CHAPTER 3

MATERIALS AND METHODS

3.1 SOURCES OF MICROORGANISMS

3.1.1 Samples

Fifty-five samples were collected during May 1998 to December 1999, from different sites, i.e., smelting area, chemical industry, electronic industry, laboratory and natural sources. Particularly, at arsenic contaminated area, Amphor Ron Phibun, Changwat Nakhon Si Thammarat, it was reported that average value of arsenic contaminated in soil and sediment was quite high 9 μg/ml (Williams et.al.,1996)., and grateful thanks to Khun Janewitt, who kindly collected samples from arsenic contaminated area, Amphor Ron Phibun, Changwat Nakhon Si Thammarat, is cordially performed here. The detail of samples was shown in **Appendix B**

3.1.2 Bacterial References Strains.

Bacterial references strain used in this study were provided by Microbiology Laboratory of Chulalongkorn Hospital, Thailand, namely, ***Bacteroides fragilis*** (ATTC 25285) which was served as arsenic-sensitive strain. For resistance tests, ***Escherichia coli*** and ***Serratia macescens*** as sensitive to arsenic, and other metal ions, that were used for resistance tests of other metal ions.

3.2 CHEMICALS, REAGENTS, GASES and SPECIAL INSTRUMENTS

3.2.1 Chemicals, Reagents and Stains

Chemicals used in this study were all analytical grade, i. e.,

Ammonium chloride, NH_4Cl, Merck, Darmsatadt, Germany;

Boric acid, H_3BO_3, Merck;

Calcium chloride, $CaCl_2.2\ H_2O$, Merck;

Calcium pantothenate, $(C_8H_{16}O_5N)\ Ca$, Merck;

Cysteine hydrochloride, $C_3H_8Cl\ NO_2S.H_2O$, Merck;

Cyanocobalamin, with B_{12}, Roussel Vclaf;

Ferrous chloride, $FeCl_2.4H_2O$, Fluka, Switzerland;

Glucose, Merck;

HEPES, Sigma, S.T. Louis, USA;

Iodine, I_2, May and Baker , Dagenham, England;

Magnesium chloride, $MgCl_2.6H_2O$, Merck;

Manganese chloride, $MnCl_2.4H_2O$, May and Baker;

Nikel chloride, $NiCl_2.6H_2O$, Merck;

Potassium antimonyl tartrate, $C_4H_4KO_7Sb0.5H_2O$, Fluka;

Potassium chloride, KCl, May and Baker;

Potassium dihydrogenphosphate, KH_2PO_4, Merck;

Potassium iodate, KIO_3, May and Baker;

Potassium iodide, KI, Merck;

Pyridoxol hydrodiloride, $C_8H_{12}ClNO_3$, Merck;

Silver sulfate, Ag_2SO_4, Merck;

Sodium arsenate, $Na_2HASO_4.7H_2O$, Hopkin and Williams,Chadwell Health Essex, England;

Sodium arsenite, $NaASO_2$, Hopkin and Williams;

Sodium chloride, NaCl, Merck;

Sodium molybdate, $Na_2MoO_4.2H_2O$, Merck;

Sodium thiosulfate, $Na_2S_2O_3.5\ H_2O$, Merck;

Sodium metabisulfite, $Na_2S_2O_5$, May and Baker;

Sodium sulfide, $Na_2S.9H_2O$, Merck;

Sodium bicarbonate, $NaHCO_3$, Merck;

Sodium hydroxide, NaOH, Merck;

Zinc chloride, $ZnCl_2$, Merck;

Thiamine hydrochloride, $C_{12}H_{18}Cl_2N_4O_5$, Merck;

Reagents i. e. :

Glycerol, $C_3H_8O_3$, Merck;

Sodium lactate, $C_3K_6O_3$, Merck;

Ethanol, C_2H_5OH, Merck;

3.5 N Sulfuric acid, H_2SO_4, Merck;

1M, 0.1M hydrochloric acid HCl, Merck;

0.1M, 1M, 2M Sodium hydroxide NaOH, Merck;

Stains i. e.:

Crystal violet, Merck;

Safranin O, BDH Chemical, Poale, England;

3.2.2 Gases

All gases supplying in to anaerobic system throughout the thesis work which were:

(i) 10% H_2 ,5% CO_2 and 85% N_2 (nitrogen balance)

(ii) 99.99% N_2

The Thai industrial Gases (TIG) limited made them.

3.2.3 Special Instruments

The special instruments used in the thesis work were as follow:

(i) Anaerobic chamber (Anaerobic system Model 1024, dual type, Forma Scientific, Marietta, Ohio, USA);

(ii) Spectrophoto meter (Spectronic 20 Genesys, USA);

(iii) 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);

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

(v) Incubator (Memmert G mbH, Model 700, Schwabach, Germany);

(vi) Oven (Memmert G mgH, Model 700);

(vii) pH meter (Model pH scan I, Singapore);

(viii) Low Temperator Incubator

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

3.3 CULTURE MEDIA

3.3.1 GENERAL MEDIA

The culture media for this study used as Freshwater minimal medium, modified from Newman, 1997 by Cysteine HCl 0.05% (w/v) was used as the reductant instead of $PdCl_2$. The formula and preparation of the culture media were found in **Appendix C.** It was used as liquid medium and semi-solid medium for general medium and also used for stock medium.

3.3.2 SELECTIVE MEDIUM

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

Eosin Methylene Blue Agar (EMB), Scharlau Microbiology, Sentmenat, Spain;

MacConkey Agar, Difco;

Nutrient Gelatin, Difco;

Shigella-Salmonella Agar, Difco;

Simmons Citrate Medium, Difco and;

Triple Sugar Iron Agar (TSI), Difco.

3.3.3 MEDIUM FOR RESISTANT TEST

Resistance of those bacterial strains was determined in Freshwater minimal medium and Brewer anaerobic medium used as culture media for ***B. fragilis***; arsenic sensitive strain.

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 morphology by microscope.

3.5 BIOCHEMICAL TESTS FOR IDENTIFICATION

Some biochemical tests were done, i. e., catalase, oxidase, indole, H_2S production, gelatinase, motility, urease, MR-VP and OF test.

Formular, preparation and procedure of some biochemical tests were found in **Appendix C.**

3.6 BACTERIOLOGICAL PROCEDURES

3.6.1 SAMPLING AND CULTIVATION PROCEDURES*

3.6.1.1 Sampling Procedures

Samples of soil, sediment and waters were collected from certain sites that have been contaminated by heavy metal(s) and also from natural sources. At each sampling site, three to five replicates were conducted. Isolation of arsenic resistance bacteria was performed either in the same or after day of collection.

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

3.6.1.2 Isolation of Arsenic-resistance bacteria

First of all, all steps here were done in anaerobic chamber. Each sample of sediments or soil or water was incubated in Freshwater minimal medium containing 700 μg/ml As(v) for six day at 35 °C. After that, each sample was spread on this medium plates containing 700 μg/ml As(v). Later, three to five days of incubation at 35°C, isolated colonies were individually picked and purified at least 2 times and then kept in the stock culture broth as same medium containing 200 μg/ml As (v). Those bacterial isolates were further proceeded.

3.6.1.3 Arsenic resistance test

Again, all steps were done in anaerobic chamber. The agar dilution method with a multiple inoculation system was chosen for the determination of high concentration resistance to heavy metal. (Nieto et. al., 1989) 10% of 4 days culture (grow in minimal medium containing As 200 μg/ml in 35°C.) of the test organisms were placed in the well of a multi-inoculator device. The inoculator was used to transfer the culture to control plates (Brewer anaerobic agar) and to the arsenic plates (minimal medium plus 800-2,400 μg/ml of As(V) incrementing by roughly 200 μg/ml of As(V). Average of each initial inoculum was about $3.8x10^{4}$ to $4.0x10^{5}$ cells/ml. Besides, all strains were tested resistance to arsenite As (III)at level of concentration as 500 μg/ml. The results were read after 3-4 days incubation at 35°C. At least 3 tested sets of replicate were performed in all experiments.

3.6.1.4 Precipitation of arsenic sulfide form test

All of isolated bacterial strains were tested the capability of precipitate as arsenous sulfide form. All steps were done in anaerobic chamber 10% inoculum of each bacterial strains were incubated in freshwater minimal medium plus 10 mM Na_2SO_4 . After several days, a yellow precipitation formed and was determined to be arsenous sulfide form. The examination was observed from disappearing of soluble arsenic in this medium by molybdenum blue method and random samples. X-ray diffraction were used for confirming the existing precipitate arsenic.

3.6.1.5 Identification of the selected bacterial strains

Three of the 219 isolated bacteria were found to be resistance to As(V) higher than 2,400 μg/ml. One of them was able to precipitate arsenic as arsenic sulfide form and two bacterial strains show that arsenic precipitation occurred by working together as consortium. They were chosen as the selected bacterial strains were test for some fundamental characteristics, e. g., size, color and appearance of colony, gram-staining and morphology of cell (size and shape). And then some biochemical tests were further proceeded.

3.6.2 RESISTANCE TO OTHER METALS

Resistance to other metal ions by selected bacterial strains was determined. Those were i. e., Cd (II), Cr(VI), Cu(II), Ni(II), Mn(II), Ag(I) and Zn(II) in the forms of $CdCl_2.H_2O$; K_2CrO_4; $CuSO_4.5H_2O$; $NiSO_4.H_2O$; $MnSO_4.H_2O$; $AgNO_3$ and $ZnSO_4.7H_2O$ at concentration of these metal ions as 100,200,400 and 800 μg/ml. Due to bacterial consortium AsR-19 and AsR-20 as facultative anaerobic bacteria, so both strains were tested

the anaerobically in 1/10, strength TSB plus each concentration of those metal ions. The procedure was performed similar to arsenic resistance test as mention in **section 3.6.1.2.**

3.6.3 EFFECT OF SOME ENVIRONMENTAL FACTORS ON GROWTH OF THE SELECTED BACTERIAL STRAINS

3.6.3.1 Effect of pH

Effect of pH on growth of 3 selected bacterial strains was tested. 10% inoculum (initial amount of each strain were 1.2×10^7 cells/ml for AsR-17 and 1.0, 2.0×10^6 cells/ml for AsR-19 and AsR-20, respectively) of these bacterial strains were cultivated separately in 50 ml of freshwater minimal medium. The pH ranged from 5.0-9.0 as set by adding of 0.1 M HCl or 0.1 N NaOH to medium under nitrogen supply. The pH of the media was measured after autoclaving. Culture medium was incubated at 35°C for 1-2 days. The number of each tested organism was determined by viable count method.

3.6.3.2 Effect of temperature

The procedure for study the effect of temperature was performed similar to the effect of pH, but the pH value of the freshwater minimal medium was adjusted to optimum pH from **section 3.6.3.1.** The temperature range studied was 25-45°C incrementing by roughly 5°C. Again, the number of each test organism was determined by viable count method.

3.7 CHEMICAL ANALYSIS PRECEDURES

3.7.1 EFFECT OF SOME FACTORS ON PRECIPITATION AS SULFIDE FORM IN THE SELECTED BACTERIAL ISOLATES

3.7.1.1 Effect of arsenic concentration

Optimum concentration of arsenic was tested in sterile freshwater minimal medium containing 100, 200 and 300 μg/ml by adding to sterile medium. The optimum pH of the medium was adjusted to 7, and anaerobically incubated at 35°C for 8 days. By spectrophotometric assay the concentration of soluble arsenic, both arsenate, As(V) and arsenite, As(III) (at least three replicates) were determined by molybdenum blue method (Johnson, and Pilson, 1972), and concentration of sulfide was examined spectrophotometrically by method of Cline (1969) from at least 5 replicates of AsR-17 and AsR-19/AsR-20 consortium, at different time interval, i. e., day 0, day 4 and day 8.

Analytical method by the molybdenum blue spectrophotometric assay; total arsenic (oxidized sample), arsenate (untreated sample), and background phosphate (reduced sample) were measured directly and arsenite was indirectly determined by measuring the difference between the oxidized and untreated samples. Determination of soluble sulfide was performed by spectrophotometric method. Procedures of determination of those methods were shown in **Appendix E.**

3.7.1.2 Effect of pH

Optimum pH for sulfide precipitation by selected bacterial strains was tested. The pH range tested was 6, 7 and 8 as set by additions of 0.1 M HCl or 0.1N NaOH to freshwater minimum medium, and after autoclaving arsenic (in arsenate form) was supply individually in the culture of AsR-17 and AsR-19/AsR-20 i. e., 100 and 200 μg/ml, respectively. Both of arsenic resistant strains were incubated at 35°C for 8 days. The concentration of soluble arsenic and sulfide were quantified as mention above.

3.7.1.3 Effect of temperature

Again, the procedure for study the effect of temperature was performed similarly to the effect of pH, but the pH value of this medium was adjusted to optumum pH of the tested organisms. Optimum temperature tested was 30, 35 and 40°C. The concentration of soluble arsenic and sulfide were measured as mention above.