

5.1. INTRODUCTION:

The study of the interactions between heavy metals and microorganisms has been specially focused on bacterial transformation and conversion of metallic ions by reduction in different polluted environments (Chang et al., 1993), the selection of metal-resistant microorganisms from polluted environments (Hiroki, 1994), and the use of resistant microorganisms as indicators of potential toxicity to other forms of life (Doelman et al., 1994) as well as on mechanisms, determinants and genetic transfer of microbial metal-resistance (De Rore et al., 1994). Many heavy metal ions are “soft” Lewis acids, which mean that their affinity for soft donor atoms such as phosphorus and sulfur are considerably higher than for hard donor atoms such as nitrogen and oxygen. Examples of soft metal ions are Hg^{2+} , Pb^{2+} , Cd^{2+} , Cu^{2+} , Ag^+ , Au^{3+} , Pt^{4+} , and Tl^+ . Many soft metal ions are of concern because they are highly toxic and are present in a variety of waste streams that can potentially contaminate the environment if released. Safe, efficient, and cost effective separation and recovery of these toxic metals are of great concern in modern day environmental scenario.

An inherent problem in bioremediation of toxic wastes is when the heavy metal contamination in the environment, over a period of time, leads to reaction with organic moieties. One of the main aims of this study is concerned with MRB-mediated removal of heavy metals like Hg, Cd and Pb from aqueous solutions. As heavy metals are not biodegradable in the same sense as carbon-based molecules, metals, unless removed completely either through precipitation, ligand binding or diffused out from a system, they will persist indefinitely (Roane, 1996). Cadmium, copper, lead, mercury, nickel and zinc are considered the most hazardous and are included on the US Environmental Protection Agency's (EPA) list of priority pollutants (Cameron, 1992). In the US,

37% of the tested sites polluted with organic compounds were also found to be polluted with metals such as arsenic, mercury, lead and zinc (Kovalick, 1991).

Mercury has no known biological function and is highly toxic to humans (Goldwater & Clarkson, 1972). It can exist in three oxidation states viz. elemental mercury, Hg^0 ; mercurous mercury, Hg^+ ; or mercuric mercury, Hg^{2+} , and the latter two can combine with other elements to form either organic or inorganic mercury compounds (Hu, 1998). The distinction between elemental, inorganic and organic mercury is much more important than oxidation states in determining toxicity as, organic mercury compounds are the most toxic (Goldwater & Clarkson, 1972; Hu, 1998). Organic mercury compounds, like the metal, are volatile at room temperature. Some of the compounds that have important industrial applications are those in which mercury is coupled with methyl or ethyl (alkyl mercury compounds), or phenyl groups (aryl mercury compounds). The alkyl mercury compounds are more toxic, particularly methyl mercury, than the aryl mercury compounds (Taylor & Francis, 1995).

Mercury exerts its toxicity by binding its ions to sulphhydryl groups in the body. These groups may be part of some enzymes, structural and functional proteins and hence mercury and its compounds are potent inhibitors of some enzymes (Livingstone, 1972; Taylor & Francis, 1995; Williams, 1999). Mercury also blocks the transport of potassium and sugars into cells. These effects are due to the binding of mercury to the S-H groups in or on the cell membrane. Once inside the cell, it may be sequestered as an inactive combination, or it may react with enzymes or other compounds to elicit toxic effects (Livingstone, 1979). Organic mercury is able to reach the CNS, where it is oxidized (alkyl mercury compounds are demethylated) to Hg^{2+} and leads to neurological damage (Taylor & Francis, 1995). Exposure of the body to mercury in any of its

forms stimulates kidneys to produce metallothionein, a metal-binding protein that affords partial protection against mercury toxicity (Hu, 1998). In recent years, there has been some concern that mercury contained in dental amalgams adversely affects human health, produces illnesses including multiple sclerosis and Alzheimer's disease, but this conjecture has not been conclusively proven (Hu, 1998; Baldwin & Marshall, 1999).

Mercury poisonings have been reported from ingestion of mercuric chloride (an inorganic compound which is used as a disinfectant), as well as from contaminated illegal drugs, for example, amphetamines. Poisoning has also occurred from exposure to fungicides containing organic mercury compounds and from industrial accidents in which mercury vapour was inhaled. Environmental discharges of mercury have also occurred, by discharge of industrial wastes (Livingstone, 1972).

Cadmium is well known for its toxicity, bioaccumulation and biomagnification through the food chain. Cadmium has no essential biological function and is extremely toxic to humans. In chronic exposure, it also accumulates in the body, particularly in kidney and the liver (Goldwater & Clarkson, 1972; Williams et al., 1999). These properties, along with its common usage in Ni-Cd batteries, make cadmium one of the commonest environmental metal poisonings. Acute poisoning from inhalation of fumes and ingestion of cadmium salts can also occur and at least one death has been reported from self-poisoning with cadmium chloride (Baldwin & Marshall, 1999).

Cadmium has a wide variety of sources in the environment and from industry. One source is from ingestion of grown foodstuffs, especially grain and leafy vegetables, which readily absorb

cadmium from the soil. It may also contaminate fish (Hu, 1998; Williams et al., 1999). In addition, being a constituent of alloys, pigments, batteries, metal coatings, plastics and fertilizers, it may occur naturally or as a contaminant. Contaminants such as sewage sludge, fertilizers, polluted groundwater and mining effluents are important sources of Cd. Occupational exposure may occur from the manufacture of these products and from welding, and smelting of lead, zinc and copper as these occur in mixed ores with cadmium. It is also found in cigarette (0.007 to 0.35 µg per cigarette) and vehicular fumes. Residential sites may be contaminated by municipal waste or leaks from hazardous waste sites (Taylor & Francis, 1995; Hu, 1998; Williams et al., 1999).

The toxic effects of cadmium are due to its inhibition of various enzyme systems. Like similar heavy metals, it is able to inactivate enzymes containing sulphhydryl groups and it can produce uncoupling of oxidative phosphorylation in mitochondria (Fassett, 1972). Cadmium may also compete with other metals such as zinc and selenium for inclusion into metallo-enzymes and it may compete with calcium for binding sites on regulatory proteins such as calmodulin (Hu, 1998). In prolonged cadmium exposure, disorders of calcium metabolism occur causing osteomalacia (Hu, 1998; Williams et al., 1999). This leads to painful fractures, hence the name given to the chronic exposure disease in Japan: Itai-itai disease (literally "ouch!-ouch!" disease; Williams et al., 1999; Hu, 1998; Taylor & Francis, 1995). Cadmium is also known to be carcinogenic, and has often been linked with lung and prostate cancers (Taylor & Francis, 1995; Hu, 1998; Williams et al., 1999).

Lead has no known biological function, is highly toxic and accumulates in humans (Williams et al., 1999). People have known about the toxicity of lead for centuries, at least since 200BC, when Dioscorides wrote, "lead makes the mind give way". Despite this, lead has been used extensively

for both industrial and domestic applications for hundreds of years (Baldwin & Marshall, 1999). The major source of lead in the environment is earth's crust. Lead enters the food and water supply (Goyer & Chisholm, 1972; Williams et al., 1999) quite naturally and is absorbed by foodstuffs (particularly green leafy vegetables) growing on soil where lead is present. Contamination from vehicle exhausts or wastes or from the areas naturally high in lead (Hu, 1998) is substantial sources of Pb. Previously, tetraethyl lead was an additive in petrol and lead was used in plumbing (Hu, 1998; Williams et al., 1999). In many countries the use of leaded petrol is being phased out, and lead piping in households is gradually being replaced, due to health concerns. Lead also used to be used in paints and some cases of lead poisoning are due to small children eating flakes of this paint (Taylor & Francis, 1995; Williams et al., 1999). Today, lead is still used in batteries, some insecticides, and is found in cigarette smoke, where there is between 0.017 and 0.98 micrograms per cigarette (Taylor & Francis, 1995; Williams et al., 1999).

Occupational exposure to lead may occur during the manufacture of the products above, but in addition to this lead is mined and smelted (Hu, 1998; Williams et al., 1999). Exposure to organic lead substances such as tetraethyl and tetramethyl lead may occur in some industries (Livingstone, 1979). There may also be exposure in people who mine gold and zinc, as lead is a contaminant of the ore (Williams et al., 1999).

Elemental lead and inorganic lead compounds are absorbed by ingestion or inhalation, but organic lead compounds e.g. tetraethyl lead may also be absorbed by skin contact. Organic lead compounds are the most toxic. Absorption of lead by the lungs is very efficient, especially when the particles are less than 1 micrometre in diameter, as may happen for example with fumes from

burning lead paint. Gastrointestinal absorption of lead varies with the age of the individual; children absorb around 50% of what they ingest, but adults only absorb 10-20% of what they ingest (Taylor & Francis, 1995; Hu, 1998). It also affects sodium, potassium and calcium ATP-ase pumps, which maintain the cells' concentration gradients of these ions. Activities of calcium-dependent intracellular messengers and functions of protein kinase C in the brain are also impaired. Lead also stimulates the formation of inclusion bodies in cells that may translocate the metal into nuclei and alter gene expression (Hu, 1998).

Microbial metal resistance mechanisms include precipitation of metals as phosphates, carbonates and sulfides; metal volatilization by methyl or ethyl group addition; physical exclusion by electronegative components in membranes and extracellular polymeric substances (EPS); energy-dependent metal efflux systems; and intracellular sequestration with low molecular weight, cysteine-rich proteins (Hughes & Poole, 1989; Gadd, 1990; Silver, 1998). In general, while microbial metal resistance includes a variety of strategies to deal with toxic metal concentrations in the environments (Roane et al., 1996), these strategies are either to prevent entry of the metal into the cell or to actively pump the metal out of the cell. Such resistance can be divided into two classes: metal dependent and metal-independent (Roane & Pepper, 1997). Unlike the mercury and arsenic resistance systems that are highly homologous in all bacteria studied, other resistance systems have evolved several times (Silver & Phung, 1996). In addition, many moieties of chromosomal DNA have been shown to be important in resistance to heavy metals. For example, Cánovas et al. (2003) reported that the genome sequence of *Pseudomonas putida* KT2440 has 61 open reading frames (ORFs) involved in different metal tolerance/resistance. Pain et al. (1998) reported that most of the TBT-resistant bacteria are also resistant to six heavy metals (Hg, Cd, Zn, Sn, Cu, Pb), which suggest that resistance to many types of toxicants may be present in the

same organism. Present investigation was planned to address such mechanisms. Several mercury-resistant marine bacteria positive for resistance primarily to toxic mercury and other toxic heavy metals as well as many xenobiotics were chosen and their detoxification of Hg, Cd and Pb. Since these toxicants are present in many aquatic environments, remediation of such environments, or the avoidance of contamination, is an area of active research interest, this study has attempted to understand the potential of MRB in dealing with the above listed toxicants.

5.2. MATERIALS AND METHODS:

5.2.1. Growth of MRB in heavy metals (Hg, Cd and Pb):

MRB were grown in seawater nutrient broth (SWNB; 5g peptone, 5g yeast extract, 500 ml seawater and 500 ml distilled water). An inoculum of 100 μ l was added to 100 ml SWNB to which individual heavy metal salt was aseptically added to a final concentration of 100, 50, or 10 ppm. Hg (as HgCl_2), Cd (CdCl_2) and Pb [$(\text{CH}_3\text{COO})_2\text{Pb}$] were the different salts used for this experiment. Suitable controls were maintained without any addition of heavy metal. The optical density was recorded at specific time intervals as a measure of growth. The OD values were transformed into cell numbers using an empirical formula of $1 \text{ OD} = 9.98 \times 10^9 \text{ cells ml}^{-1}$ (Ramaiah, 1989).

5.2.2.1. Detoxification of mercury by volatilization:

Eleven mercury-resistant bacterial isolates viz. GP15 (*A. faecalis*), CM10 (*Bacillus* sp.), CH07 (*P. aeruginosa*), GP08 (*B. iodinium*), GP13 (*B. iodinium*), GO02 (*A. faecalis*), GP16 (*A. faecalis*), GP17 (*A. faecalis*), GP14 (*B. iodinium*), GP06 (*A. faecalis*), CH13 (*B. pumilus*), one mercury-sensitive (unidentified) and *P. putida* KT2442::mer73 (as positive control) were grown

in marine broth for 24 hours and the cells were pelleted by centrifugation at 10,000 rpm. The cells were washed with phosphate buffer and placed in wells of microtitre plates. Mercury stock solution was added to the phosphate buffer to a final concentration of 10 ppm and then added to wells containing the cells. The whole plate was covered with Kodak XAR film and the plate was incubated at 30° C in dark for 4 hours. After incubation, the XAR film was removed and developed in a darkened room.

5.2.2.2. Kinetics of Hg removal:

Figure 5.2.2.1 shows the set up for online system to measure the kinetics of mercury transformation by bacteria. It comprised of an atomic absorption spectrophotometer (AAS), a three-reaction vessels-assembly, one magnetic stirrer, a water bath (with temperature control), and one motor with timer and channels for carrying air and mercury. Accumulated metallic mercury was blown out from time to time into the AAS by airflow through the reaction vessel. A magnetic stirrer was used for complete mixing. The reaction vessels had a volume of 50 ml. Each lid had a plastic tube for inflow and outflow of air and a needle to couple the syringe. Air tubes had unidirectional valves above the reaction vessels so that there was no back-flow into the vessels. Direction of the airflow was always from the reaction vessels directly into the AAS. Therefore the reaction vessel was airtight when the inflow valve was closed. The reaction vessels were maintained at a temperature of 25°C.

5.2.2.2.1. Bacterial cultures:

CH07 (a marine *P. aeruginosa*), *P. putida* (*P. putida* KT 2442 mer::73) and Bro12 (a *P. aeruginosa* isolated from chloralkali wastewater) were grown overnight (approx. 16 hours) in modified M9 minimal medium (10x salts: Na₂HPO₄·7H₂O, 70 g; KH₂PO₄, 30 g; NaCl, 25 g;

NH₄Cl, 10.0 g; distilled water 1 litre), trace elements(MgO, 10.75 g; CaCO₃, 2.0 g; ZnSO₄, 1.44 g; MnSO₄, 1.12 g; CuSO₄, 0.25 g; CaSO₄, 0.28 g; H₃BO₃, 0.06 g; MgSO₄, 120 g; FeSO₄.7H₂O, 10 g; conc. HCl, 51.3 ml; distilled water, 1 litre) and glucose (4g l⁻¹) with 1 mg l⁻¹ HgCl₂ at 30°C on a rotary shaker. “Preculture” was made by adding this overnight grown culture to fresh medium and allowing them to grow to a desired cell density in the exponential phase. This was examined by frequent measurements of OD. For reliable comparison between the experimental runs, adequate care was taken to transfer the precultures at the right time to meet with the rigor that these precultures always came from the same stage of the exponential growth phase.

5.2.2.2.2. Hg biotransformation assay:

For this assay 20 ml of minimal medium (including carbon source) was prepared with the defined mercury concentration to be tested (for example 1, 2, 5, 10, 15 mg Hg l⁻¹). Mercury transformation was allowed to take place in 3 parallel reaction vessels where 600 µl culture containing approximately same cell numbers/protein concentration was added to 5.6 ml minimal medium (M9) amended with defined mercury concentration. Effect of NaCl concentration on Hg removal was done with all three isolates at a fixed Hg concentration of 8 ppm. NaCl concentrations of 5gm l⁻¹ to 30 g l⁻¹ were used for this experiment. A standard solution of 1000 ppm (1 g l⁻¹) Hg(NO₃)₂ was used to prepare different concentrations for calibration curve throughout the experiment.

5.2.2.2.3. Hg Measurement and protein analysis:

Aliquot of 1 ml from the culture was taken simultaneously for protein detection. This sample was centrifuged (13,000 rpm, 5 minutes, 4°C) and the resulting pellet was stored – 20°C till analysis of total protein following Bradford method (Bradford, 1976).

5.2.2.2.4. Calculation:

The highest peak area was taken from each vessel and the background level was subtracted from the last cycle of the pre-blowouts. This value was calculated together with the calibration curve into the total amount of mercury in ng per cycle. The kinetics of Hg biotransformation (v) was expressed as $\text{Hg}^{2+} \text{ cell}^{-1} \text{ min}^{-1}$.

$$v = \text{Hg}^{2+} (\text{ng}) / 0.6 \text{ ml culture} * \text{cells (no. ml}^{-1}) * 3.2 \text{ minutes} = \text{ng Hg}^{2+} \text{ cell}^{-1} \text{ minute}^{-1}$$

5.2.3. Removal of cadmium and lead by MRB:

Two sets of separate experiments were carried out for determining the removal of Cd and Pb. CH07, GP06 were used in the experiment with Cd whereas CH07, GP13 and S3 were used in the experiment with Pb. A sensitive strain CH05 was used in all cases as a negative control.

Once every 24 hour, one ml sample was withdrawn aseptically into two sets. One set of sample was centrifuged at 13,000 rpm for 15 minutes at 24°C. The supernatant was filtered through preweighed 0.22 μ membrane filters and the filtrate was diluted (10 x dilution) with 10% HNO_3 for estimation of the heavy metals (either Cd or Pb) from the medium. The pellets were treated overnight using 1N HCl and further treated including a sonification step twice for 45 seconds followed by centrifugation at 10,000 rpm for 5 minutes. The supernatant was collected and diluted with 10% HNO_3 for estimation of heavy metals (either Cd or Pb) accumulated by the cells. The cell pellets were dried for 48 h at 70 °C and weighed for noting bacterial biomass. The Cd concentrations were determined by inductively coupled plasma -atomic emission spectrometry (ICP-AES) and Pb was measured using atomic absorption spectrophotometry (AAS) following manufacturer's protocols. Their concentrations were calculated using proper

blanks (the growth medium after diluting with 10% HNO₃ in the same way as was done for the samples) and several standards ranging from 5 ppm to 20 ppm were used for calibration.

5.2.3.1. Biomass measurement:

One ml sample was always withdrawn aseptically when taking samples for Hg concentration determination and was centrifuged at 10,000 rpm for 5 minutes at 4° C. The dry weight of the cell mass was used in calculating accumulation of heavy metals per unit biomass and also the removal rate of Cd and Pb by cell mass was calculated using the results obtained from the ICP-AES and AAS analyses.

5.2.4. SEM and EDS analyses:

Ten ml broth culture was centrifuged at 10,000 rpm at 4° C for five minutes. The pelleted cells were washed twice with 0.1 M phosphate buffer solution (PBS; pH 7) and were fixed for overnight in 2% glutaraldehyde (Roane, 1996). The cells were washed again with PBS and then distilled water before further processing. The cells were dehydrated through a series of different concentrations (10% to absolute) of ethanol by holding them in each concentration for half an hour. The cells were fixed onto a brass stub, sputter-coated with gold and scanning electron microscopy (SEM) was carried out. Energy dispersive x-ray spectroscopy (EDS) was carried out to detect Cd and Pb and their compounds adsorbed to the cell surface, precipitated on the cell membrane or entrapped in the extracellular polymeric substances (EPS) produced by the cell.

5.2.5. Protein concentration and profile of MRB exposed to Hg and Cd:

In order to quantify the protein concentration and to qualitatively detect the expression, if any, of metal binding proteins, the following experiments were carried out. Cultures of GP06, GP14,

GP15, GP16, CH07, GO02, were grown in the medium amended either with Hg (1 and 25 ppm) or Cd (10, 50 ppm) with suitable control with no metal solutions added to the medium. Three ml of the broth culture was removed from each flask and centrifuged at 10,000 rpm at 4°C for 5-10 minutes. The supernatant was discarded and 1 ml lysis buffer (QC buffer) was added to the pellet. The pellets were sonicated for a total of 2 minutes (4 times for 30 seconds each time with a gap of 30 seconds between successive sonications). The sonicated sample was centrifuged at 5000 rpm at 4° C for 2 minutes. Two hundred microlitres of this supernatant was incubated with 1600 µl acetone (always stored at -20° C) for 30 minutes at 4° C. The precipitated protein was dried under the clean bench for nearly 20 minutes. Five hundred microlitres PBS was added to this precipitated protein for brining it into solution. The protein was estimated using 1mg ml⁻¹ bovine serum albumin (BSA) with BIORAD protein estimation kits following manufacturer's protocol. The BIORAD protein estimation kit used during the Hg experiment was based on the Bradford method (Bradford, 1976). Whereas, method of Lowry (1970) was followed for protein estimation from experimental cultures exposed to Cd. Equal amount of protein samples were loaded and SDS- PAGE (Lamelli, 1951) was run using 15-150 KDa and 10-225 KDa protein markers (perfect protein marker, Novagen). The gels were stained overnight in comassie blue, fixed in 10% acetic acid and destained in distilled water prior to scanning for documentation.

5.3. RESULTS:

5.3.1. Growth of MRB in heavy metals:

In medium amended with mercury:

The MRB showed a diauxic growth response when exposed to mercury. Details of these observations are already presented in chapter 3 (see Figure 3.1).

In medium amended with cadmium:

The growth curves of both the isolates (CH07 and GP06) showed similar growth response (Figure 5.1.1). Both the isolates showed a lag phase of nearly 6 h and they reached the stationary phase around 48 hours after having an exponential growth phase for ca. 18 h. There was no growth in the negative control (isolate CH05) in presence of Cd even after 48 hours *vis a vis* normal growth in medium without Cd. Toxic effect of Cd was hardly discernible on the growth of two MRB isolates tested.

In medium amended with lead:

All three MRB isolates (CH07, GP13 and S3) grew quite spontaneously in presence of Pb in the SWNB (Figure 5.1.2). All the isolates reached the stationary phase around 24 h and there was almost no difference in the test condition from that of the controls. The negative control (CH05) did not grow in presence of lead also as was the case with the other two heavy metals.

5.3.2.1. Volatilization of Hg:

The foggy areas on the XAR plate denoted the reaction of silver (Ag) reduction by the Hg vapor as seen in the photograph (Figure 5.2.1). All isolates tested during this study (*viz.* GP15, CM10, CH07, GP08, GP13, GO02, GP16, GP17, GP14, GP06 and CH13) volatilized mercury from the assay medium though all of them were not positive for *merA*. The negative control showed indication of no Hg vapour.

5.3.2.2. Kinetics of Hg removal:

CH07 was able to remove mercury by means of volatilization from the M9 assay medium containing 20 ppm ($\approx 100 \mu\text{M}$) mercury. The highest removal rate was in the reaction with 1 ppm

Hg concentration though the removal rate was quite good up to 8 ppm (i.e., 40 μM) Hg in the medium (Figure 5.2.2.2) with removal concentrations as high as 1.16×10^{-6} ng cell⁻¹ min⁻¹. *P. putida* showed the fastest removal rate when compared to the other two isolates (Figure 5.2.2.3) and it was discernible that all three isolates had high removal rates at 1 ppm (5 μM) Hg concentration. More importantly, Hg removal rate increased with increasing concentrations of NaCl (Figure 5.2.2.4.). The marine pseudomonad CH07 was most efficient at a high salt concentration of 30 g l⁻¹. Bro12 (*P. aeruginosa*) was most efficient at salt concentrations of 20-25 g l⁻¹ NaCl whereas *P. putida* (*P. putida* KT2442 mer::73) had its best removal efficiency at moderate salt concentrations of 12-15 g l⁻¹ NaCl.

5.3.3. Removal of Cd and Pb:

The pseudomonad CH07 removed Cd at a faster rate from the medium during the first 48 hours. After the third day, the removal of Cd by both the isolates (viz, CH07 and GP06) was almost similar (Figure 5.3.1.1). In medium amended with 100 ppm Cd, the concentration in the medium was reduced to 17.41 ppm by CH07 and 19.19 ppm by GP06 (*A. faecalis*) in about 72 h and the maximum accumulation of Cd in the biomass was observed within 72 hours corroborating with the removal rate (Figure 5.3.1.2). GP06 removed >70% Cd and, CH07 was also equally efficient removing >75% from growth media containing 100 ppm Cd.

All three strains of MRB were able to remove Pb from the growth medium. In case of CH07 the concentration of Pb in medium amended with 100 ppm Pb reached as low as 1.8 ppm (>98% removal) in 96 h and it was found to be entrapped in the extracellular polymeric substances (EPS) as revealed by the SEM and EDS (Figure 5.4.3.2. and 5.4.6). GP13 (*B. iodinium*) and S3 (*B.*

pumilus) precipitated lead in the sulfide form (5.3.2.3) removing more than 87 and 88% during the same period.

5.3.3.1. Biomass:

The dry weight of the bacterial biomass changed in accordance with the growth phase. The biomass in the later stage showed marginal increases in case of CH07 probably due to the production of EPS and entrapment of heavy metals into the biomass and also due to precipitation of the heavy metals on the cell surface. The biomass of CH07 reached as high as 19.2 mg ml⁻¹ in the SWNB with Cd whereas it was higher in case of GP06 with 21.3 mg ml⁻¹. Increased concentration of toxic metals bore no specific effect on biomass. In the experiment with Pb, the maximum biomass of 5.5 mg ml⁻¹ was attained by CH07 whereas, GP13 and S3 had a biomass each of 4.8 and 2.3 mg ml⁻¹ respectively.

5.3.4. SEM and EDS:

From the SEM photographs and EDS signature it was evident that the Cd was mostly entrapped in the EPS in case of both the isolates (Figures 5.4.1.1 to 5.4.2.2). This could be due to efflux commonly seen in gram-negative bacteria as a detoxification measure. Extracellular matrices with negative charges form metal complexes so as to confer resistance to the cell. Lead was precipitated as sulfide in case of GP13 and S3 (Figure 5.3.2.3) whereas, it was entrapped in the EPS of CH07 (Figure 5.4.3.1 and 5.4.3.2). The EDS of the cell masses showed the concentration of Pb as high as 21% and the Cd had a percentage signal of 19%, when the added concentrations in the growth medium was 100 ppm (0.01%). Bacteria grown in medium without these heavy metals showed no signals of either of Cd or Pb in the EDS analyses.

5.3.5. Protein concentrations and profiles:

As a detoxification response, there were changes in protein concentrations in case of all the isolates exposed to 1-25 ppm Hg. There was an increase in the total protein concentration in the medium with mercury though the amount did not increase with further increasing concentration of Hg (Figure 5.5.1.1). The SDS-PAGE revealed that one fragment of protein corresponding to 75 KDa disappeared from all the tested isolates when exposed to Hg (Figure 5.5.1.2 and 5.5.1.3). The two isolates exposed to Cd also showed a similar trend (Figure 5.5.2). There was an increase in the total protein concentration when there was Cd in the medium, but similar to the observations from Hg effect, protein concentration did not increase with increase in the concentration of Cd. The protein profile showed appearance of new band in the test condition, which were absent in case of the control condition (Figure 5.5.2).

5.4. DISCUSSION:

Microorganisms have a high surface area-volume ratio because of their small size and therefore provide themselves with a large contact area that can interact with matter in the surrounding environment. There are many approaches for the use of bacteria to remove heavy metals from the environment: bioaccumulation and bioabsorption, oxidation and reduction, methylation and demethylation, and ligand degradation by bacteria (Bolton & Gorby, 1995). Lower costs and higher efficiency at low metal concentrations make biotechnological processes very attractive in comparison with physicochemical methods for heavy metal removal (Gadd, 1992). Among the principal pollutant transformation processes, microbial degradation/biotransformation may be the most efficient way for removal of chemical pollutants and their toxicity from the environments. Until recently, the interest was focused on technologies that could be applied to achieve *in situ*

immobilization of metals (Roane et al., 1996). However, within the last few years, the focus has begun to shift towards the actual metal removal, because it is difficult to guarantee that metals will remain immobilized indefinitely (Roane et al., 1996).

Silver (1996) summarized the mechanisms of bacterial resistance to heavy metals as follows:

- i) bacteria have specific genes for resistance to the toxic ions including Ag^+ , AsO_2^- , Cd_2^+ , and Hg^{2+} and there is no general resistance mechanism to all heavy metals;
- ii) metal-ion resistance have been found on plasmids as well as chromosomes of all eubacterial group tested;
- iii) the mechanisms of resistance are generally efflux pumping (removing toxic ions that enter the cell by means of transport systems evolved for nutrient cations or anions) and enzymatic detoxification (generally redox chemistry) converting a more toxic to a less toxic or less available metal-ion species. It seems that the metabolic penalty for having more specific uptake pump is greater than the genetic cost of having plasmid genes in the population that can spread and become induced. Efflux pumps are the major currently known group of plasmid resistance systems.

Several studies have shown that metals are more toxic when they are in liquid medium than in agar medium (Chang et al., 1993; Inhoue et al., 1996). The pH dependence of microbial metal accumulation can be entirely different, both between metals and between organisms (Ledin, 2001). The pH-dependence of anions is generally significantly different from that of cations. As pH increases, functional sites on the microbial surface become deprotonated and may bind cationic metals instead. This results in the common trend of increasing accumulation of metals with increasing pH. However, in many cases, the accumulation at higher pH is smaller (Ledin,

2001). So, in all the experiments carried out in this thesis, a near neutral pH (7-7.5) was maintained to minimize this effect of pH.

Bacterial chromosomes contain genes for resistance to many heavy metal cations and oxyanions, as do plasmids or other genetic elements (Silver & Phung, 1996). Detoxification of mercury by enzymatic reduction was proposed more than three decades ago (Summers & Silver, 1972) and, it has been realized later that the *mer* operon -conferring both resistance and detoxification capabilities to its possessor- is almost universally distributed in resistant bacterial populations (Summers & Silver, 1978; Robinson & Tuovinen, 1984; Osborn et al., 1997; Barkay et al., 2003). Okino et al. (2002) studied the effect of initial cell concentration of removal of HgCl₂ and concluded that the removal rate increased with increasing initial cell concentrations. Rasmussen et al. (1997) reported that the expression of *mer* operon was increased at lower cell density due to a reduction in the number of cellular binding sites that may compete with the MerR for binding of the inducer, Hg²⁺. In the present study, it was found that the removal rate was the highest at low Hg concentration, and it reduced with increasing Hg concentration. However, the removal rate was quite effective upto a concentration of 8 ppm of Hg from the minimal medium used in all the detoxification experiments for this study. Genetically engineered *E. coli* strain with Hg²⁺ transport system and metallothionein has been used to bioaccumulate mercury from wastewater (Deng & Wilson, 2001). High concentrations of NaCl, causing less bioavailability of metal usually inhibit the volatilization of mercury by bacteria (Selifonova & Barkay, 1994; Barkay et al., 1997). At the NaCl concentrations of 35ppt all the *merA* positive isolates vaporized Hg. This is pointing to the possibility of marine MRB potential to deal with Hg toxicity. Indeed, there are many halophiles tolerant to Hg (Harithsa et al., 2002), which might be accomplishing the survival in their extreme environment. The 'multiutilitarian' *Deinococcus radiodurans* has been

engineered to remediate radioactive mixed waste containing mercury as a contaminant (Brim et al., 2000), which detoxifies Hg through vaporization.

There was no clear relation between the percentage of Cd bioabsorbed by the microorganisms and the Cd concentration in the medium. This phenomenon may be explained by the role of the microbial metabolism onto the bioabsorption (Nies, 1999). The Cd concentrations play a major role in bioabsorption. Although molecular analyses examining the Cd resistance mechanisms were not attempted during this study, it is quite likely that one or several of the following mechanisms reported in literature do operate in the marine MRB isolates examined during this study.

Liesegang et al. (1993) reported that *Alcaligenes eutrophus* CH34 harbours numerous heavy metal resistance determinants including three for mercury resistance, one for chromate resistance and two for divalent cations called *czc* (for Cd²⁺, Zn²⁺ and Co²⁺ resistances) and *cnr* (for Co²⁺ and Ni²⁺ resistances). *Czc* is an efflux pump (Nies et al., 1989) that functions as a chemio-osmotic divalent cation/proton antiporter (Nies, 1995; Nies & Silver, 1995). Homologues of the *czc* genes, called *czt*, which conferred cadmium and zinc resistance, were recently identified in *Pseudomonas aeruginosa* genomic DNA and appear to be highly conserved in environmental isolates of that species (Hassan et al., 1999). Mutational analysis indicated that *cadA* and *cadR* are fully responsible for cadmium resistance and partially for zinc resistance (Lee et al., 2001).

P. aeruginosa CW-96-1 was shown to grow well aerobically upto 5 mM Cd in a medium containing citrate and S₂O₃²⁻ (Wang et al., 1997). A *Klebsiella planticola* strain (Cd-1) has been reported to grow anaerobically at high Cd concentration (15 mM) and precipitate CdS (Sharma et

al., 2000). Lebeau et al. (2002) was unable to grow the bacterium ZAN-044 at a Cd concentration of 10 mg l⁻¹ (ppm). But, Bang et al. (2000) achieved 98% removal of Cd from medium with 150 μM Cd and 91% from medium with 200 μM Cd by using a strain of *Escherichia coli*. *P. aeruginosa* PU21 has been able to uptake 58 mg Cd g⁻¹ dry wt (Chang et al., 1997). Two most resistant isolates *Bacillus* H9 and *Pseudomonas* H1 were highly resistant upto 275 and 225 μg ml⁻¹ of soluble Cd respectively (Roane & Pepper, 2000).

The resting cells of *P. aeruginosa* PU21 (Rip64) have been reported to take up upto 110 mg Pb g⁻¹ dry cell mass whereas, the inactivated cells could absorb 70 mg Pb g⁻¹ dry cell (Chang et al., 1997). The *P. aeruginosa* PU21 (Rip64) was reported to adsorb Hg²⁺ upto 400 mg Hg g⁻¹ dry cell mass and this adsorption might have originated from the cysteine-rich transport proteins associated with the cell membrane and the adsorption efficiency of the biomass reached its maxima at a low pH of 2 (Chang et al., 1997). As mentioned earlier, it is quite likely that the multi-metal resistant strains such as CH07, GP14, GP15, GP16 and GP06 possess the genetic components for dealing with many toxic metal ions.

From the SEM photographs and EDS signal it was evident that the Cd was mostly entrapped in the EPS in case of both the isolates. This could be due to efflux commonly seen in gram-negative bacteria as a detoxification measure as reported by Nies (1999). The EDS was not successful in detecting Hg as, the sputter coating by gold (Au), overlapped the Hg signal.

There are numerous practical reasons for selectively separating heavy metal ions of all types from aqueous media. A few obvious examples are the remediation of hazardous or radioactive wastes, remediation of contaminated groundwater, and recovery of precious and/or toxic metals from

industrial processing solutions. A variety of well-known techniques are available to the chemists or engineers for these tasks, including solvent extraction, ion-exchange chromatography, and precipitation. In modern applications of these techniques, recovery and re-use of the extractant materials is becoming more and more important. This is being driven by tougher environmental regulations, the high initial costs of new, more effective and more selective extractants, and the need to minimize the volume of waste destined for permanent disposal. Already proven to be economical, bioremediation by marine bacterial means for detoxification of metals holds promise. Results from the present study clearly demonstrate the possibilities of employing them for metal detoxification/recovery and even sequestration under special circumstances. Further, leads from this study can be explored to adapt marine bacteria for bioremediation under various situations.

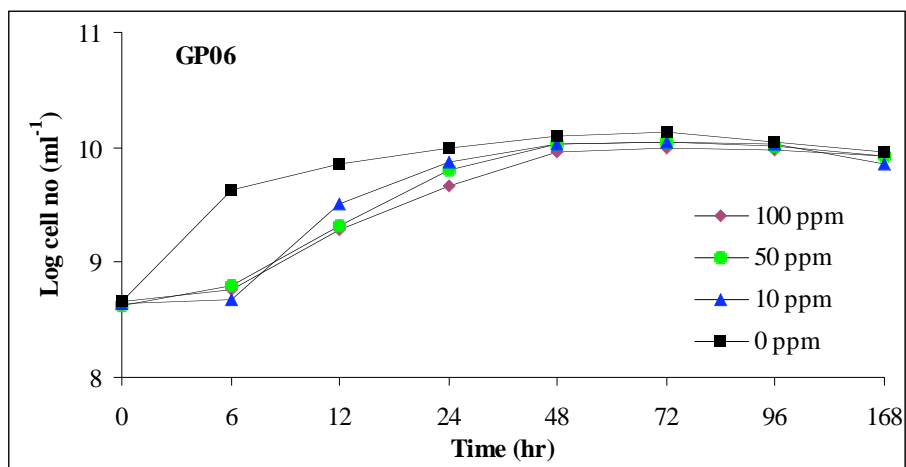
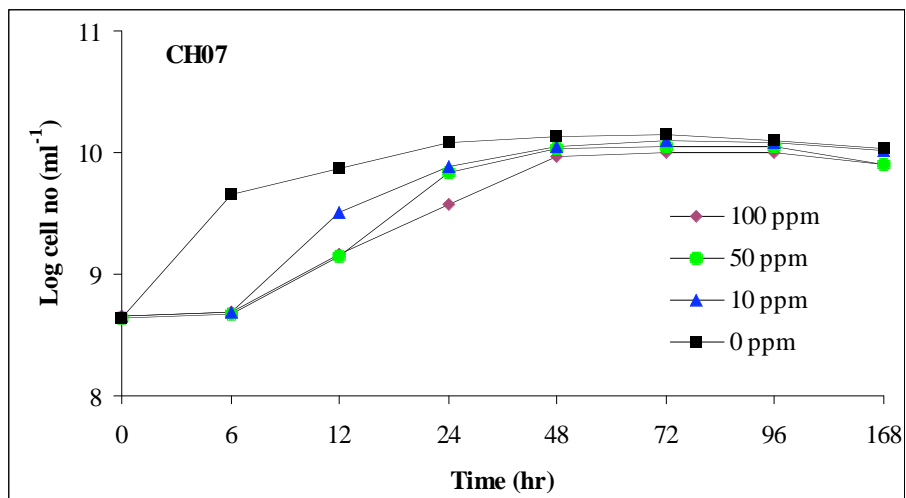


Figure 5.1.1. Growth patterns of the isolates subjected to different concentrations of Cd.

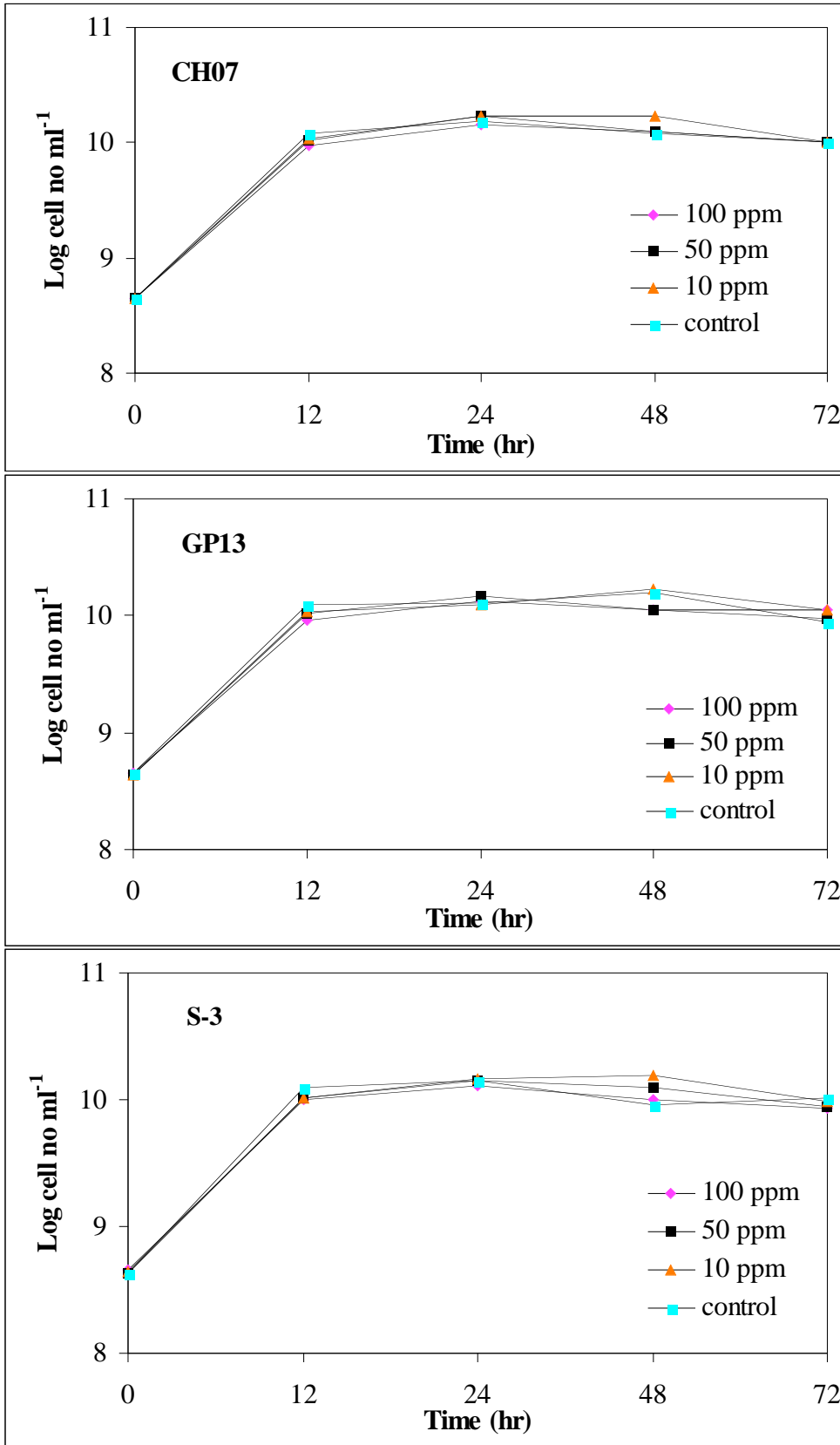


Figure 5.1.2. Growth patterns of the isolates subjected to different concentrations of Pb

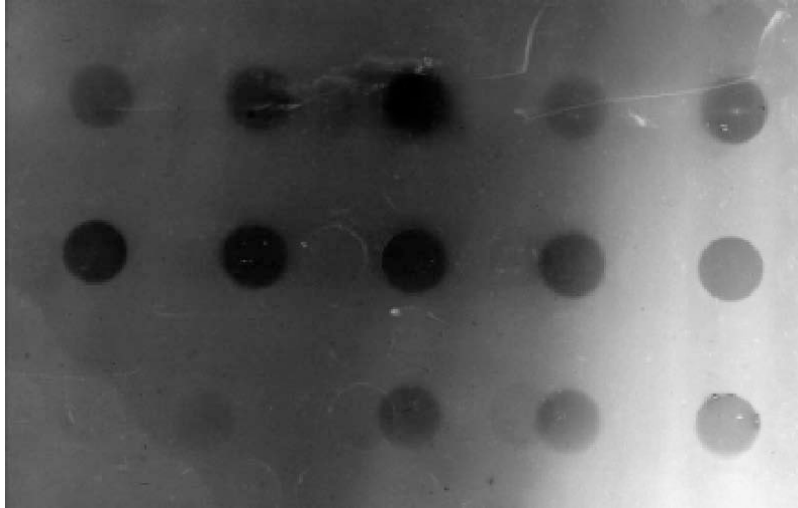


Plate 5.2.1. Mercury volatilization by MRB as visualized on Kodak XAR film

Upper row (from left to right): GP15 (*A.faecalis*); CM10 (*Bacillus* sp.); CH07 (*P.aeruginosa*); GP08 (*B.iodinium*); +ve control (*P. putida* KT2442::mer73); Middle row: GP13 (*B.iodinium*); GO02 (*A. faecalis*); GP16 (*A. faecalis*); GP17 (*A. faecalis*); GP14 (*B. iodinium*); Lower row: mercurated PBS used in the experiment, non MRB isolate(-ve control), 3C (*Virgobacillus panto*), GP06 (*A. faecalis*), CH13 (*B.pumilus*)

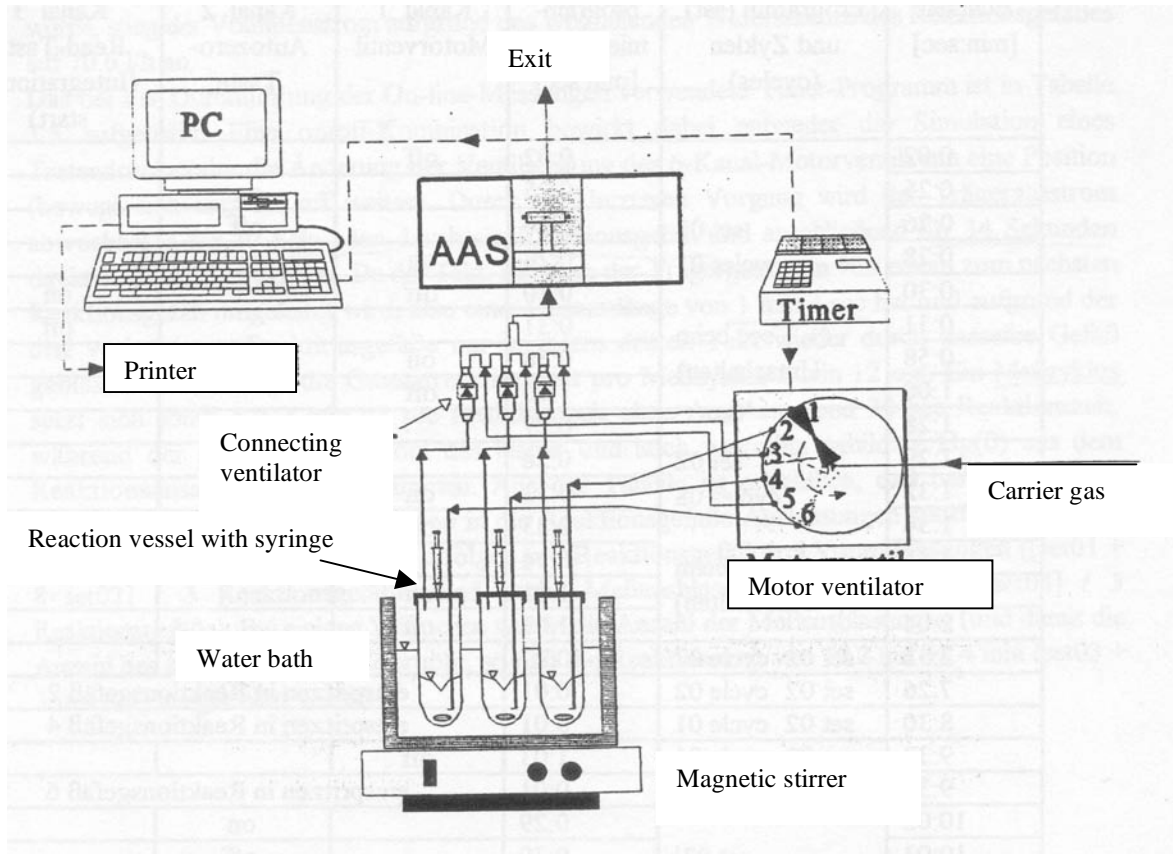


Figure 5.2.2.1. Set up of the Hg-kinetics system

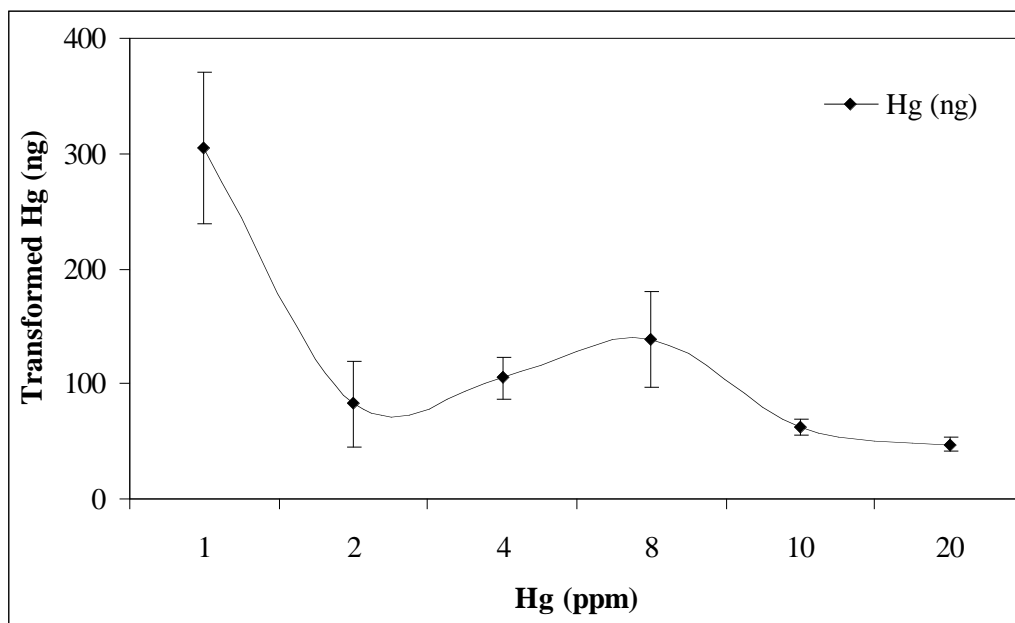


Figure 5.2.2.2. Hg biotransformation by CH07

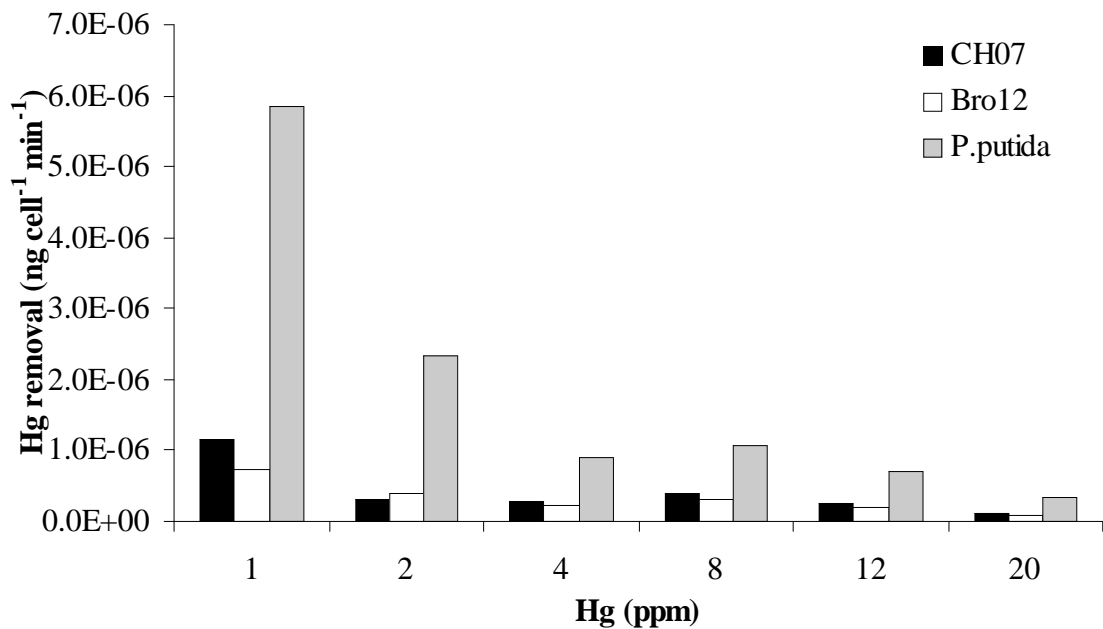


Figure 5.2.2.3. Rates of Hg removal by CH07, *P.putida* KT 2442 mer::73, Bro12

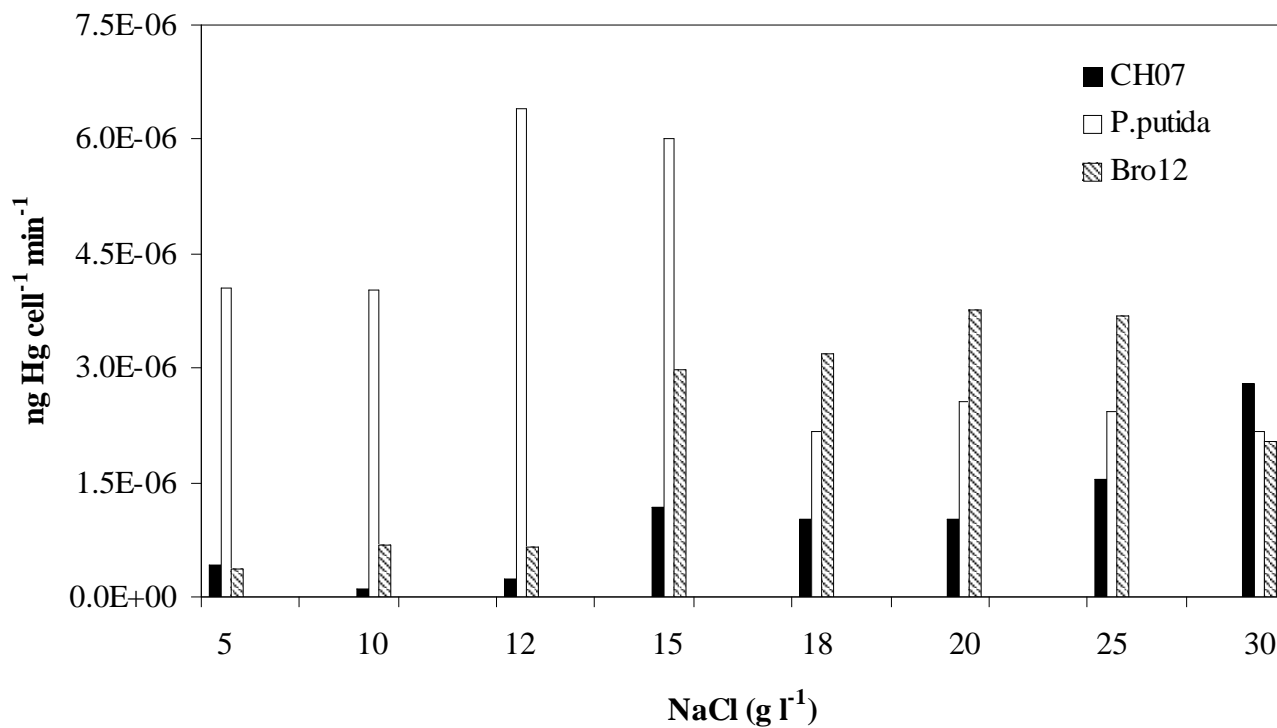


Figure 5.2.2.4. Effect of NaCl on Hg removal rate by different experimental strains

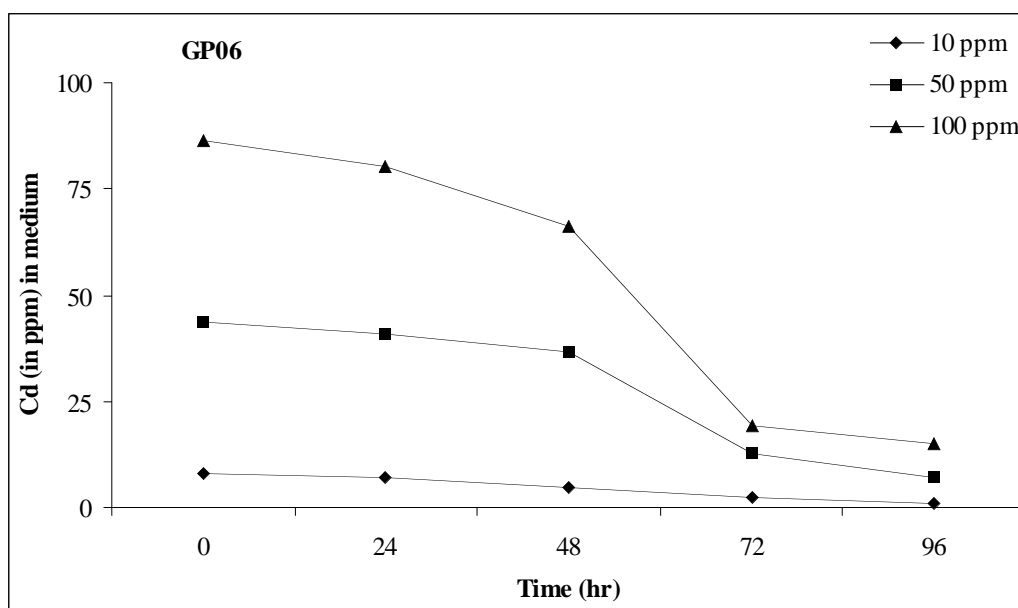
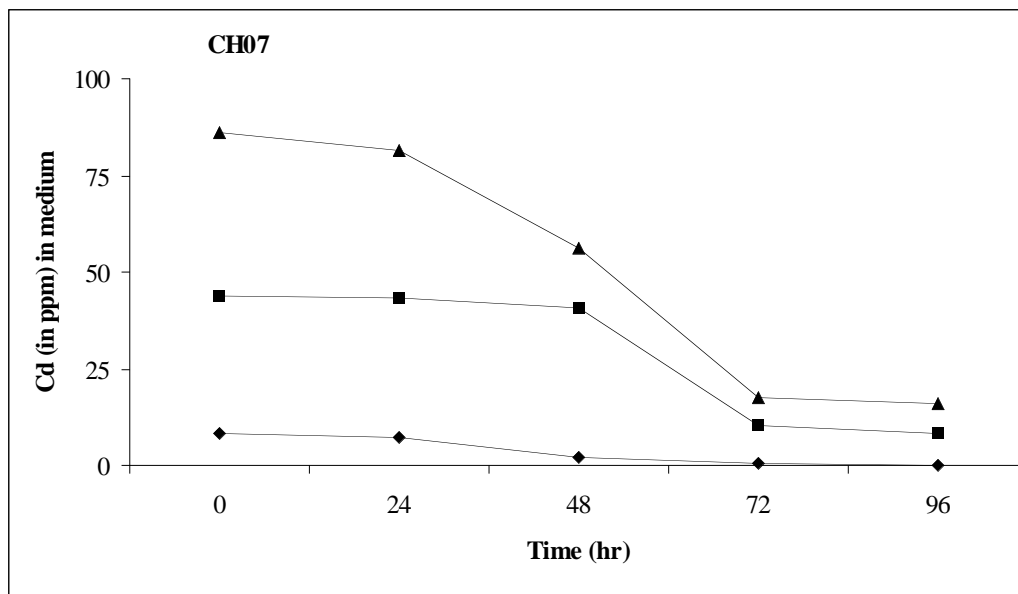


Figure 5.3.1.1. Removal of Cd from medium by different MRB when grown in different concentrations

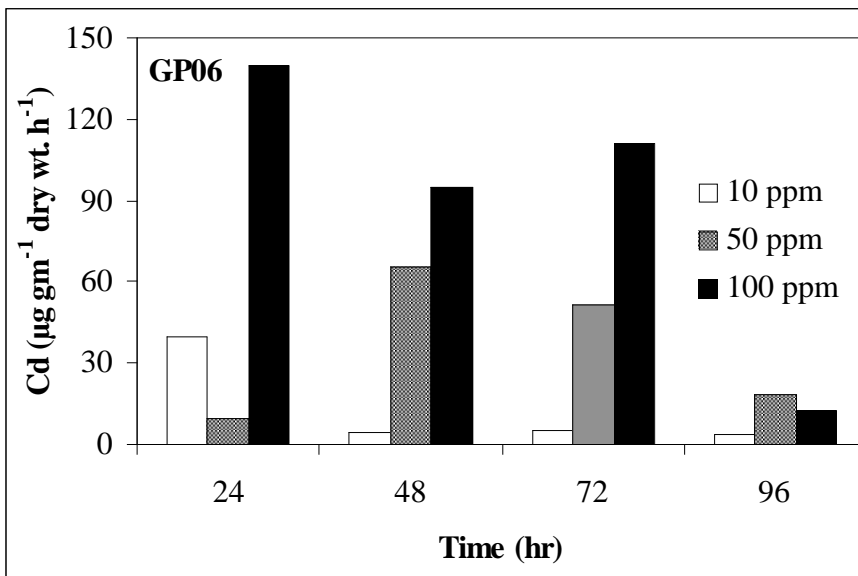
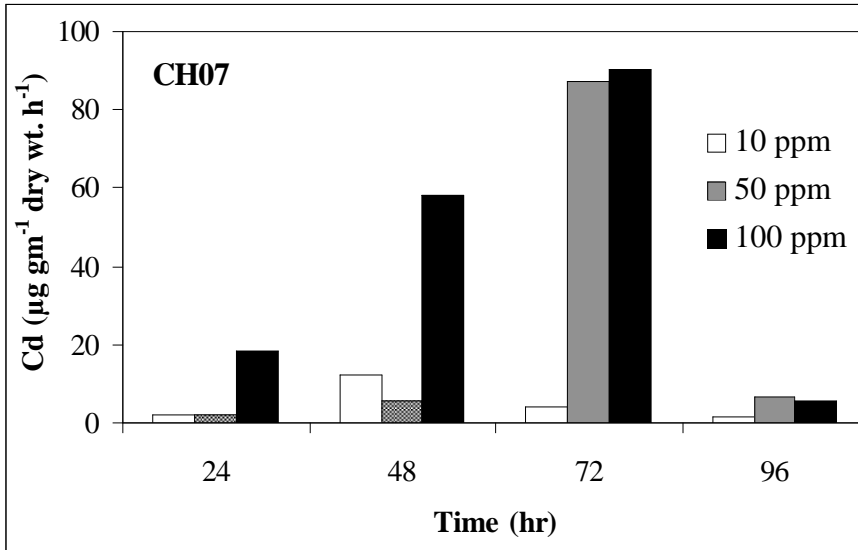


Figure 5.3.1.2. Cell biomass associated quantities of Cd ($\mu\text{g g}^{-1}$ dry wt. h^{-1}) by marine MRB strains from the media containing different concentrations of this toxic metal.

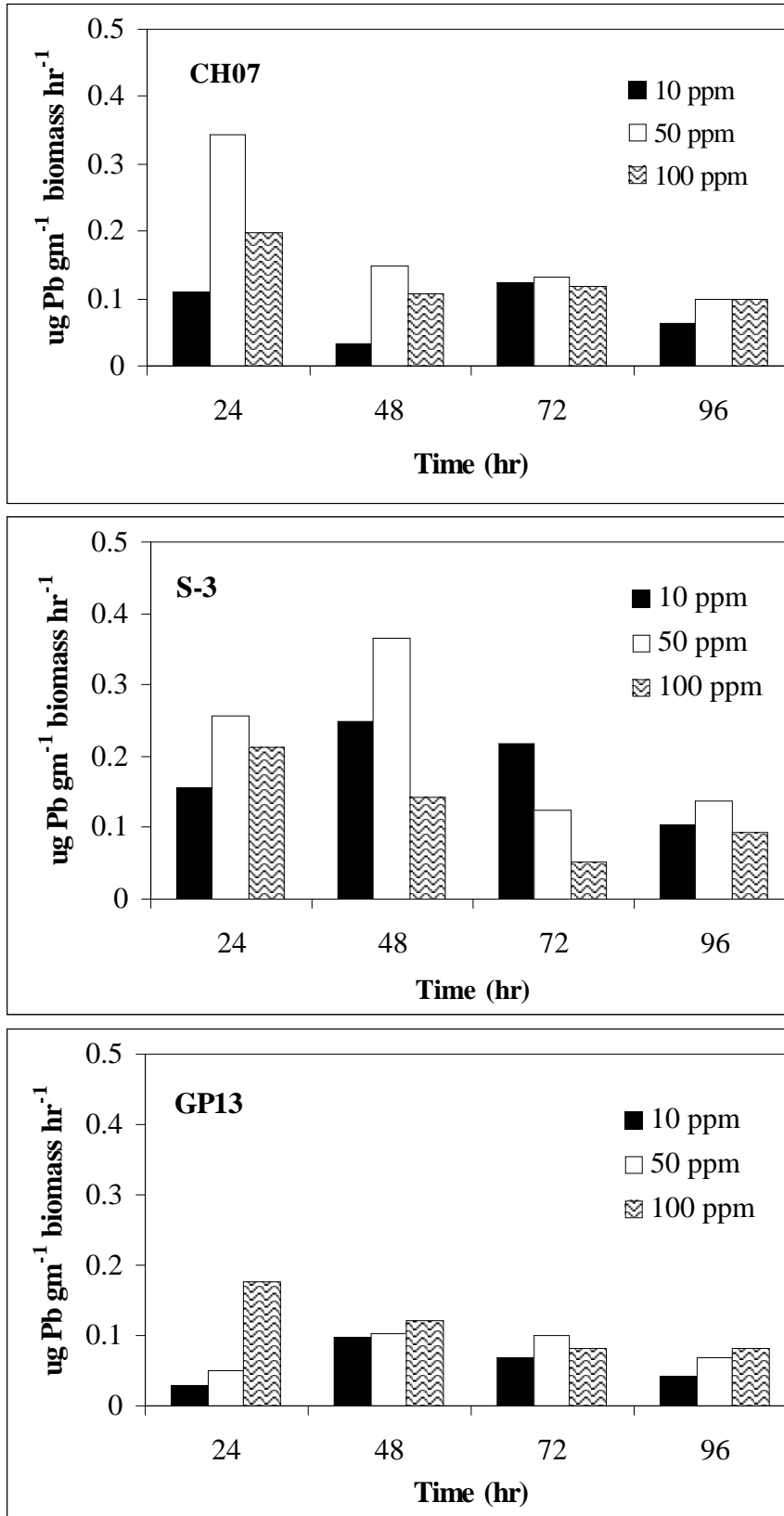


Figure 5.3.2.1. Cell biomass associated quantities of lead by different MRB strains grown in media with various concentrations of Pb.

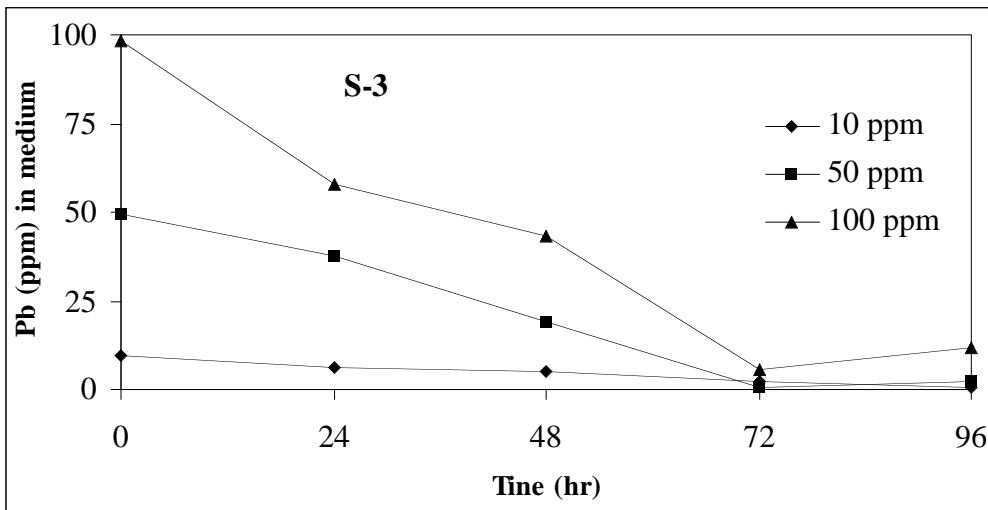
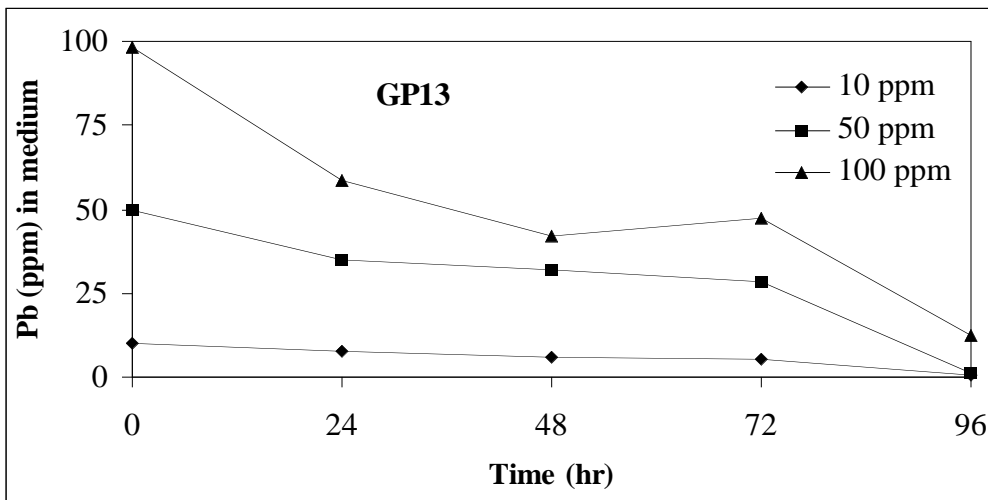
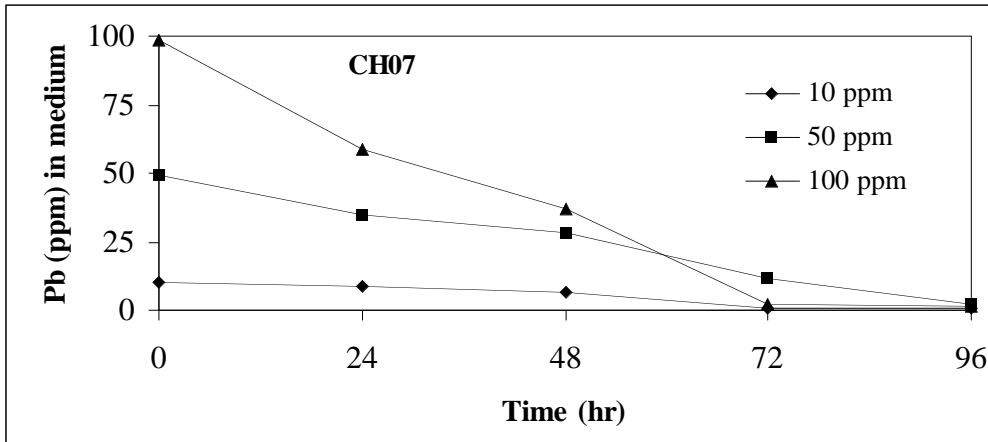


Figure 5.3.2.2. Removal of Pb from medium by different MRB when grown in different concentrations



a

b

c

Figure 5.3.2.3. Culture filtrate exposed to air showing formation of PbS as a measure of detoxification of Pb by MRB. a) GP13; b) CH07; c) S-3. Formation of sulfide was prominently visible in media inoculated with GP 13 and S-3, unlike that with CH07.



Figure 5.4.1.1. SEM photograph of CH07 grown in normal seawater nutrient broth (i.e.,) without

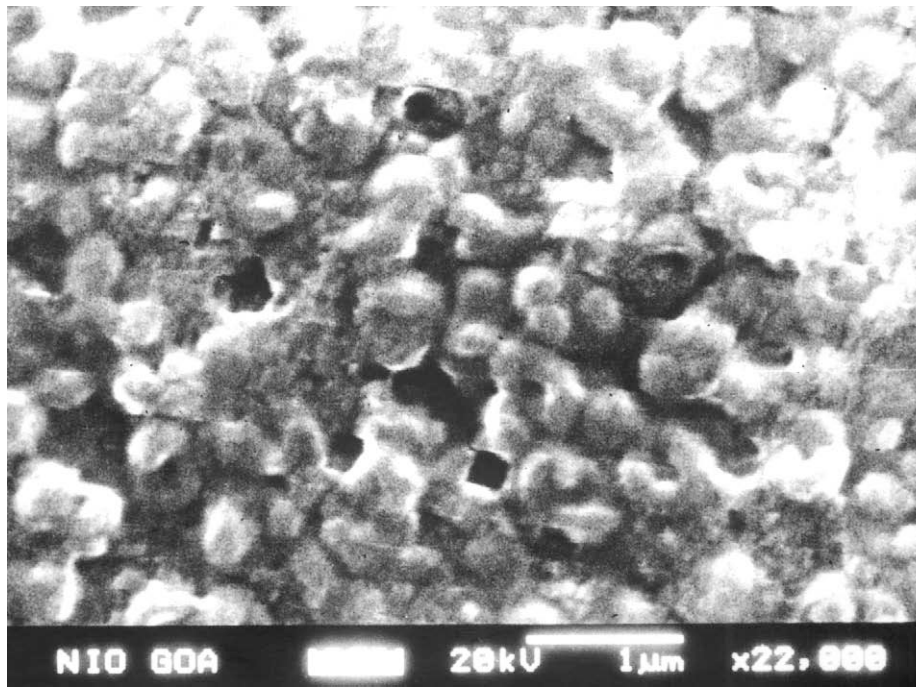


Figure 5.4.1.2. SEM photograph of CH07 grown in seawater nutrient broth containing 100 ppm Cd.

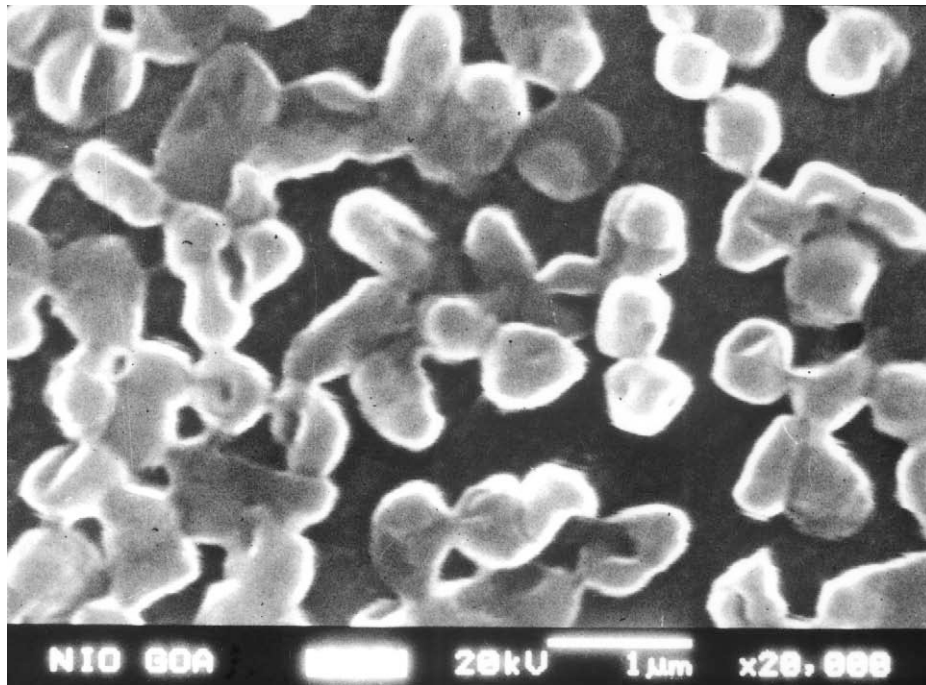


Figure 5.4.2.1. SEM photograph of GP06 grown in normal seawater nutrient broth

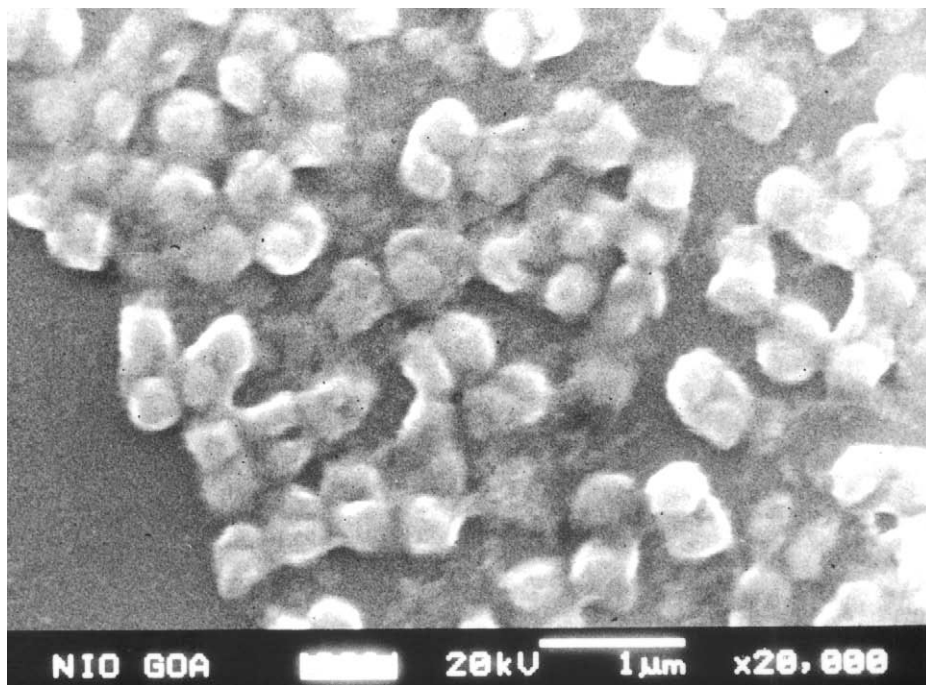


Figure 5.4.2.2. SEM photograph of GP06 grown in seawater nutrient broth containing 100 ppm Cd.

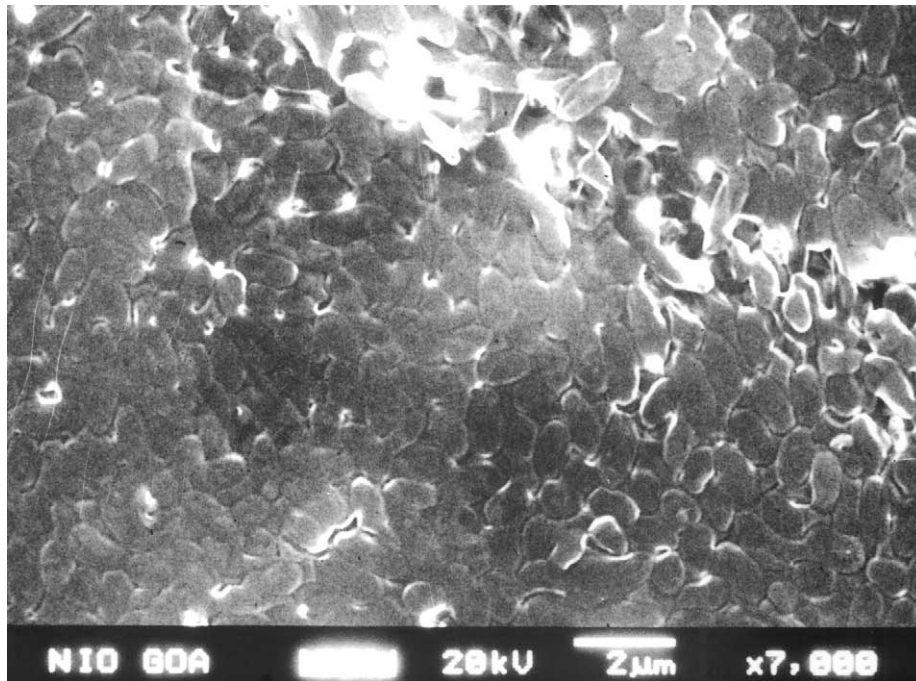


Figure 5.4.3.1. SEM photograph of CH07 grown in normal seawater nutrient broth

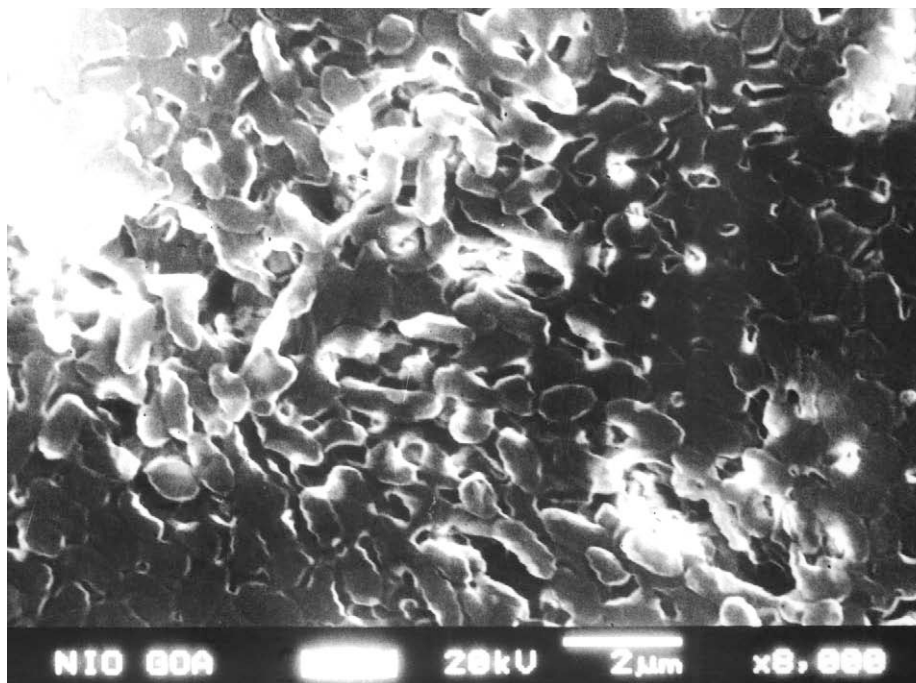


Figure 5.4.3.2. SEM photograph of CH07 grown in seawater nutrient broth containing 50 ppm Pb.

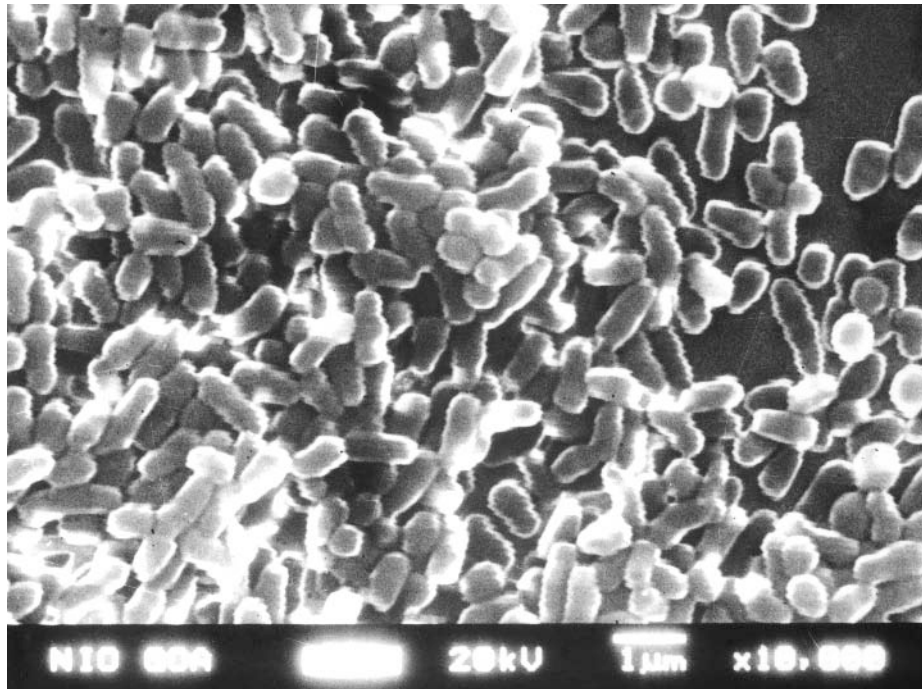


Figure 5.4.4.1. SEM photograph of GP13 grown in normal seawater nutrient broth

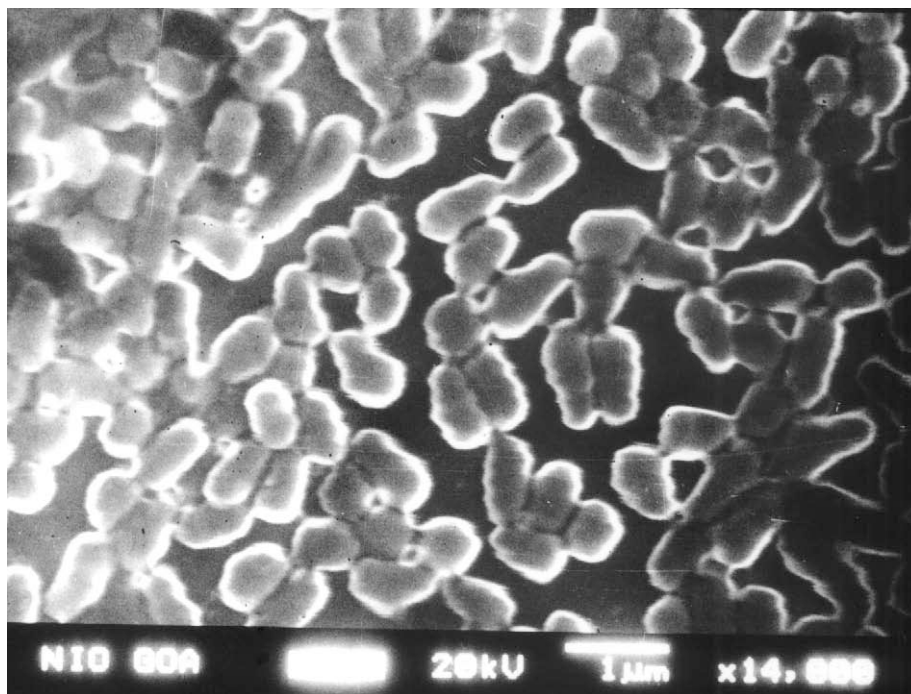


Figure 5.4.4.2. SEM photograph of GP13 grown in seawater nutrient broth containing 50 ppm Pb.

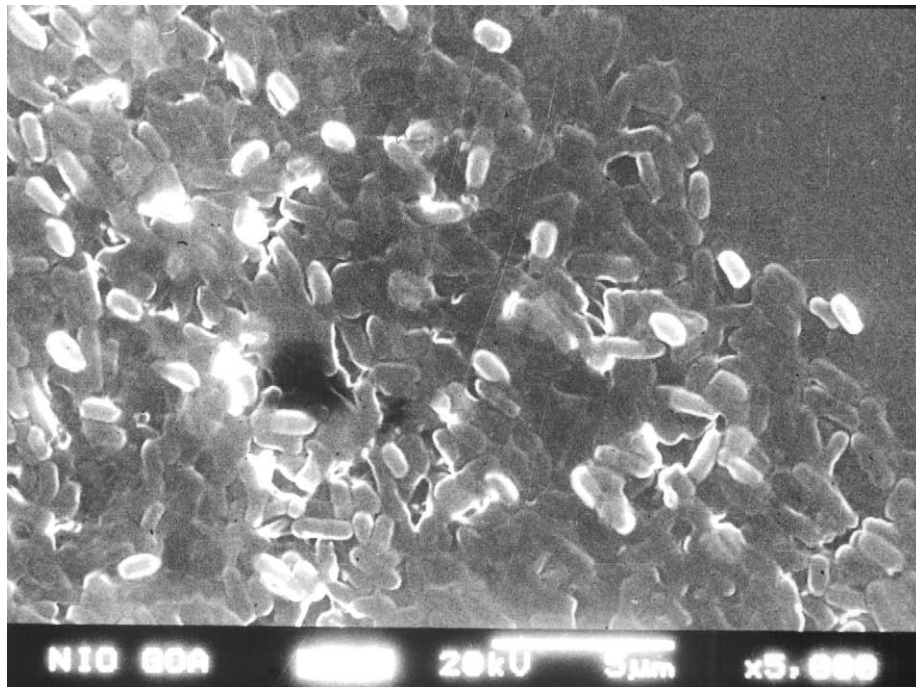


Figure 5.4.5.1. SEM photograph of S3 grown in normal seawater nutrient broth

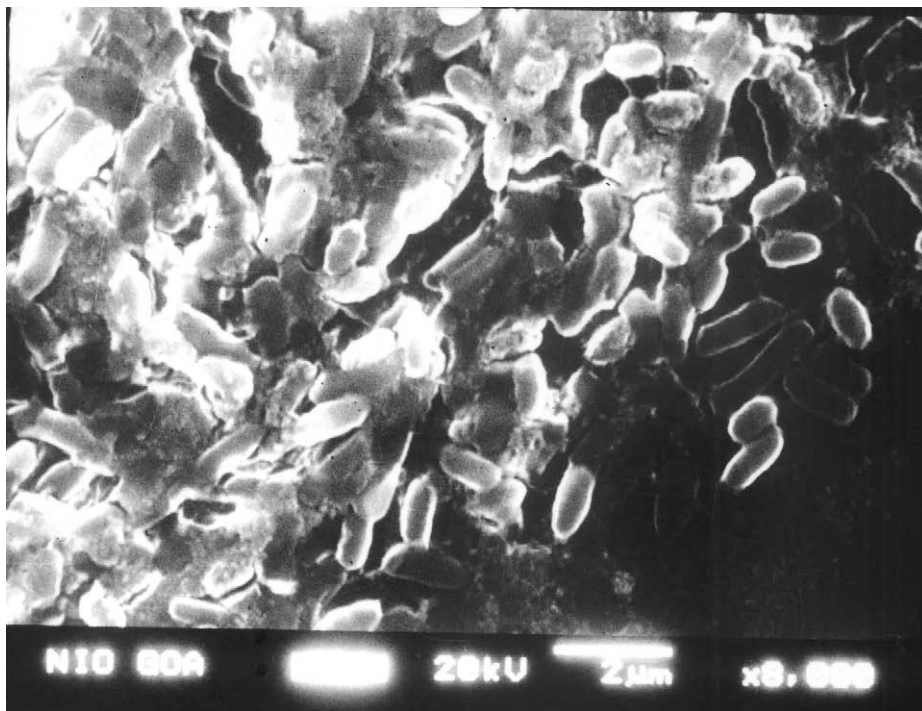


Figure 5.4.5.2. SEM photograph of S3 grown in seawater nutrient broth containing 50 ppm Pb.

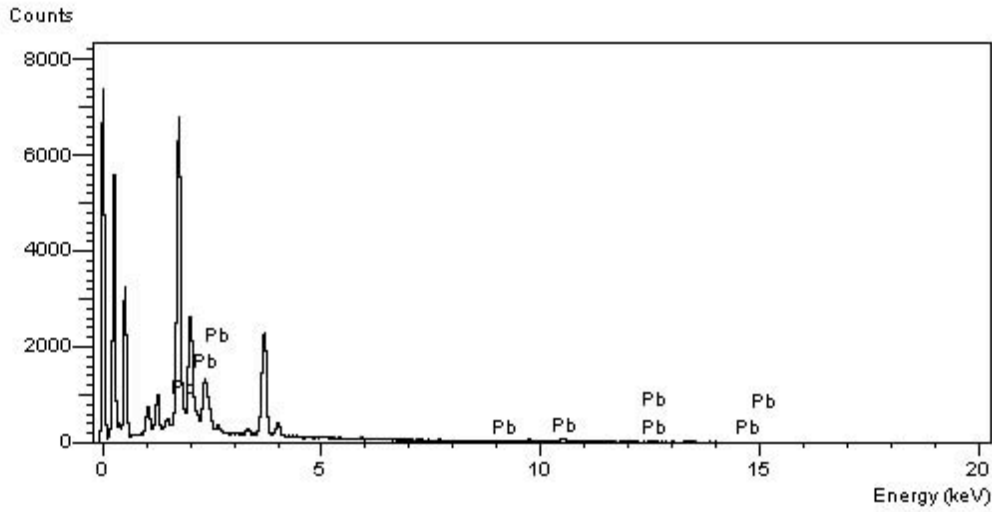


Figure 5.4.6. EDS showing presence of Pb in the cell precipitate of GP13

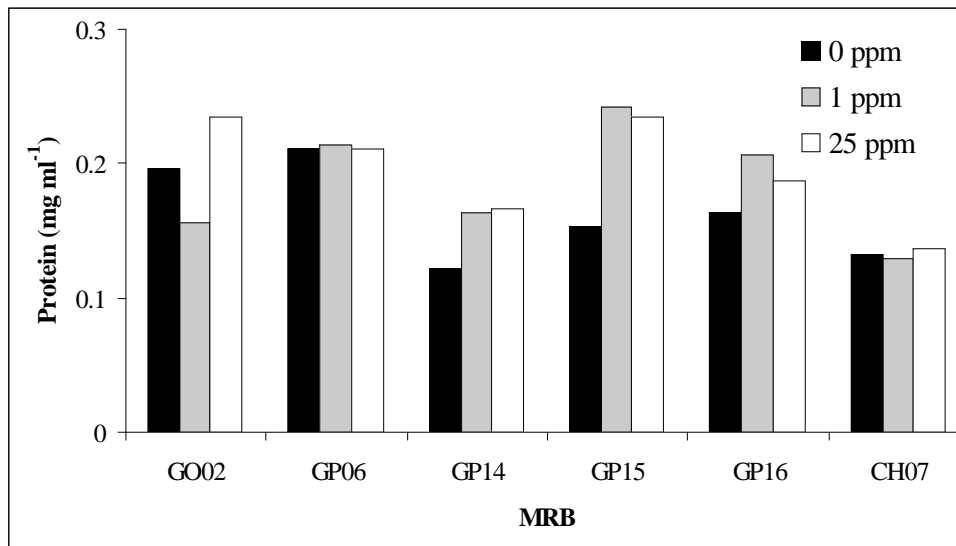


Figure 5.5.1.1. Change in total protein concentrations in MRB grown in different concentration of Hg

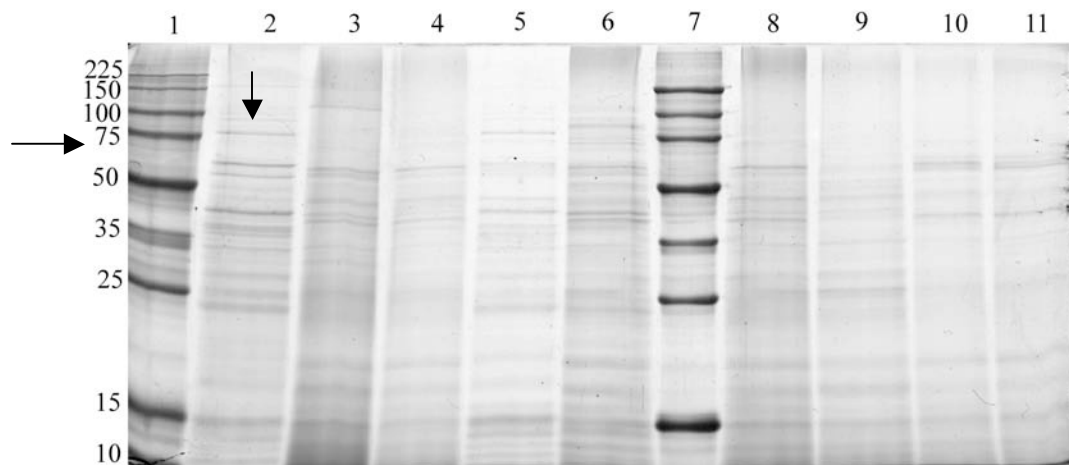


Figure 5.5.1.2. SDS-PAGE protein profile of MRB showing effect of Hg. From left to right; lane 1, marker (10-225 KDa); 2-4, GP15 (no Hg, 1 ppm Hg, 25 ppm Hg); 5-6, GP16 (no Hg, 1 ppm Hg); 7, marker (15-150 KDa); 8, Gp16 (25 ppm Hg); 9-11, CH07 (no Hg, 1 ppm Hg, 25 ppm Hg)

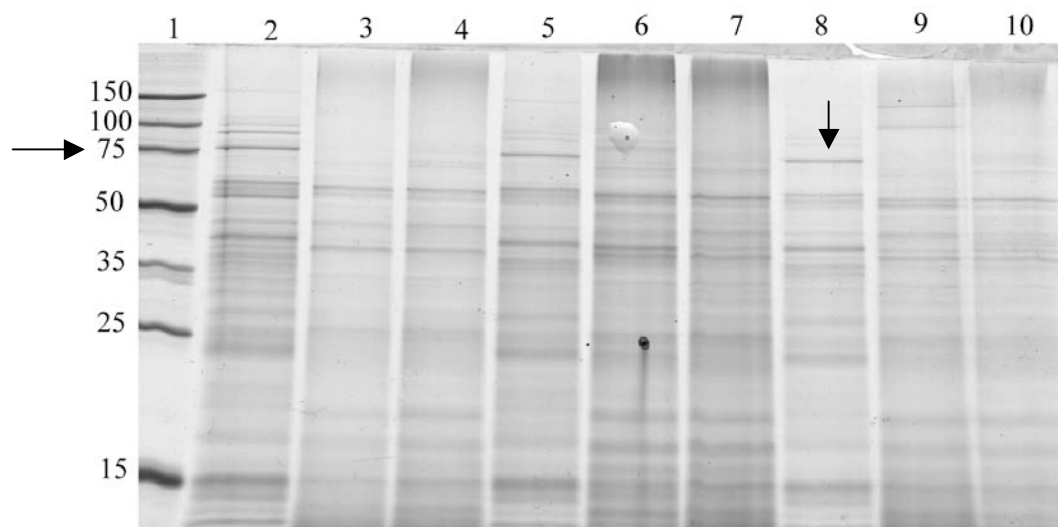


Figure 5.5.1.3. SDS-PAGE protein profile of MRB showing effect of Hg. From left to right; lane 1, marker (15-150 KDa); 2-4, GO02 (no Hg, 1 ppm Hg, 25 ppm Hg); 5-7, GP06 (no Hg, 1 ppm Hg, 25 ppm Hg); 8-10, GP14 (no Hg, 1 ppm Hg, 25 ppm Hg)

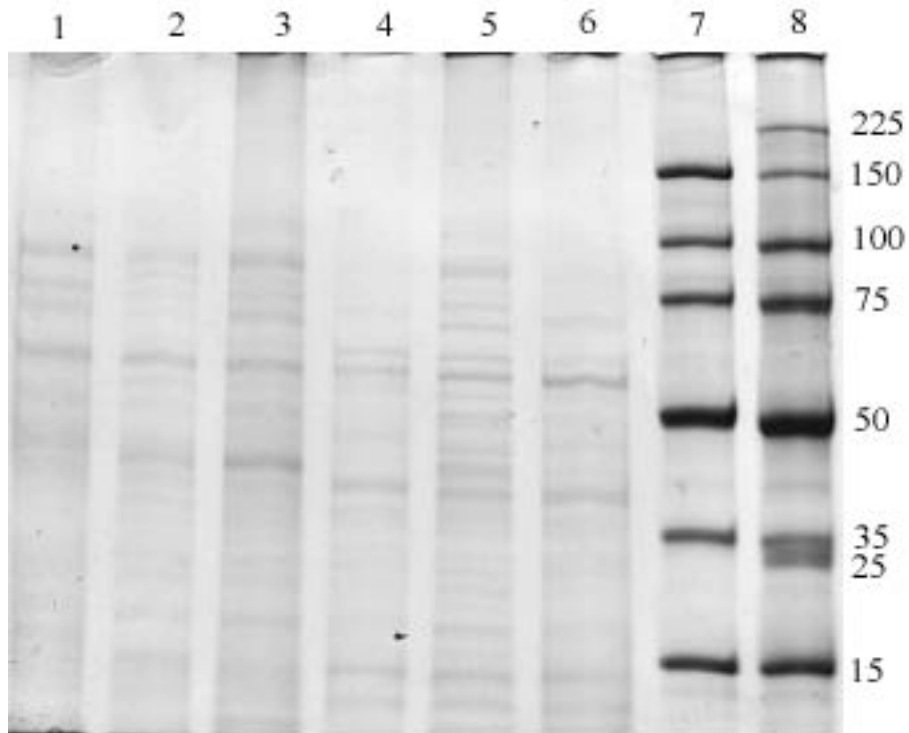


Figure 5.5.2. SDS-PAGE protein profile of two MRB showing their response to Cd. From left to right; lanes 1-3,GP06- no Cd, 10 ppm Cd, 100 ppm Cd and 4-6, CH07- no Cd, 10 ppm Cd, 100 ppm Cd, 7, 15-150 KDa marker (Perfect protein marker, Novagen), 8, 10-225 KDa, marker (Perfect protein marker, Novagen)