$$

(Total Cr and Cr(VI)

$$\text{Cr(III)} = \text{Total Cr} - \text{Cr(VI)}$$

d. Phenol

1. Pipet 500-ml sample, adjusted pH ~ 4 with H_3PO_4;
2. distillation, used 500-ml cylinder keep distilled solution;
3. distilled, stopped when 450-ml distilled solution;
4. added distilled water 50 ml;
5. continue distilled, final amount 500 ml;

6. pipet 100-ml distilled solution into 250-ml beaker;
7. prepared the standard phenol solution; i. e., 0.1, 0.2, 0.3, 0.4 and 0.5 mg in 100-ml volumetric flask, added distilled water to 100 ml (used 100-ml distilled water as blank);
8. put in the 250-ml beaker;
9. added 2.5-ml NH_4OH, adjusted pH 7.9±0.1 with phosphate buffer;
10. added 1-ml 4-aminoantipyrine solution, mixed;
11. added 1-ml $K_3Fe(CN)_6$, mixed;
12. for 15 minutes, read absorbency of the sample and the standard solution compared with blank at 500 nm and
13. Calculation

$$\text{mg phenol/dm}^3 = \frac{\text{mg phenol, read from the curve} \times 1000}{\text{amount of sample (ml)}}$$

or

$$\text{mg phenol/dm}^3 = \frac{\text{mg std. phenol soln.} \times \text{abs. of sample} \times 100}{\text{abs. std. phenol soln.} \times \text{amount of sample (ml)}}$$

3.7.1.2 Effect of Low Concentrations of Chromium Detoxification and Phenol Degradation on Nine Cocultures and Three Single Cultures

Nine cocultures (CrR- + PhR-), i. e., CrR-2+PhR-26, CrR-2+PhR-33, CrR-2+PhR-64, CrR-14+PhR-26, CrR-14+PhR-33, CrR-14+PhR-64, CrR-15+PhR-26, CrR-15+PhR-33, CrR-15+PhR-64 and three single cultures (CPR-), i. e., CPR-4, CPR-16, CPR-17, were mixed into BM medium with various concentrations : 100, 200, 300 and 400 μg/ml, incubation period 6-hr., at contact time 15 min. At the end of contact time, centrifugation 5,000xg for 5 min. was the separation

method. The supernatant was decanted and used for analysis by spectrophotometer.

3.7.1.3 Effect of High Concentrations of Chromium Detoxification and Phenol Degradation on Three Cocultures and Three Single Cultures

Three cocultures (CrR- + PhR-), i. e., CrR-2+PhR-26; CrR-14+PhR-33; CrR-15+PhR-64 and three single cultures (CPR-), i. e., CPR-4, CPR-16, CPR-17 were studied the efficiency of high concentrations was studied by adding equal amounts of organisms (10^8 cells/ml, incubation period 6 hr.) to solution with various concentrations : 500, 1000, 1500 and 2000 μg/ml. At the end of exposure time (15 min.), centrifugation 5,000xg for 5 min. was the separation method. The supernatant was decanted and used for analysis of remaining concentration by spectrophotometer.

3.7.2 EFFECTS OF SOME ENVIRONMENTAL FACTORS ON CHROMIUM DETOXIFICATION AND PHENOL DEGRADATION

3.7.2.1 Effect of pH

Three cocultures (CrR- + PhR-), i. e., CrR-2+PhR-26; CrR-14+PhR-33; CrR-15+PhR-64 and three single cultures (CPR-), i. e., CPR-4, CPR-16, CPR-17 were cultivated in 5-ml BM medium culture tubes. The pH values of the culture medium were adjusted to be 4, 5 and 6 by adding of 0.1N HCl and 8, 9 and 10 by adding 0.1N NaOH. Certain amounts of the test organisms were inoculated into these sterile BM medium in culture tubes and then incubated at 37°C for 24-hr. 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.

3.7.2.2 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 BM medium was adjusted to 7. Certain amounts of the test organisms were inoculated into those sterile BM medium 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.

3.7.3 EFFICIENCY OF PHENOL DERIVATIVE DEGRADATION BY THE SELECTED BACTERIAL STRAINS

Three PhR-resistant bacterial isolates, i. e., PhR-26; PhR-33; PhR-64 and three CPR-resistant bacterial isolates, i. e., CPR-4, CPR-16, CPR-17 were studied degradation to derivative of phenol, p-cresol; *p-chlorophenol* and *p*-nitrophenol, concentration 50 μg/ml, withdrawn 2-ml samples at weekly intervals and stored in the refrigerator. At the end of the experiment (3 weeks), the samples were quantified by high-performance liquid chromatography, with a methanol-water (60:40) mobile phase (Bossert and Young, 1986) flowing through a C18 analytical column at 2 ml/min. A flow through UV detector measured the derivative of phenol at detection wavelength between 270 and 310 nm, retention time 3.5 min (Boyd et.al., 1983).