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APPENDIX A

BACTERIAL SOURCE

In this present study. 61 samples collected from different site. These samples were collected during December, 1998 to June, 1999.and were categorized into various groups as follows:

Type of samples	Site	Characteristic
Municipal waste (sediment and waste water)	-M1 -M2 -M3 -M4	-Sand and mud -Mud+humus, black -Mud+humus, black -Mud+water, black
Natural water	-N1 -N2 -N3	-Turbidity -Black water -Turbidity
Sediment	-S1 -S2 -S3 -S4 -S5 -S6 -S7 -S8 -S9 -S10 -S11 -S12 -S13 -S14 -S15 -S16 -S17 -S18 -S19 -S20 -S21 -S22 -S23 -S24 -S25 -S26	-Sand+clay, black and odor -Clay+mud, black -Clay+mud, brown -Clay+sand, brown -Sand+clay+humus,black and odor -Mud,black and oil odor -Mud,black and odor -Clay+sand, brown -Clay+mud, almost black -Clay+sand, black -Sand+humus -Clay, brown -Mud,black and odor -Clay+sand, brown -Sand+clay, black -Mud, black -Mud, black -Mud, black -Mud, black -Mud, brown -Mud, brown -Sand, black -Sand, black -Sand, black -Sand, black -Mud, brown

APPENDIX A (CONT.)

Type of samples	Site	Characteristic
Sediment	-S27	-Sand+humus, black
	-S28	-Sand+clay, black
	-S29	-Sand+clay, black
	-S30	-Clay+sand, black
	-S31	-Clay+sand, black
	-S32	-Clay+sand, black
	-S33	-Sand, brown
	-S34	-Sand, brown
	-S35	-Clay+sand, black
	-S36	-Clay+sand, black
	-S37	-Clay+sand, black and oil odor
	-S38	-Sand+clay, black
	-S39	-Sand+clay, brown
	-S40	-Clay, black
	-S41	-Clay+sand, brown
	-S42	-Clay, black and oil odor
	-S43	-Clay, brown
	-S44	-Sand, black
	-S45	-Clay+sand, black and oil odor
	-S46	-Clay+sand,brown and black
	-S47	-Mud, black
	-S48	-Humus, black
Sludge (Sl)	-Sl_1	- Sludge, brown
Soil (So)	-So1	-Clay, brown
	-So2	-Clay, brown
Industrial waste water (W)	-W1	-Browny clear water+black sediment
	-W2	-Browny clear water
	-W3	-Blackly clear water+ humus

APPENDIX B

MEDIA

Basal salt medium (Nakamura, 1986)

NaCl	5g
KCl	0.7g
$MgCl_2.6H_2O$	10.8g
$MgSO_4.7H_2O$	5.4g
$CaCl_2.2H_2O$	1g
Yeast extract	1g
Bacto peptone	5g
Agor	15g

Dissolve each of them in distilled water.

Eosin Methylene blue Agar (E.M.B.Agar)

Gelatin peptone	10g
Lactose	10g
Dipotassium phosphate	2g
Eosin yellow	0.4g
Methylene blue	0.065g
Agar	15g

PH 7.1 (approx)

Directions:

Suspend 37.5 in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°c for 20 minutes.

MacConkey Agar

Bacto peptone	17g
Bacto proteose peptone	3g
Bacto Lactose	10g
Bacto Bile Salts No.3	1.5g
Sodium chloride	5g
Bacto agar	13.5g
Neutral Red	0.03g
Bacto Crystal Violet	0.001g

Final pH 7.1± 0.2 at 25°C

Directions

Suspend 50 grams in 1 liter distilled or deionized water and buil to dissolve completely. Sterilize at 121-124°c for 15 minutes. Avoid overheating.

Nutrient broth

Bacto Beef Eztract	3g
Bacto Peptone	5g

Final pH 6.8±0.2 at 25°C

Dissolve 8 grams in 1 liter distilled or deionized water. Sterilize at 1221-124°c for 15 minutes.

Nutrient Gelatin

Bacto Beef Extract	3g
Bacto Peptone	5g
Bacto Gelatin	120g

PH 6.8 at 25°c

To rehydrate the medium, suspend 128 grams in 1000 ml. cold distilled water. Warm to about 50°c to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°c)

Simmons Citrate Agar

Magnesium Sulfate	0.2g
Ammonium Dihydrogon Phosphate	1g
Diyotassium Phosphate	1g
Sodium Citrate	2g
Sodium Chloride	5g
Bacto Agar	15g
Bacto Brom Thymol Blue	0.08g

Final pH 6.8 at 25°c

Directions

To rehydrate the medium, suspend 24.2 grams in 1000ml.cold freshly distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°c)

SS Agar

Bacto Beef Extract	5g
Bacto Proteise Peptone	5g
Bacto lactose	10g
Bacto Bile Salts No.3	8.5g
Sodium citrate	8.5g
Sodium thiosulfate	8.5g
Ferric citrate	1g
Bacto agar	13.5g
Brillant green	0.33g
Neutral Red	0.025g

Final pH 7.0±0.2 at 25°c

Directions

Suspend 60 grams in 1 liter distilled or deionized water and boil carefully for no more than 2-3 minutes to dissolve completely. Avoid overheating. DO NOT AUTOCLAVE.

Triple sugar iron agar

Bacto Beef extract	3g
Bacto Yeast extract	3g
Bacto Peptone	15g
Proteose Peptone,Difco	5g
Bacto Dextrose	1g
Bacto Lactose	10g
Sauharose,Difco	10g
Ferrous Sulfate	0.2g
Sodium chloride	5g
Sodium thiosulfate	0.3g
Bacto agar	12g
Bacto Phenol Red	0.024g

Final pH 7.4 at 25°c

To rehydtate the medium suspend 65 grams in 1000 ml.cold distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 pound pressure (121°c). Allow the tubes to solidify in a slanting position in a manner, which will give a generous bult.

Tryptic soy broth

Bacto Tryptone Pancreatic Digest of Casein	17g
Bacto Soytone Papaic Digest of Soybean Meal	3g
Bacto Dextrose	2.5g
Sodium Chloride	5g
Dipotassium Phosphate	2.5g

Final pH 7.3±0.2 at 25°c

Suspend 30 grams in 1 liter distilled or deionized water and warm slightly to dissolve completely. Sterilize at 121-124°c for 15 minutes. To prepared 1/10 TSB: dissolved grams in 1 liter distilled of deionized water.

APPENDIX C

THE DETERMINATTION OF MERCURY WITH DITHIZONE

Reagents

Dithizone stock solution Chloroform	: Prepare a 0.05 % W/V solution in
Dilute dithizone	: Dilute 2 ml of the stock solution in 100 ml with chloroform
Hydrochloric acid 0.1 N water	: Prepare 8.28 ml HC1 in 250 ml deionized
Sodium nitrite solution	: Dissolve 12.42g $NaNo_2$ in 250 ml deionized water
Urea solution	: Dissolve 25.08g $Co(NH_2)_2$ in 250 ml deionized water
EDTA solution	: Dissolve 6.25 g EDTA (disodium salt dihydrate) in 250 ml deionized water
Acetic acid solution 4N	: Prepare 14.28 ml CH_3COOH in 250 ml deionized water
Standard mercury stock solution	: Dissolve 0.0135 g of mercuric chloride in 100 ml of 0.1 N hydrochloric acid 1 ml of Solution ≡ 100 μof mercury
Hydroxylammonium chloride solution	: Preparre a 20 % W/V solution in deionized water

Produre

Separation and determination of mercury

I) Transfer the solution to a separating funnel of suitable capacity.

II) Add 10 ml of dilute dithizone, shake foe 1 minute, allow to separate, run the lower layer into the second separating funnel.

III) Continue the extraction with successive 1ml portions of dithizone solution until two successive extracts remain green, and combine the extracts in the second separating funnel.

IV) Add 10 ml of 0.1 N HC1 and 1 ml of $NaNo_2$ solution, shake 1 minute

V) Discard the lower layer

VI) Add 1 ml of hydroxylammonium chloride solution and set aside for 15 minutes, shaking occasionally.

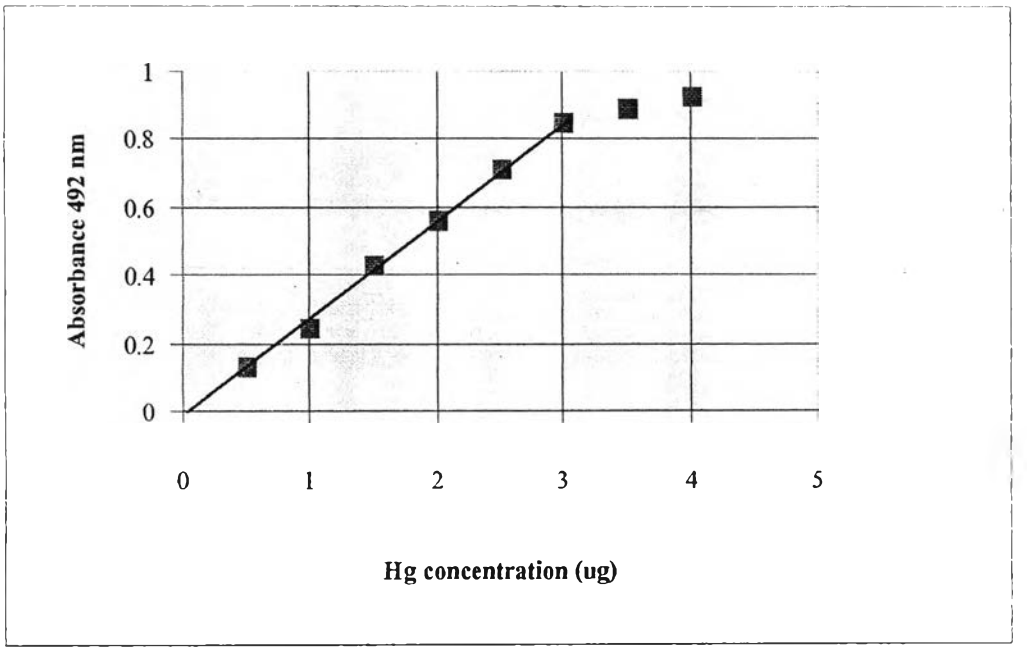
VII) Add 1 ml of urea solution and 1 ml of EDTA solution
VIII) Add 0.5 ml of dilute dithizone from a 10 ml burette, shake 10 seconds
IX) Run the lower layer into another separating funnel containing 5 ml of 4 N acetic acid, repeat the operation until the separated layer is greenish-orange; shaking time 30 seconds and dithizone solution reduced to 0.2 ml.
X) Continue the titration and separation, combining the extracts, until the organic layer has a greyish mixed color: note the volume of dithizone solution required.
XI) Adjust the volume of the extract to 4.0 ml with chloroform.
XII) Run the lower layer into a 1 cm glass spectrophotometer cell.
XIII) Measure absorbance at 492 nm.

Preparation of calibration graph

I) Transfer aliquots of dilute standard mercury solution to cover the range 0.5 to 10.0 μg of mercury to a series of separating funnels.
II) Add 10 ml of 0.1 N HC1.
(Transfer 10 ml of 0.1 N HC1 to another separating funnel to be used as a blank solution)
III) Add 1 ml of $NaNo_2$ solution and 1 ml of hydroxylammonium chloride solution mix, and sit aside for 15 minutes.
IV) Add 1 ml of urea solution and 1 ml of EDTA solution and follow VIII) to XIII) as described under "separation and determination of mercury".

Reference : Johnson, W.C., Gage, J.C., Gorsuch, T.T., Johnson, E.I., Johnson, E.M., Milton, R.F., Newman, E.J., Sharples, W.G., Thackray,G.B., Tyler, J.F.C. and Shallis, P.W. 1965. The Determination of Small Amounts of Mercury in Organic Matter. Analyst, 90: 515-530.

Standard curve of mercury concentration by colorimetric method with dithi zone



SLOPE = 0.2437

APPENDIX D

Some characteristic of the selected bacterial isolates; HgR-11 and HgR-14

Some characteristics	The selected bacterial strains	
	HgR-11	HgR-14
1.Source of sample	Sediment 42	Sediment 44
2.Type of organism	Aerobe	Aerobe
3.Colony	~2-3 mm, entire, circular	~2-3 mm, entire, circular
4.Morphology	Rod, Gram-negative, 0.6 by 0.4 μm.	Rod, Gram-negative, 0.6 by 0.4 μm.
5.Expected genus	***Acinetobacter*** sp.	***Acinetobacter*** sp.
6.Maximum resistance to mercury (μg/ml)	250	250
7.Maximum resistance to other metals (μg/ml)		
-Ag	less than 100	less than 100
-Cd	less than 100	less than100
-Cr	200	100
-Cu	200	200
-Mn	800	800
-Ni	100	100
-Zn	400	200
8.Optimum for growth		
-pH	8	8
-Temperature	35°C	35°C
9.Type of organism to volatilization	by itself	by itself
10.Condition for volatilization		
-pH	7-9	7-9
-Temperature	25°-40°C	25°-40°C
11.Percentage of mercury removal		
-pH	~98-99%	~98-99%
-Temperature	~98-99%	~98-99%
12.Percentage of mercury removal by trap solution in 5 day	95.38%	98%

The percentage of mercury removal by bacterial compared to the former investigations.

Organisms	Max. Resist[a] µg/ml	Initial [b]Conc. of Hg (µg/ml)	% removal	Product	Ref.
Pseudomonas sp.	450	NI[c]	ND[d]	Hg(0)	Tonomura, 1968
Escherichia coli	NI	10	95	Hg(0)	Summers, 1972
Bacillus sp.	50	20	ND	Hg(0)	Timoney, 1978
E.coli KP245 pRR130	NI	70	98	Hg(0)	Hansen, 1984
Azotobacter chroococccum	NI	12.5	64	Hg(0)	Ghosh,1996
Azotobacter beijerinckia	NI	12	79.92	Hg(0)	Ghosh,1997
P. putida FB1	NI	NI	99.2-99.8	Hg(0)	Baldi, 1993
P. aeruginosa PU21	NI	NI	>80	Hg(0)	Chang,1998
Acinetobacter sp.	**250**	**50**	**98-99**	**Hg(0)**	**This study**
Acinetobacter sp.	**250**	**50**	**98-99**	**Hg(0)**	**This study**

a: max.resist.=maximum resistance
b: Initial Conc.= Initial concentration of mercury
c: NI= no information
d: ND= not determine

BIOGRAPHY

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