BIOACCUMULATION OF MERCURY IN MARINE BACTERIA: A NOVEL APPROACH OF MERCURY REMEDIATION

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CERTIFICATE

This is to certify that the project report titled **"BIOACCUMULATION OF MERCURY IN MARINE BACTERIA: A NOVEL APPROCH OF MERCURY REMEDIATION"** submitted by **Ms. Roma Sinha** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfilment of the requirement for the degree of Master of Science in LIFE SCIENCE is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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Last but not the least; I bow my head before Almighty for his blessings.

DATE: 04 May, 2012 ROMA SINHA

Rourkela

DECLARATION

I hereby declare that the thesis entitled "**BIOACCUMULATION OF MERCURY IN MARINE BACTERIA: A NOVEL APPROCH OF MERCURY REMEDIATION**", submitted to the Department of LIFE SCIENCE, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Surajit Das, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date: 04 May, 2012 Roma Sinha

Place: Rourkela

List of Symbols and Abbreviations Used

gm	Gram
hr	Hour
l	Litre
µl	Microlitre
^{0}	Degree
C	Centigrade
ml	Mili litre
min	Minute
LB	Luria Bertani
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
ppm	Parts per million
%	Percentage
MRMB	Mercury resistant marine bacteria
SWNA	Sea water nutrient agar
SD	Standard deviation
+	Positive
-	Negative
No.	Number

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ABSTRACT

Most of the environmental bacteria that are continuously exposed to mercury present in soil and sediments of lakes and rivers might adopt the heavy metal genotype to sustain in the toxic environment. The most widely studied genetic mechanism of mercury involves *mer* operon mediated mechanism. *mer* operon present in mercury resistant bacteria harbours certain functional genes like *mer*A, *mer*B, *mer*T, *mer*P etc. *mer*B codes for organ mercurial lyase which cleaves the C-Hg bond in organo mercurial compounds which is subsequently converted to non toxic metallic mercury by mercuric ion reductase encoded by *mer*A gene. In the present study, for the first time an attempt has been made to understand the non *mer* mediated mercury resistance mechanism in potent marine bacterial isolates and their role in bioremediation of mercury contamination in the environment. Ten strains from four different sites of Odisha coast showing minimum inhibitory concentration of 25-50 ppm were isolated, which were further studied in order to deduce non *mer* mediated mercury resistance mechanism. The potent mercury resistant isolates when grown under mercury stress were studied to understand bioaccumulation and thus their role in mercury bioremediation in the environment. From the above results it was concluded that marine bacteria play a significant role in detoxification of mercury in the environment either by reducing it to non toxic or by accumulating it inside their cells and therefore they are a key regulator in reducing the environmental pollution.

Keywords: mercury, bioremediation, bioaccumulation, marine bacteria

1. Introduction

Microbial world is invisible to unaided human eyes. So what goes on in their world is not easily comprehended, unless some direct measurements and analyses are carried out. Comprised of bacteria, yeasts, fungi, protozoans, phytoplankton, and other microfauna within 500 μm sizes, microbial communities perform immense tasks mainly to keep themselves perpetuating in their ecosystems. Although it is for their survival, reproduction, and growth the microbial activities of photosynthesis (by microscopic phytoplankton), respiration by all living organisms and breaking down of organic matters into simpler moieties and finally to inorganic molecules by all heterotrophic beings are the pivotal roles that help the earth's ecosystems function and achieve, as far as possible a dynamic equilibrium. All together sunlight is the only external input the life on earth requires. All other matters are produced, consumed and recycled by an array of organisms inhabiting the earth in her varied and often extreme habitats. In essence, the environmental functioning and stability are continuously aided and maintained by microscopic organismic activities. Any instability largely due to their activities through human and natural effects adversely affects the ecosystems.

Pollution due to anthropogenic activities is the greatest problem all the ecosystems were subjected to right from the beginning of human dominance through settled agricultural, the hunting-gathering to modern industrialized civilizations. Within the last two millennia or so, the rise in human population growth and indiscrete consumptions of earth's non-renewable resources have brought about rapid changes to the extent that there are already innumerable degraded/retarded habitats spoiling the Mother Earth rather undesirably. The long term outcomes of retarded habitats are too numerous including societal conflicts, shifts in human settlements, diseases, shifts in community and species diversity, economic losses, global changes and health effects. The one "adjustment" natural organism community makes in the face of all and a life-threatening ill effect of pollution in their metabolic potential is modification in its life-style so much so that some of its representatives "go-on". This adjustment can be termed variously as ***tolerance*** or ***resistance***. While many components of organism communities have the potential for such adjustment, the focus of this study will be the bacterial resistance.

Mercury is a naturally occurring heavy metal. It is unique because it is liquid at atmospheric temperatures and it uniformly expands and contracts in response to changes in temperature and pressure. Mercury occurs naturally and is found in very small amounts in oceans, rocks and soil. Mercury naturally cycles through the environment when rocks break down, volcanoes erupt, and soil decomposes. It then circulates and is distributed throughout the environment. In its metallic form, mercury is a silvery white liquid that reflects light like a mirror.

Mercury is a chemical element with the symbol **Hg** and atomic number 80. A heavy, silvery d-block element, mercury is the only metal that is liquid at standard conditions for temperature and pressure. With a melting point of −38.83 °C and boiling point of 356.73 °C, mercury has one of the narrowest ranges of its liquid state of any metal. It is the 6^{th} most toxic and 12^{th} rarest element. Most commonly, pure mercury is called elemental mercury. Because elemental mercury has high surface tension, it forms small, compact, spherical droplets when it is released into the environment. Although the droplets themselves are static, the high vapour pressure of mercury compared with other metals causes the mercury to evaporate.

1.1. Origin of mercury

Elemental mercury can be produced for human use from an ore called cinnabar, which contains high concentrations of mercury sulfide. Because mercury is a chemical element, it can neither be created nor be destroyed through ordinary chemical or physical means. Mercury is a metal occurring naturally throughout the earth's crust, usually in chemical combination with sulfur or other elements in rocks and minerals. For example, mercury occurs in coal and in ores containing economically important minerals such as copper and zinc. Recovery, purification and use of these materials may release mercury into the environment, as an instance, in process waste streams or combustion emissions. Eroding rocks and minerals form soils that release mercury by venting to the atmosphere and by water transport. Mercury is one of the most toxic elements as it binds to the sulfhydryl groups of enzymes and proteins, thereby inactivating crucial cell functions (Sheffy, 1978).

1.2 General Uses of Mercury

A number of common products contain mercury or mercury compounds.

a. Mercury in medical devices:

Mercury-containing devices have long been used in hospitals and health care settings. This includes thermometers, blood pressure measuring devices (sphygmomanometers), and oesophageal dilators.

b. Mercury containing switches and batteries:

Several kinds of electrical switches contain mercury. These include tilt switches, float switches, thermostats, relays that control electronic circuits, and others. Tilt switches have been commonly used in automobiles to control lamps in trunks and at other locations. Each switch contains, on average, 1.2 g of elemental mercury. Tilt switches have also been used in many other products, although their use has become less prevalent in recent years. These products include washing machines, clothes dryers, freezers, clothes irons, space heaters, television sets, furnace fan limit control switches, security and fire alarm systems, children's novelty shoes with blinking lights, and many others.

Mercury is also used in making batteries. The main use of mercury in batteries is to prevent a build up of hydrogen gas that can cause the battery to bulge and leak. Mercury has also been used as an electrode in mercuric oxide batteries.

c. Mercury in Measuring Devices:

Mercury expands and contracts evenly with changes in temperature and pressure. This characteristic has made mercury useful in scientific, medical, and industrial devices that measure temperature and pressure. Thermometers and sphygmomanometers are the most common mercury-containing measuring devices. Thermometers are used in a variety of applications such as fever thermometers as well as other types of thermometers used in homes and in industrial, laboratory, and commercial applications.

Other mercury-containing measuring devices include the following:

- **Barometers** measure atmospheric pressure. (Each may contain 400 g to 620 g of mercury.)
- **Manometers** measure differences in gas pressure. (Each may contain 30 g to 75 g of mercury.)

- **Psychrometers** measure humidity. (Each may contain 5 g to 6 g of mercury.)
- **Flow meters** measure the flow of gas, water, air, and steam.
- **Hydrometers** measure the specific gravity of liquids.
- **Pyrometers** measure the temperature of extremely hot materials. (They're primarily used in foundries.)

d. Mercury in dental amalgam:

Dental amalgam is a material used by dentists to fill dental caries, or cavities, caused by tooth decay. Dental amalgam fillings are also sometimes called silver fillings because they have a silver-like appearance. The amalgam is a mixture of metals that contains elemental mercury and a powdered alloy composed of silver, tin, and copper. By weight, approximately 50 percent of dental amalgam is elemental mercury.

e. Mercury containing Pesticides and biocides:

Both inorganic and organic mercury compounds have been used as pesticides for a number of applications. The compounds have been used in seed treatments, to control algae and slime in cooling towers and pulp and paper mills, as additives in marine paints and water-based paints and coatings, in wound dressings, in protection for seed potatoes and apples, for fabric and laundry uses, and others.

1.3 Mercury affecting environment

Although mercury is a natural element, the waste and residual contamination from past use of the metal and the ongoing burning of fossil fuels for energy continue to emit mercury into the environment. Mercury can turn into airborne when coal, oil, wood, or natural gas is combusted as fuel or when mercury containing garbage is incinerated. Once in the air, mercury can fall down to the ground with rain and snow. This can then contaminate soil, bodies of water, and the creatures living there.

As, in industrial areas both metal and organic pollution is of vital concern, interest in bacterial resistance to metals, especially when associated with degradative activities, is of practical significance. In industrial areas, organic pollution (fossil fuels or their derivatives, pesticides, PCBs and TBT among others) is often accompanied by inorganic

ones mainly of heavy metals (mercury, cadmium, lead, etc). Without effective retention technologies, toxic chemicals including Hg are permitted to get discharged into the environment, threatening ecosystems and public health.

1.4 Forms of mercury in the environment

Mercury occurs in two primary forms: elemental mercury (Hg^0) and inorganic mercury (Hg^{2+}) fig. 1. Elemental mercury is the pure silvery-white form found in rocks and minerals. It does not blend with other chemicals. Inorganic mercury can combine with other chemicals to form compounds. Combustion may release both elemental and inorganic mercury from materials containing them. Combustion also releases fine particles that may carry small amounts of mercury bound to their surfaces.

In addition to elemental and inorganic mercury, there are other organic mercury compounds such as methylmercury (MeHg). Exposure to methylmercury is the main cause of public health concern about mercury.

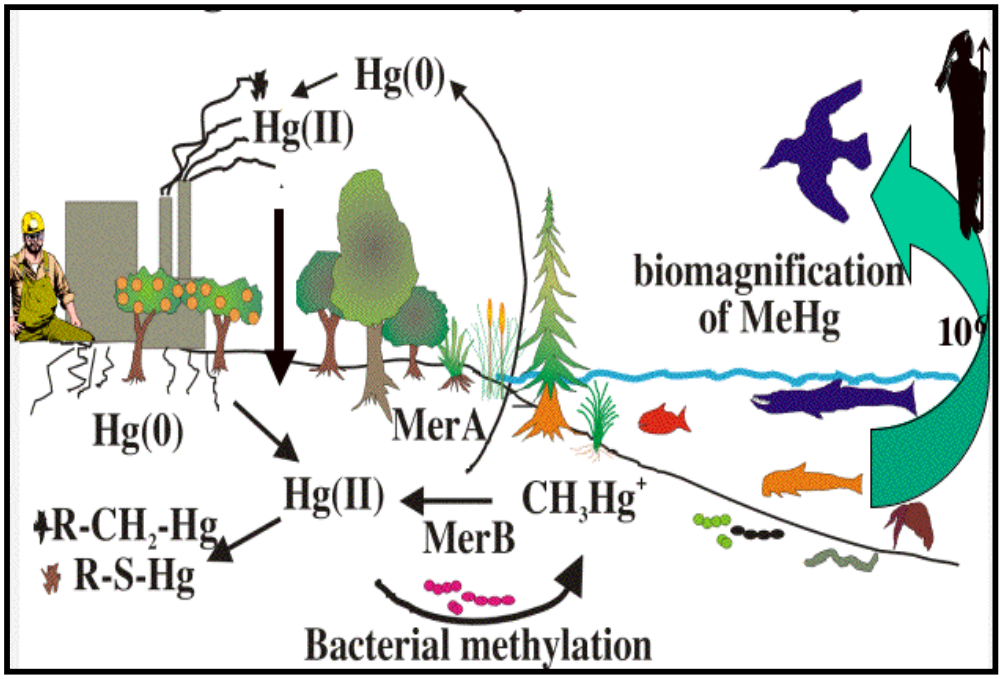


Fig 1. Biogeochemical cycle of mercury in the environment

1.5 Mercury Pollution

Mercury pollution of the environment by mining activities and industrial has resulted in worldwide contamination of large areas of soils and sediments and let to elevated atmospheric mercury levels (Baldwin and Marshall, 1999). Because of lack of suitable cleanup technologies, efforts to cope with polluted sites are directed toward the mechanical removal of contaminated material and its deposition elsewhere. Such treatments are costly and frequently result in remobilization of toxic mercury compounds during the degrading process (Bogdanova et al., 1992). Sources of mercury pollution include:

- Dental filling
- Production of electrical apparatus
- Chloro-alkali industries
- Agricultural industries fungicides
- Gold extraction
- Coal fired power plants
- Treatment of syphilis
- Steel industry

1.6 Effects of Mercury on Human Health

Mercury is one of the most toxic elements as it binds to the sulfhydryl groups of enzymes and proteins, hence inactivating crucial cell functions (Sheffy, 1978). After discharge of mercury into the environment, mercury enters the sediments where it remains for many decades. It is acquired by aquatic organisms in the form of highly toxic methyl mercury and is subsequently biomagnified through the food chain and thus the health of top predators, e.g., birds, fish, seals, and man, is ultimately threatened.

At high concentrations, mercury vapour inhalation produces acute necrotizing bronchitis and pneumonitis, which is result in death due to respiratory failure. Long term exposure to mercury vapour primarily affects the central nervous system and it also accumulates in kidney tissues, straight away causing renal toxicity, including proteinuria or nephritic syndrome. High concentration of Hg^{2+} causes impairment of pulmonary function and kidney, chest pain and dyspnousea (Carrier, 2001). Mercury poisoning causes a decreased ability to see, hear, talk and walk. It can lead to personality changes,

natural depression, irritability, nervousness, and the inability to concentrate. Mercury is a serious problem particularly for pregnant women and children. Foetuses and young children suffer the greatest risk because their nervous systems are still developing. They are 4-5 times more sensitive to mercury than adults. Therefore, the discharge of mercury into the environment needs to be prevented by efficient and cost-effective end-of-pipe treatment technologies for mercury emitting industries (Kleinert and Degurse, 1972). Cleaning of areas polluted by heavy metals such as mercury is difficult, because the metals cannot be altered into harmless elements.

Over a few decades, community is devoting concentrated efforts for the treatment and removal of heavy metals in order to face this problem. Various types of technology is available for removing of mercury in water and wastewater including chemical precipitation, conventional coagulation, reverse osmosis, ultrafiltration, magnetic filtration, ion exchange and activated carbon adsorption and chemical reduction (Wood, 1972).

1.7 Bacterial Transformation of Mercury in the Environment

Biological systems have been thought to be adapted for removal of toxic heavy metals. Bioremoval in biological systems for removal of metals ion from polluted water has the potential to achieve greater performance at lower cost than non-biological wastewater treatment. Developments in the area of environment biotechnology indicate the bacteria, fungi, yeasts and algae can withdraw heavy metals from aqueous solution by adsorption.

Bacteria have a unique important role in the mercury biogeochemical cycle. Earlier it was generally assumed that discharges of elemental mercury remained relatively inert in the environment. More recent epochs have shown that inorganic mercury can be methylated by organisms present in the soil and sediment of rivers and lakes. Their most substantial contribution is the conversion of inorganic mercury to methylmercury by the insertion of a covalent bond between the carbon and mercury atoms. This reaction allows the mercury to penetrate cell membranes more rapidly and accumulate within the cells by complexing with important proteins, enzymes and nucleic acids. These reactions demonstrate that all mercury compounds represents a potential threat to living beings, since in the presence of bacteria, any compound containing mercury cab be transformed to the highly poisonous organic substances

which are readily incorporated into living tissues. The end products of bacterial reactions (fig.2) with mercury are either a gas or an insoluble precipitate which is removed from their immediate environment (Baldi, 1997).

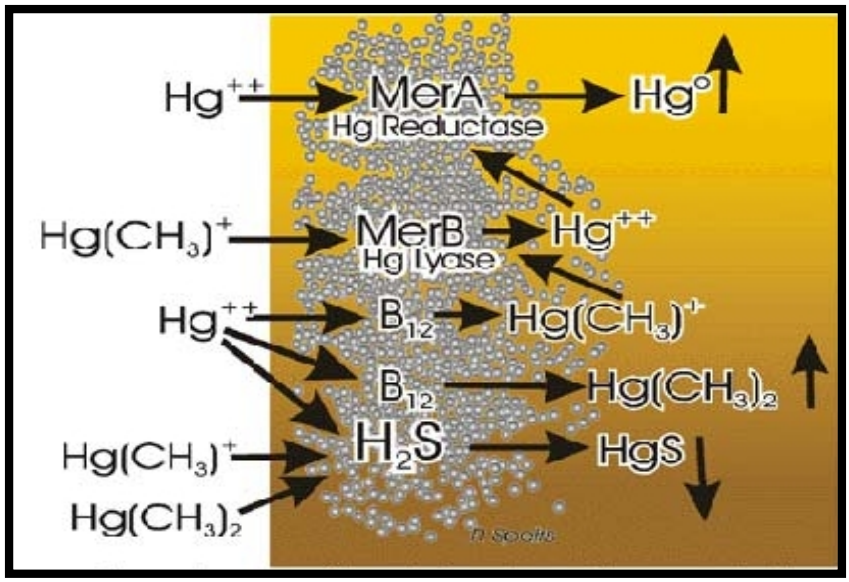


Fig 2. Bacterial Transformation of Mercury in the Environment

1.8 Bacterial Resistant to Mercury by *mer* Operon

As a response to toxic mercury compounds globally distributed by geological and anthropogenic activities, microbes have acquired a surprising array of resistance mechanisms to overcome mercury toxicity. The best studied resistance system primarily based on clustered genes in an operon (i.e. *mer*), allows bacteria to detoxify Hg2+ into volatile mercury by enzymatic reduction (Komura & Izaki, 1971; Summers, 1986; Mishra, 1992; Silver, 1996; Osborn et al., 1997 and Barkay et al., 2003). Resistance against mercury compounds in the environment, mediated by the microbial *mer* operon was discovered in the early 1970's (Summers and Lewis, 1973). Since then, several "archetypical" *mer* operons (Liebert et al., 1999) have been well studied in depth with respect to structure, function and regulation of the individual gene products (Ji and Silver, 1995 and Barkay et al., 2003). Genes conferring resistances to mercury compounds are clustered in an operon in most known naturally occurring systems (Silver and Phung, 1996 and Barkay et al., 2003).

The *mer* resistance elements can be subgrouped into three categories based on their functional roles:

- transporters of Hg(II) into the cells,
- converters (enzymatically) of toxic mercury compounds into a relatively nontoxic form [Hg(0)], and
- regulators of operon expression.

Most *mer* operons contain atleast the mercury-resistance genes *mer*R, *mer*D, *mer*T, *mer*P and *mer*A (Silver & Phung, 1996 and Osborn et al., 1997). Expression of *mer* operon is regulated by the products of *mer*R and *mer*D and is inducible by Hg (II). The product of *mer*R represses operon expression in the absence of inducer and activates transcription in the presence of inducer. The product of *mer*D coregulates expression of operon (Misra, 1992; Silver & Phung, 1996). Products of periplasmic *mer*T and inner membrane-*mer*P take part in the transport of metal across the cell membrane. Products of *mer*C and *mer*F, (membrane proteins) were found to act as mercury transport system (Kusano et al., 1990). Bacteria that are resistant solely to Hg(II) have a so-called "narrow spectrum resistance", whereas others that are resistant to both Hg(II) and certain organomercurials are with broad-spectrum resistance. Resistance against organomercurials depends on the organomercurial lyase (encoded by ***merB***) that cleaves the carbon-mercury bond of the organomercurials, and the resultant product Hg (II) can be subsequently reduced by the mercuric reductase (Silver & Mishra, 1984). In some cases, the *mer* operon contains different functional genes. The *mer*G product renders phenylmercury resistance, presumptively by reducing the in-cell permeability to phenylmercury (Kiyono & Pan-Hou, 1999).

Although the presence of mercuric reductase is essential for enzymatic detoxification and hence resistance to inorganic mercury, expression of *mer*A gene (and *mer*R), has been reported in a very high proportion of gram-positive environmental strains sensitive to mercury, suggesting the existence of non-functional *mer* operons in which the mercury transport genes are either absent or non-functional (Bogdanova et al., 1992).

Although the physical arrangement of the *mer* operons may vary, all contain the requisite genes but surprisingly, only limited studies have sought to characterize mercury

resistance at the molecular level in marine bacterial isolates (Barkay et al., 1989 and Rasmussen & Sorensen, 1998).

1.9 Alternative Mechanism of Mercury Resistant in Bacteria

There are certain groups of bacteria capable of tolerating high concentration of mercury but does not have *mer* operon in their genotype. Hence definitely an alternative mechanism of mercury resistance exists in those group of microorganisms which not only convert the toxic form of mercury to non toxic form (*mer* operon mediated), but accumulation of mercury in the bacterial cell mass which reduces the concentration of mercury in the environments. Hence, mercury bioaccumulation results in gradual decrease of mercury concentration in the polluted sites when mercury accumulating bacteria are employed in those sites. Though mercury is highly toxic to any living organisms by readily binding with the sulphur containing amino acids of the proteins, hence mercury bioaccumulation provides a new discovery regarding mercury bioremediation. Bioaccumulation of other heavy metals in bacterial cytoplasm have been reported by many workers (Kotrba et al., 1999; El-Hendawy et al., 2009; Ahemad and Malik, 2012), however so far there is no report regarding bioaccumulation of mercury in bacterial cell. Hence the present work will provide a new line of discovery regarding mechanism of mercury resistance in marine bacteria which can be potentially utilized in the contaminated sites for remediation of the same for a better future.

2. Review of Literature

Mercury and its compounds are distributed widely all around the earth. Many of the chemical forms of mercury are toxic to all living organisms. However, bacteria have developed mechanisms of resistance to several of these totally different chemical forms, and thus play a major role in the global cycling of mercury in the natural environment. Five different mechanisms of resistance to mercury compounds have been described, of which resistance to inorganic mercury (Hg^R) is best studied, both in terms of the mechanisms of resistance to mercury and resistances to heavy metals in general.

i) Reduced uptake of mercuric ions.

This has been reported in a strain of *Enterobacter aerogenes* where resistance is believed to be due to the expression of two plasmids encoded proteins which cause a reduction in the cellular permeability to Hg^{2+} ions (Pan et. al., 1981).

ii) Demethylation of methylmercury followed by conversion to mercuric sulphide compounds.

In *Clostridium cochlearium* T-2P two plasmids encoded genetic factors are believed to be responsible for the demethylation of organomercurial compounds which are subsequently inactivated by reaction with hydrogen sulphide to form insoluble mercuric sulphide (Pan et. al., 1981).

iii) Sequestration of methylmercury.

In *Desulfovibrio desulfuricans* API, methylmercury is maintained at subtoxic levels by the continuous production of hydrogen sulphide, from the dissimilative reduction of sulphate, which reacts with methymercury to form insoluble dimethylmercury sulphide (Baldi et. al., 1993).

iv) Mercury methylation.

Although methymercury is generally considered to be more toxic than Hg^{2+}, in some bacteria methylmercury may be the less toxic form, possibly due to subsequent sequestration or volatilisation from the cell. Methylation has been identified in bacteria

from sediment, water, soil and the gastrointestinal tract, and is both plasmid and chromosomally encoded (Trevors, 1986). In *Desulfovibrio desulfuricans* LS the methylation of mercury occurs as a two step process which involves the transfer of a methyl group from methyltetrahydrofolate to methylcobalamin to Hg (Choi et al.,1994)

v) Enzymatic Reduction of Hg^{2+} to Hg^{0}.

Reduction occurs both in Gram-negative and Gram-positive aerobic bacteria from a variety of natural and clinical environments across the globe, and as such has become the best studied of the mercury resistance mechanisms. Mercury resistance is often located on conjugative plasmids and/or transposons (Radford et al., 1981, Kelly et al., 1984, Jobling et al., 1988a and Peters et al., 1991) and in particular is often borne on class II transposable elements, typified by that carried by Tn2*I* (Grinsted et al., 1990). Furthermore, such Hg^{R} plasmids or transposons often carry resistances to other heavy metals and/or antibiotics.

Resistance to inorganic mercury can be located on transposons, plasmids and bacterial chromosomes and is encoded by the genes of the *mer* operon. Such systems have a worldwide geographical distribution, and moreover, they are found across a wide range of both Gram-negative and Gram-positive bacteria from both natural and clinical environments. The presence of *mer* genes in the bacteria from sediment cores suggests that *mer* is an ancient system. DNA sequences analysis from *mer* operons and genes have revealed genetic variation both in operon structure and between individual genes from different *mer* operons, although analysis of bacteria which are sensitive to inorganic mercury has identified number of vestigial non-functional operons. It is speculated that *mer*, due to its ubiquity with respect to environment, species range and geographical locations is an ancient system and that ancient bacteria carried genes conferring resistance to mercury in response to increased levels of mercury in natural environments, possibly resulting from volcanic activity. The *mer* operons from both gram-positive and gram-negative bacteria have been cloned and sequenced. Biochemical and Genetic studies have advanced our knowledge, leading to in- depth understanding of gene evolution, resistance mechanism, and regulation of expression of the *mer* genes (Mishra, 1992).

Mercury resistant bacteria (MRB) are those bacteria that grow in presence of 10 ppm mercury (as $HgCl_2$) in the seawater nutrient agar medium (SWNA). Izaki (1981) had used 2 ppm mercury (as $HgCl_2$) to isolate mercury-resistant bacteria whereas Baldi et al. (1989) had used 10 ppm Hg for the same. Many of those MRB were able to grow at an Hg (as $HgCl_2$) concentrations of 25 ppm or higher and were termed as bacteria highly resistant to mercury (Nascimento et al., 2003).

2.1 Mercury in the environment:

Mercury, the only metal in liquid form at room temperature is the most toxic of the heavy metals (Dechwar et al., 2004) and the sixth most toxic chemical in the list of hazardous compounds (White et al., 2005). Erupted from the core of earth by volcanic activity it exists as mineral (mostly as cinnabar-HgS), as mercuric oxide, oxychloride, sulfate mineral (Kiyono and Pan Hau, 2006) or also as elemental mercury. It also exists as gas due to its high vapour pressure.

In a biogeochemical cycle (Fig. 3) mercury is globally diffused undergoing many physical and chemical transformations (Barkay et al., 2003):

- in the atmosphere elemental mercury is photo-oxidized to ionic mercury ($Hg2^{+}$).
- rain precipitates the inorganic mercury on the surface of the earth, where carried out mainly by microorganisms in aquatic systems,
- it is reduced back to its elemental form or
- methylated.
- elemental mercury evaporates into air where the cycle begins anew.

Mercury is rapidly adsorbed on top of sediments, which can serve well as a source of mercury for years (Kornad, 1971; Matsumura et al., 1972; McDuffie et al., 1976). For this reason, most of the mercury available for cycling in freshwaters is present in bed sediments.

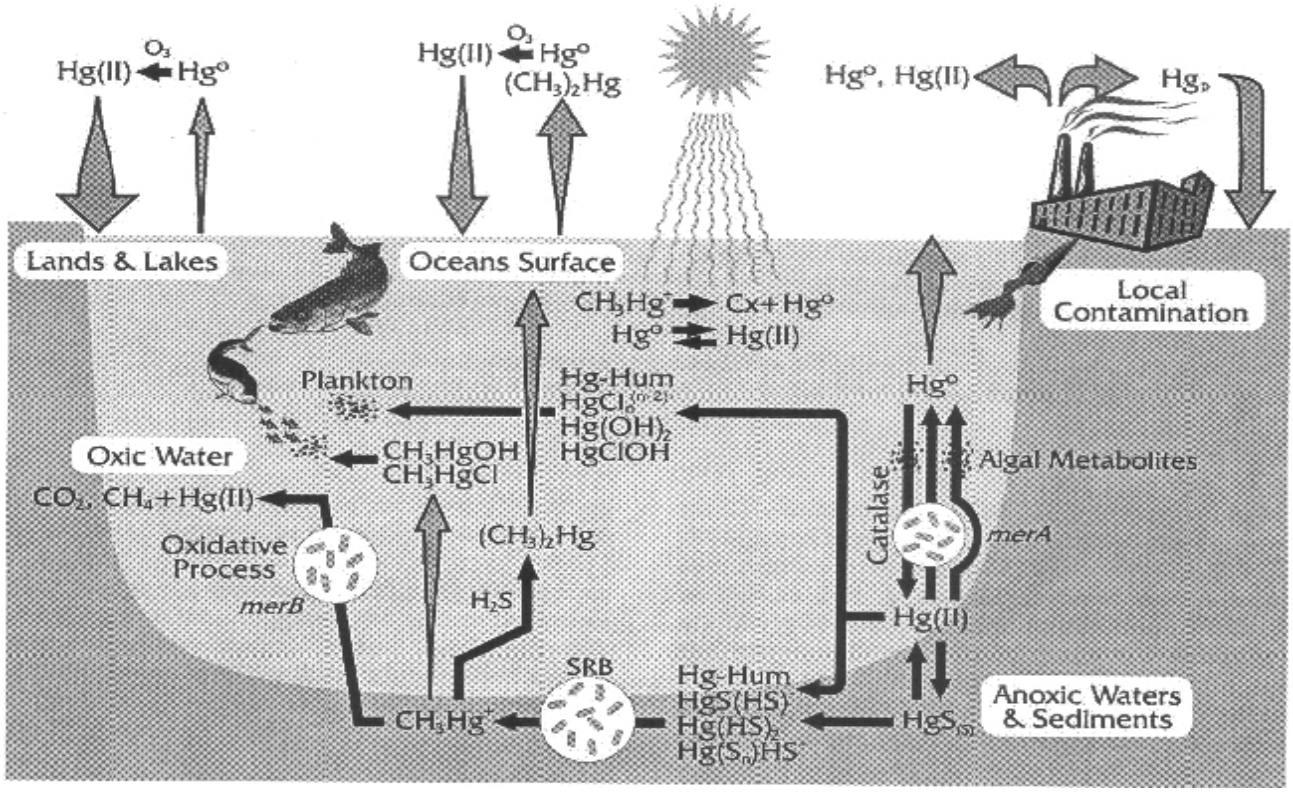


Fig 3. Fate of mercury in the environment

The major public health concern with mercury is the bioaccumulation and biomagnificattion of methyl mercury (CH_3Hg^{2+}) by the aquatic food chain. These in turn, are determined by rates of Hg transformation and transport in the environment. Black arrow depicts the transformation and gray arrow depicts the transfer pathways (Barkay et al., 2003).

2.2 Bacterial bioremediation of mercury contamination:

There are two main types of reactions in the mercury cycle that convert mercury through its various forms: oxidation-reduction and methylation-demethylation. In oxidation-reduction reactions, mercury is either oxidized to a higher valence state (e.g. from relatively inert Hg^0 to the more reactive Hg^{2+}) by the loss of electrons, or mercury is reduced, the reverse of being oxidized, to a lower valence state.

i) Mercury Oxidation

The oxidation of Hg^0 in the atmosphere is an important mechanism involved in the deposition of mercury on water and land. Elemental mercury (Hg^0) can volatilize relatively easily and be emitted into the atmosphere, where it may be transported onto the

wind currents for a year or more and be re-deposited in the environment for further cycling. In contrast, Hg^{2+} has an atmospheric residence time of less than two weeks due to its low volatility, solubility in water and reactive properties. Therefore, when (Hg^{0}) is converted to Hg^{2+}, it can be rapidly taken up in rain water, snow, or adsorbed onto small particles, and be subsequently deposited in the environment through "wet" or "dry" deposition.

ii) Mercury Methylation

In the environment, mercury is transformed into methylmercury when the oxidized or mercuric species (Hg^{2+}), gains a methyl group (CH_3). The methylation of Hg^{2+} is mainly a natural, biological process leading to the production of highly toxic and bioaccumulative methylmercury compounds ($MeHg^{+}$) that build up in living tissue and increase in concentration of mercury in the food chain, from microorganisms to small fishes, then to fish eating species like loons and humans (Fig.4).

Understanding the variables influencing the formation of methylmercury is critically important due to its highly toxic, bioaccumulative and tenacious nature. Many microorganisms, particularly methanogenic (methane producing) and sulfate-dependant bacteria are thought to be involved in the conversion of Hg^{2+} to MeHg under anaerobic (oxygen poor) conditions found, for example, in rivers sediments and wetlands, as well as in certain soils. Methylation mainly occurs in aquatic, low pH (acidic) environments with high concentrations of organic matter.

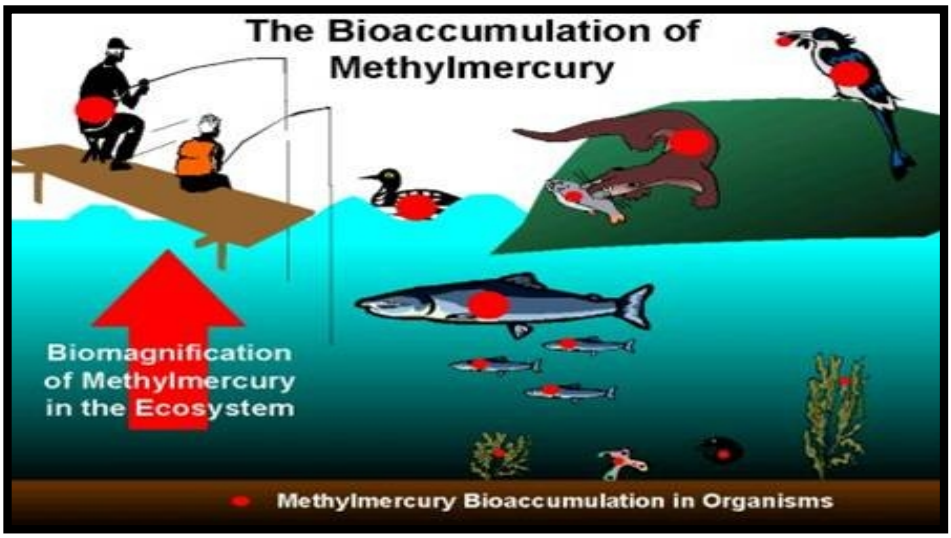


Fig. 4 The bioaccumulation of methylmercury in organisms.

2.3 Toxicity of Mercury

Even small amounts of mercury are toxic for all organisms. Mercury binds to the sulfhydryl groups of enzymes and proteins, hence inactivating vital cell functions (Dobler et al., 2000b).The most noted examples of environmental contamination with mercury occurred in Japan between 1953 and 1970 (Irukayama, 1966; Tsubaki, 1968). In Minamata, between 1953 and 1961, 121 fishermen and their families were stricken with a mysterious illness characterized by cerebellar ataxia, constriction of visual fields, and dysarthria. Of these 121 cases, a total of 46 deaths resulted. Additional cases of mercury induced poisoning, termed "Minamata Disease," were assured in the coastal town of Niigata and in the riverside villages along the Agano River between 1965 and 1970 (Konrad, 1971). Six persons died and another forty-one were irreversibly poisoned. In both incidents, the disease broke off mainly among fishermen and their families, and also among other people who fished frequently and/or liked to eat locally caught aquatic produce. Characteristically, the patients in Minamata as well as in Niigata had eaten a great amount of fish and/or shellfish from contaminated waters.

2.3.1. Inorganic Mercury Compounds

The toxicity of heavy metals is a result of their binding to active sites of important enzyme systems in the cells and their binding to 1igands in the cell membrane thereby resulting in a variety of toxic effects (Passow et al., 1961). Inorganic mercury compounds concentrate in the liver, spleen and kidney. They are readily egested, however, and do no damage unless the threshold tolerance level of the organ is exceeded (D'Itri, 1972). Prolonged exposure to inorganic mercury compounds is required for toxic symptoms to develop. The symptoms of inorganic mercury poisoning develop gradually. The first clear physical symptoms are numbness of the fingers and toes and then of the tongue and lips (D'Itri, 1972). Weakness, anorexia, loss of weight, fatigue and disturbances of gastrointestinal functions are associated with fully developed clinical forms of chronic poisoning (Friberg and Vostal, 1972). Late phases are characterised by mercurial tremor, psychic disturbances, and changes in personality (Friberg and Vostal, 1972). Prolonged exposure to high concentrations of inorganic mercury can result in death.

2.3.2 Organic Mercury Compounds

Organic mercury compounds are most toxic forms of mercury and can be divided into two categories: those in which the mercury atom is bonded to one organic radical and those in which it is bonded to two organic radicals. The first type dissociates in water to yield the R-Hg+ cation and the X- anion, making it soluble in water. Mercury is covalently linked to a carbon atom in organic mercury compounds (Nordberg, 1976). Methyl mercury can be formed from mercuric ion by a variety of microorganisms, including anaerobes, aerobes, and facultative anaerobes. Hence, the potential for microbial methylation exists under both aerobic and anaerobic conditions. Most organic mercury compounds are rapidly excreted and therefore, pose no serious health problems (Jugo, 1979). However the short-chain alkyl mercury compounds, such as methyl mercury, are formed in aquatic environments via methylation of inorganic mercury. Methylmercury is amongst the most toxic of all mercury compounds (Cassidy and Furr, 1978; D'Itri, 1972).

The mercury-carbon bond in methyl mercury is extremely stable and the attachment of the alkyl radical increases solubility of lipids. This helps in penetration of the blood brain barrier and cell membranes (Felton et al., 1972). Nervous tissue tends to accumulate the greatest concentrations of methyl mercury (Chang and Hustman, 1972). Methyl mercury rapidly diffuses through the cell membrane and enters the cell where it is rapidly bound by sulfhydryl groups. Inside the cell, methyl mercury suppresses protein and RNA syntheses (Jugo, 1979). Methyl mercury concentrates in the body during a latent period during which no symptoms are observed. After threshold levels are exceeded, serious effects on the central nervous system might occur (D'Itri, 1972). Symptoms of methyl mercury poisoning include headache, numbness of the extremities, fatigue, blurred vision that can lead to blindness, and poor muscular coordination (Jugo, 1979).

2.4 Sources of Mercury:

The four main natural processes that leads to Hg emission are:

i. degassing from geological mineral deposits,
ii. emissions from volcanic activities,
iii. photoreduction of divalent mercury in aquatic systems and
iv. biological formation of elemental and methyl mercury.

Although it is undisputed that mercury occurs naturally and toxic concentrations in some locations, mercury emissions owing to anthropogenic activities (mainly through chloralkali electrolysis and chlorine production), mining and burning fossil fuel or waste incineration are immense, contributing considerably to the mercury pool participating in the biogeochemical cycle (Komura et al.,1971). However, the concentrations of mercury in various compartments from natural and anthropogenic sources are highly variable.

2.5 Effects of Mercury Contamination on Microorganisms:

a. Effects on microbial activities:

Few studies have been attempted to determine the effect of mercury contamination on other microbial activities. Pedersen and Sayler (Nordberg, 1976) found that $HgCl_2$ had no significant effects on methanogenesis. Research by Winfrey (unpublished) confirmed these resu1ts. The sediment environment may protect the methanogenic population from the toxic effects of mercury (Pederson and Sayler, 1981). Effects of mercury on other microbial activities have apparently not been investigated.

b. Ecology of mercury resistant bacteria:

Many bacteria possess a variety of resistance mechanisms to the toxic effects of mercury. Resistance depends on the strain, species, and genus of bacteria. Nelson and Colwell (Nelson and Colwell, 1975) showed that H_2S production is not an exclusive property of mercury resistant bacteria.

2.6 Bacterial resistance to mercury:

As a response to toxic mercury compounds globally distributed by geological and anthropogenic activities, microbes have developed a surprising array of resistance mechanisms to overcome Hg toxicity (Pahan et al., 1990). However, some bacterial communities residing in the mercury contaminated areas can exchange mercury resistance genes between each other, because of continually exposure to the toxic levels of mercury. After the acquisition of resistance genes, those bacteria will be resistant to mercury (Nascimento and Souza, 2003). An extensively studied resistance system based on clustered genes in an operon (i.e. *mer*), allows bacteria to detoxify Hg^{2+} into volatile mercury by enzymatic reduction (Deckwer et al., 2004; White et al., 2005; Kiyono and Pan Hau, 2006). It appears that bacterial resistance to mercury is an ancient

mechanism, probably acquired even before anthropogenic usage of mercury. Since the same biotransformation that constitute the Hg biogeochemical cycle can take place inside the human body, understanding its external transformations and transport processes will help in figuring out which of these processes can exacerbate or ameliorate Hg toxicity in humans (Barkay et al., 2003).

2.6.1 Biochemical Basis and Molecular Basis of Bacterial Mercury Resistance:

a. Formation of insoluble HgS:

In the presence of hydrogen sulfide, mercuric ions (Hg^{2+}) spontaneously precipitate as mercuric sulfide (HgS) (Furukaura et al., 1969). Under anaerobic conditions, the formation of mercuric sulfide effectively reduced availability of mercuric ion for biological conversions. In the presence of oxygen, mercuric sulfide may be converted to methyl mercury by bacteria; however, this occurs at a rate 100-1000 times slower than mercuric ion methylation (Fagerstrom and Jernelov, 1971). Therefore, the presence of sulfide reducing bacteria prevents methyl mercury [$(CH_3)_2Hg$] and mercuric sulfide in the presence of hydrogen sulfide. Mercuric ion may also be reduced to the volatile elemental mercury by resistant bacteria. This reaction results in the release of mercury from aquatic systems (Colwell et al., 1976).

Mercury volatilization might be expected to occur readily than methylation due to the large numbers of bacteria capable of carrying out this reaction in aquatic sediments (Colwell et al., 1976) and the kinetics of volatilization in bacterial cultures compared to methylation.

b. Enzymatic reduction Hg^{2+} to Hg^0 and volatilization:

The biochemical basis of resistance to inorganic mercury compounds such as $HgCl_2$ appears to be quite similar in several different species (Canovas et al., 2003). It involves the reduction of Hg^{2+} to volatile Hg^0 by an inducible enzyme, mercuric reductase. This reductase is a flavoprotein, which catalyzes the NADPH-dependent reduction of Hg2+ to Hg0. Since mercury has such a high vapor pressure, it volatilizes and the bacterial environment is left mercury free. This mercuric reductase is found intracellularly (Furukawa and Tonomura, 1972; Summers1972; Schottel, 1978).

As a response to toxic mercury compounds globally distributed by geological and anthropogenic activities, microorganisms have developed a surprising array of resistance systems to overcome the poisonous environment (Canstein et al., 1999). An extensively studied resistance system, based on clustered genes in an operon (*mer* operon), allows bacteria to detoxify Hg2+ into volatile metallic mercury by enzymatic reduction (Komura and Izaki, 1971; Summers, 1986; Misra, 1992; Silver, 1996; Osborn et al., 1997). Mercury-resistance determinants have been found in a wide range of Gram-negative and Gram-positive bacteria isolated from different environments. They vary in the number and identity of genes involved and are encoded by *mer* operons, usually located on plasmids (Summers and Silver, 1972; Brown et al., 1986; Griffin et al., 1987; Radstrom et al., 1994) and chromosomes (Wang et al., 1987; Inoue et al., 1991); they are often components of transposons (Misra et al., 1984; Kholodii et al., 1993) and integrons (Liebert et al., 1999).Two main *mer* determinant types have been described: narrow-spectrum *mer* determinants confer resistance to inorganic mercury salts only, whereas broad-spectrum *mer* determinants confer resistance to organomercurials such as methyl mercury and phenyl mercury, as well as to inorganic mercury salts (Misra, 1992; Silver and Phung, 1996; Bogdanova et al., 1998). The functions of *mer* operon are as follows:

i. Transport of Hg^{2+} into the cell
ii. Enzymatic NADPH dependent conversion of the ionic mercury into relatively less toxic elemental mercury (Hg^0)
iii. Regulation of the functional genes
iv. Cleavage of mercury from the organic residue and the resistance is termed as “Broad spectrum”

The genes involved in *mer* operon are shown in Fig.5**:**

a) *mer T*, *mer P* (Transport)
b) *mer A* (Mercury reduction)
c) *mer B* (Cleavage of mercury from organic residue)
d) *mer R* and *mer D* (regulation)
e) *mer C* and *mer F* (Membrane proteins, conferring transport functions)
f) *mer G* (resistance to phenyl mercury)

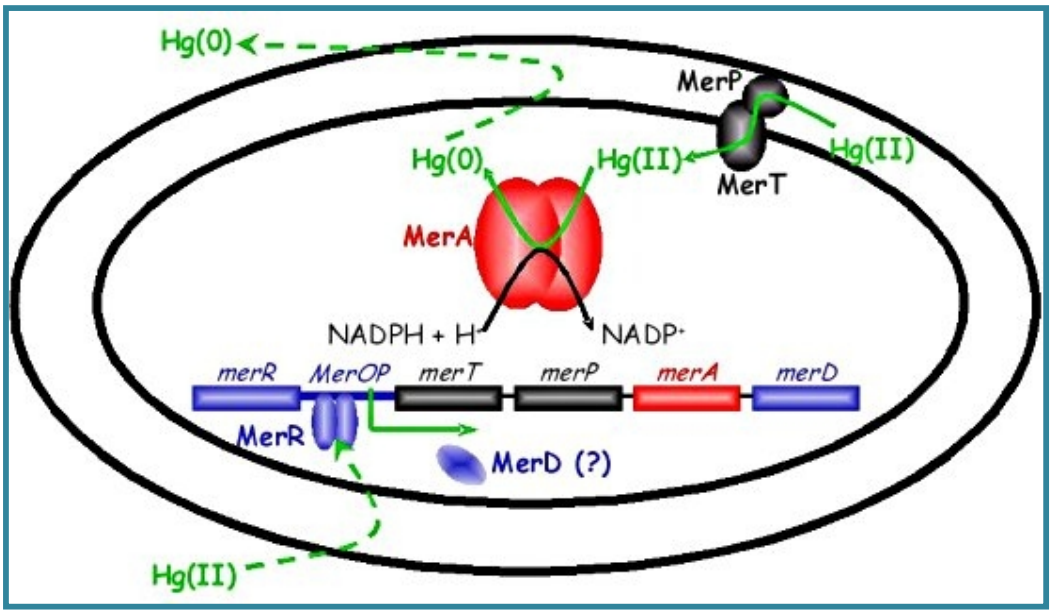


Fig. 5 The *mer* operon including the regulators (*merR* and *merD*), transporters (*merP* and *merT*), mercuric reductase (*merA*) and organomercurial lyase (*merB*).

Different mer genes in mer operon play different roles. The functions of these genes are as follows:

1. ***mer R*:** Metalloregulatory DNA binding protein that acts as a repressor of both its own and structural gene transcription in the absence of Hg (II). In addition it acts as a positive effector of structural gene transcription when Hg (II) is present.
2. ***mer B*:** Organomercury lyase, catalyzes the protonolytic fragmentation of organomercurials to the parent hydrocarbon and Hg(II) by S_E2 mechanism.
3. ***mer A*:** Mercuric ion reductase, is an FAD containing and redox active disulfide containing enzyme with homology to glutathione reductase.This enzyme reduces Hg^{2+} compounds to the metallic mercury Hg^0 which is obviously less toxic to them (Deckwer et al., 2004). It has the unique capacity to reduce Hg(II) to Hg(0) and thereby complete the detoxification scheme.

   Based on a comparison with other bacterial periplasmic binding, protein-dependent transport systems, it has been proposed that Hg2+ diffuses across the outer membrane (Brown, 1985). Mercuric ions are transported outside the cell by a series of transporter proteins. This mechanism involves the binding of Hg^{2+} by a pair of cysteine residues on the *merP* protein located in the periplasm(Chang et al., 1993). Hg2+ is then transferred to a pair of cysteine residues on *merT*, a cytoplasmic membrane protein, and finally to a cysteine pair at the active site of MerA (mercuric

reductase) (Hamlett et al., 1992). Next, Hg^{2+} is reduced to Hg0 in an NADPH-dependent reaction. The non-toxic Hg0 is then released into the cytoplasm and volatilizes from the cell.

4. ***mer* D:** A small, cysteine-rich open reading frame (ORF) lying just beyond the *mer* A gene of Tn501. Purified *mer* D binds to mer O although with a lower apparent affinity compared to *mer* R. Thus, *mer* D appears to be an antagonist of *mer* R function, perhaps replacing it at *mer* O although other mechanisms or roles for *mer* D have not been ruled out. *Mer* D is also unique protein with no homologs with identified functions.
5. ***mer* P:** Although *mer* P does not resemble any periplasmic transporter involved in normal metabolism, the *mer* P motifs appears to be quite ancient and widely disseminated in proteins involved in both membrane transiting and cell interior trafficking of thiophilic metal cations. Interstingly, *mer* P is not essential for Hg (II) uptake as *mer* T alone will suffice.
6. ***mer* T:** *mer* T is the other player in Hg (II) transport in both Gram-positive and Gram-negative bacteria (excepting *Acidothiobacilli* which apparently use only *mer* C) (Hamlett et al., 1992 and Lund and Brown, 1987). There are no reported physical studies on *mer* T, largely owing to the difficulty of such studies on membrane proteins. Possible homologs of the *mer* inner membrane proteins with known functions have not been spotted, although doing so might be difficult.
7. ***mer* C:** This 161-residue membrane-bound protein with four predicted transmembrane helices is the largest of the generally small mer operon encoded membrane proteins. Its appearance in only one of the first two, otherwise very similar *mer* operons sequenced was the first hint of the mosaic character of the opeon (Summers, 1986). Studies (Liebert et al., 2000) concluded that *mer* C is evolving differently than genes immediately adjacent to it in the operons where it occurs and may be also evolving in different hosts. It has been suggested that *mer* C may be needed under conditions of very high Hg(II) exposure (Olson et al., 1992), but this point has not been explicitly tested. *mer* C is not uniquely associated with *mer* B (Liebert and Summers, 1997) or *mer* G (Kholodii et al., 2002).

8. ***mer* F:** The *mer* F gene was first noted between the *mer* P and *mer* A genes in a plasmid-borne *mer* operon in an environmental pseudomonad (Hobman et al., 1994). Nigel Brown's group (Wilson et al., 2000) demonstrated that *mer* F is located in a crude membrane fraction derived from radiolabelled maxicells. Expression of *mer* F facilitated volatilization of Hg(II) but this activity was not enhanced by *mer* P.

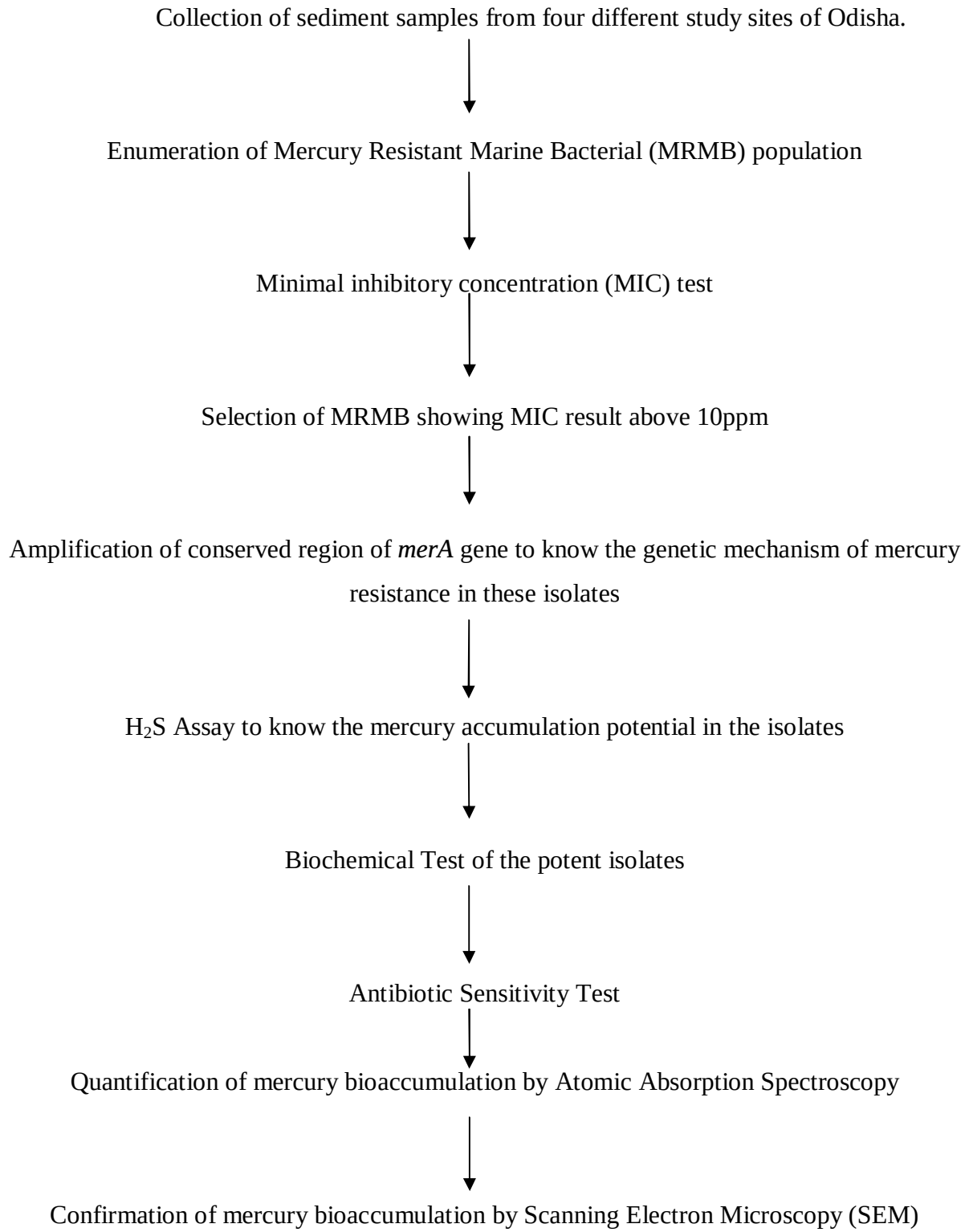
3. OBJECTIVES AND PLAN OF WORK

Keeping in mind the above views, present research is based on the following objectives:

3.1 Objectives

- To study the pollution status in study sites of Bay of Bengal by enumerating mercury resistant marine bacterial populations as indicator organisms
- To determine minimum inhibitory concentration (MIC) of the isolates
- To know the mechanism of mercury resistance in these isolates
- To deduce an alternative mechanism in the isolates other than *mer* operon mediated mercury resistance mechanism in mercury resistant marine bacteria

3.2 Plan of Work



4. MATERIALS AND METHODS

4.1. Sample Collection

Water samples were collected from the study sites of four different places of Bay of Bengal along the Odisha coast. The four study sites include Chilika (19°44.582' N & 85°12.768'E), Bhitarakanika (20°44.33'N & 086°52.06'E), Gopalpur (19°19.218'N & 084° 57.730'E) and Rushikulya (19°22.647'N & 85°03.165'E) (fig.6). The samples were collected in sterilized falcon tubes and transported to the laboratory by keeping them on ice and processed immediately in the laboratory for enumeration of mercury resistant marine bacteria in the population as soon as possible.

Fig 6. Study sites [A: Bhitarakanika, B: Chilika, C: Gopalpur and D: Rushikulya]

4.2 Enumeration of Mercury Resistant Marine Bacterial (MRMB) populations

Samples from different locations were plated onto Sea Water Nutrient Agar (SWNA) amended with 10ppm Hg (≡50µM; as $HgCl_2$). For enumeration of MRMB, 10-15ml water sample was filtered through 0.22µm filters, plates were incubated at temperature 37^0C and final counts of colony forming units (CFUs) taken after 48h. Total viable counts (TVC) from each sample were also enumerated by plating aliquots in triplicates on SWNA without added Mercury (Jayashankar and Ramaiah, 2006).

A total of ten mercury resistance isolates were selected for further characterization based upon their observable distinguished colony morphology.

4.3 Colony Morphology

Size, shape, color, elevation and margins of the isolated bacterial colonies were observed for 24 hours incubated cultures, on the Sea Water Nutrient agar (SWNA) media plates supplemented with 10ppm $HgCl_2$.

4.4 Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration is the lowest concentration of metal that completely prevented bacterium growth (De and Ramaiah, 2007; Gupta et al., 2005). MIC test was done by micro dilution technique as per Cinical Laboratory Standard Institute (CLSI) guide lines. (CLSI, 2006) which is as follows:

1. 300µl of 100ppm $HgCl_2$ in MHB was taken in the wells of the first column and 150 µl was taken in the 12^{th} column.
2. 150 µl of sterilized MHB as taken in rest all the wells.
3. 150 µl from the first well was transferred to the second well and subsequent transfer was done till the 10^{th} well (serial dilution).
4. 150 µl was discarded from the 10^{th} well.
5. 2-3 colonies were mixed with 1-2ml of sterilized distilled water and the turbidity was compared with 0.5 Mc Farland standards.
6. 20 µl of the culture was transferred to each well except the 12^{th} well which acts as negative control.

7. The plate was incubated at 37^{o}C for 24 hours and absorbance was taken at 595nm in an ELISA Plate Reader.
8. OD_{595} at each well was checked with the negative control and the MIC was determined accordingly.

4.5 Determination of Cell Morphology under oil immersion microscope by Gram's staining

To study the gram's stain i.e. Gram (+ve) or Gram (–ve) characters of the isolates, diluted suspensions of the bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 4 to 5 times. The slides, were teemed with crystal violet solution for one minute, washed with water and flooded with Gram's iodine for one minute.

The slide were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle till no violet colour was visible from drain off solution. The slides were washed with water and counter stained with safranin stain for about 30 second and washed with water. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter. Cells were then identified as purple for Gram positive and pink or red for Gram negative cells by the colour observed.

4.6 Amplification of *merA* gene in the resistant isolates

merA gene was amplified by the following primers: F1merA-5' TCGTGATGTTCGACCGCT3'; F2 merA-5' TACTCCCGCCGTTTCCAAT3' (Sotero-Martins *et al.*, 2008). The amplification reactions were performed in a total volume of 20µL by using a thermal cycler (BioRad). The PCR mixture contained 1U/µL Taq polymerase (Sigma), 1X Enzyme buffer, 200µM of each dNTP (Sigma), 1.25 mM $MgCl_2$ and 0.5µM of each primer. The optimized amplification conditions included a pre denaturation step at 94°C for 1 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and an extension step at 72°C for 1 min and final extension at 72°C for 7 minutes. The PCR product was analyzed using gel electrophoresis (1.5% agar) and visualized in Gel Documentation System (BioRad). A mercury resistant strain of PW-05 was used as the positive control.

4.7 Study of mercury bioaccumulation by H_2S assay of the *mer* negative isolates

Lueria Bertani (LB) broth (5 ml) was prepared. As per MIC of each strain mercury was supplemented in the broth and pure cultures were used as inoculums. The colonies were transferred and incubated at 35 ± 2^0C till the development of moderate turbidity. Samples (5ml) of broth cultures from test flasks with Hg were centrifuged at 7,500xg at 4^0C for 10 min. Supernatant and pellets were collected in different eppendorf tubes. The cells were washed with PBS before exposing the pellets to H_2S gas. The cell mass of each strain was exposed to H_2S gas for 10 min (Fig. 7).

Fig 7. Set-up for the determination of mercury bioaccumulation in *mer* negative isolates by H_2S assay

4.8 Biochemical Characterization of the isolates

Commercially offered systems cut back the need for preparing a variety of test media and reagents and the time required for interpretation of results, thereby making the identification of various bacterial species more plausible in the routine laboratory. Hi Media Rapid Biochemical Identification kit, *Bacillus* Identification Kit [KB013 Hi25®] and Motility Test kit (KBM001 to KBM003) are a number of methods used worldwide. Hi Media provides a range of Biochemical Identification test kit (KB001 to KB012) involving single step procedure of inoculation that leads to final identification of test for the organism being studied. The Biochemical Identification test kit is a standardized

colorimetric identification system utilizing conventional biochemical tests and carbohydrate utilization tests. The tests are primarily based on the principle of pH change and substrate utilization by the organism. On incubation organisms undergo metabolic changes that are indicated by a colour change in the media that is either interpreted visually or when a reagent is added.

4.9 Antibiotic Susceptibility testing of the isolates

Antibiotic susceptibility test was done by six different antibiotic discs of 30 mcg each [Amoxycillin(AM^{30}),Chloramphenicol(C^{30}), Gentamycin(GEN^{10}), Kanamycin(K^{30}), Neomycin (N^{30}) and Tetracycline(T^{30})].

- Mueller Hinton Agar (MHA) plates were prepared for performing the antibiotic susceptibility test of the potent isolated strains.
- The pure culture colonies used as inoculums were transferred to Lueria Bertani (LB) broth (5 ml), incubated at 35 ± 2^0C till the development of moderate turbidity.
- A sterile non toxic cotton swab is dipped into the inoculum in the broth and rotated firmly against the upper inside wall of the tube to express excess fluid. The entire agar surface was streaked with the swab for 3 times turning the plate at 60^o angle between each streaking. The inoculums were allowed to dry for 5 mins.
- The discs were dispensed using aseptic technique at least 24 mm apart. Petri plates were incubated immediately at 37^0Cand examined after 16-18 hours. The zones showing complete inhibition were measured and the diameters of the zones were measured to the nearest millimetre.
- By the antibiotic zone scale, the area of inhibition was measured for each antibiotic. Sensitivity of the isolates to each antibiotic was determined according to the chart provided by Himedia, Mumbai.

4.10 Quantification of mercury accumulation by Atomic Absorption Spectroscopy

Lueria Bertani (LB) broth (5 ml) was prepared. As per the MIC of each strain mercury was supplemented in the broth and pure cultures were used as inoculums. The colonies were transferred and incubated at 35 ± 2^0C till the development of moderate turbidity. Samples (5ml) of broth cultures from test flasks with Hg were centrifuged at 7,500xg at 4^0C for 10 min. Supernatant and pellets were collected in different eppendorf tubes.

The corresponding cell pellets were resuspended with lysis buffer [100 mM NaH_2PO_4, 10 mM Tris–Cl, and 8 M urea (pH 8.0)] for 1 hr and then centrifuged at 13,000 rpm for 10 minutes to collect cell associated mercury.

4.11 Confirmation of bioaccumulation of mercury by Scanning Electron Microscopy (SEM)

Samples (10ml) of broth cultures from test flasks with Hg were centrifuged at 8,000xg at 4^oC for 5min. The cells were washed twice with 0.1 M phosphate buffered saline (PBS; 15 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4 at 25^oC) and fixed overnight in 2% glutaraldehyde (prepared in 0.1 M PBS). The cell mass were washed with PBS and distilled water before dehydration through an ethanol series (10% to absolute), held at each concentration for 30 min. Samples were placed on a brass stub, sputter-coated with gold or platinum, and was examined by scanning electron microscopy (SEM) (De et al., 2008).

5. RESULTS

5.1 Isolation of Mercury Resistant Marine Bacteria (MRMB)

A total of 10 bacterial strains, showing visible distinguished colony morphology, capable of tolerating mercury were isolated when isolated from the environmental samples by plating on SWNA+10 ppm $HgCl_2$ plates. The isolates were then characterized further for their resistance mechanism towards mercury the result of which has been given below. The pure cultures of the isolates were obtained by repeated streaking on the same plate and were stored on soft agar tubes for preservation.

5.2 Phenotypic Characterization of the Isolated Colonies

The observed colony morphology characteristics pertaining to their colour and shape have been collectively displayed in Table 1 and Fig.8.

Table 1. Colony Morphology of the Isolated Strains

Sl. No.	**Strain Name**	**Color**	**Shape**
01	CW601	Yellowish Orange	Round
02	GW702	Yellow	Small rounded
03	RW404	White	Rough rounded
04	CW302	Yellow	Round
05	RW402	Yellow	Round
06	RW403	Yellowish Orange	Round
07	CW501	Whitish yellow	Small rounded
08	RW203	Yellow	Round
09	BW02	Yellow	Round
10	GW601	White	Very small round

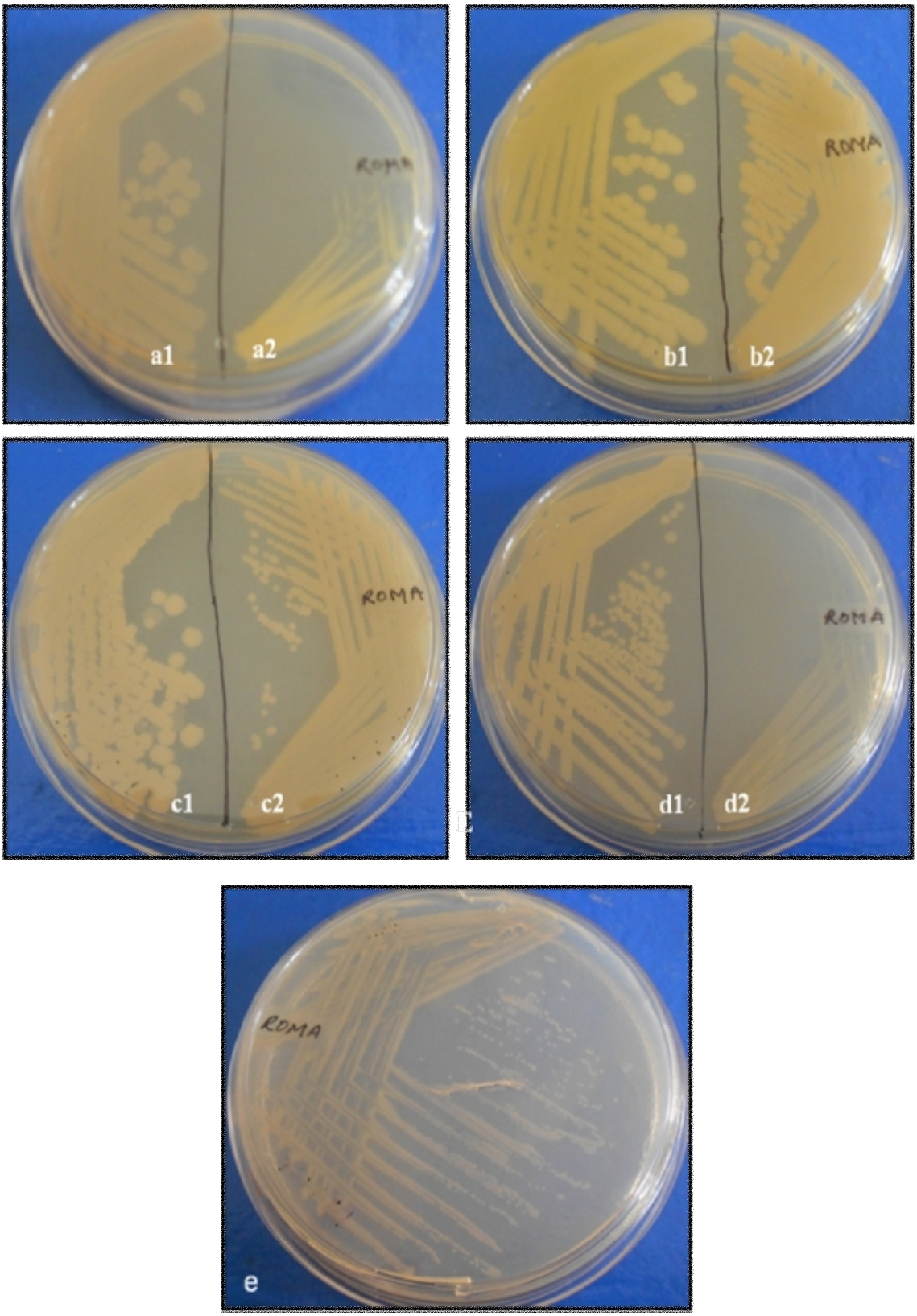


Fig 8. Isolated colonies on SWNA+10 ppm $HgCl_2$ plates [a1-CW-302, a2-RW-402; b1-BW-02, b2-CW-601; c1-RW-404, c2-GW-702; d1-CW-501, d2-RW-203; e-GW-601]

5.3 Cell Morphology

Cell morphology of strains was studied by gram staining and observing under oil immersion microscope, the result of which has been given in the Table 2 and Fig. 9.

Table 2. Cell Morphology of the Isolated Strains

Sl.No.	**Strain Name**	**Color**	**Gram Staining**	**Shape**
01	CW601	Pink	-ve	Cocci
02	GW702	Pink	-ve	Rods
03	RW404	Pink	-ve	Filamentous
04	CW302	Pink	-ve	Rods
05	RW402	Pink	-ve	Cocci
06	RW403	Pink	-ve	Rods
07	CW501	Pink	-ve	Cocci
08	RW203	Pink	-ve	Rods
09	BW02	Pink	-ve	Cocci
10	GW601	Pink	-ve	Rods

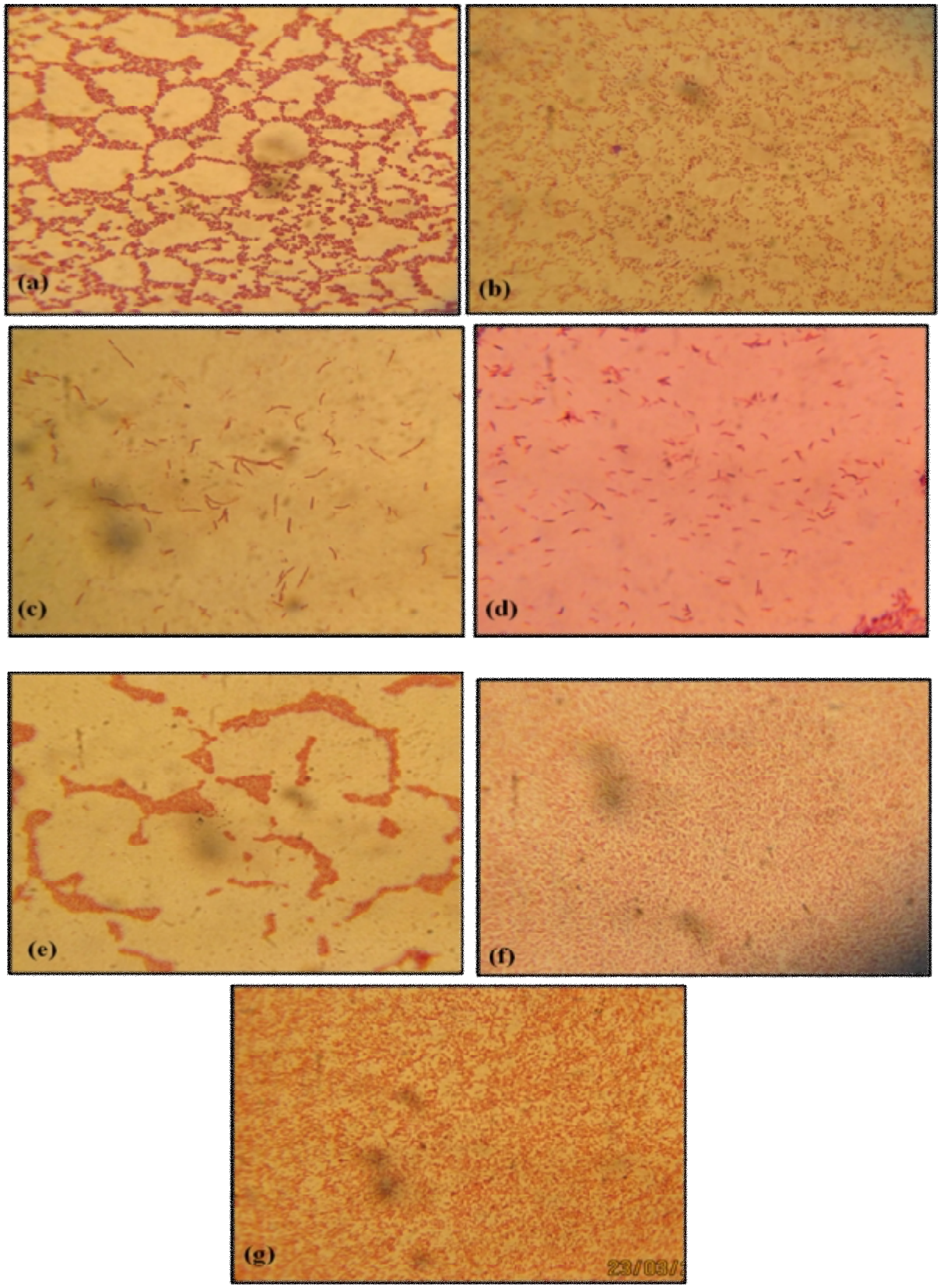


Fig 9. Gram Staining results of the isolates [a-CW-601, b-GW-702, c-RW-404, d-CW-302, e-RW-402, f- Rw-203, g-GW-601]

5.4 Minimum Inhibitory Concentration Test (MIC)

Minimum Inhibitory Concentration refers to the minimum concentration of mercury at which bacterial growth can be inhibited. After the test it was concluded that all the 10 isolates gave the result well above the MIC value in the range of 25-50 ppm. The results of Minimum Inhibitory Concentration Test (MIC) of $HgCl_2$ for 10 strains have been shown in Table 3 and Fig.10.

Table 3. MIC Results of Bacterial Isolates

Sl. No.	**Sample Name**	**MIC (ppm)**
01	CW601	25
02	GW702	50
03	RW404	50
04	CW302	25
05	RW402	50
06	RW403	50
07	CW501	25
08	RW203	50
09	BW02	50
10	GW601	25

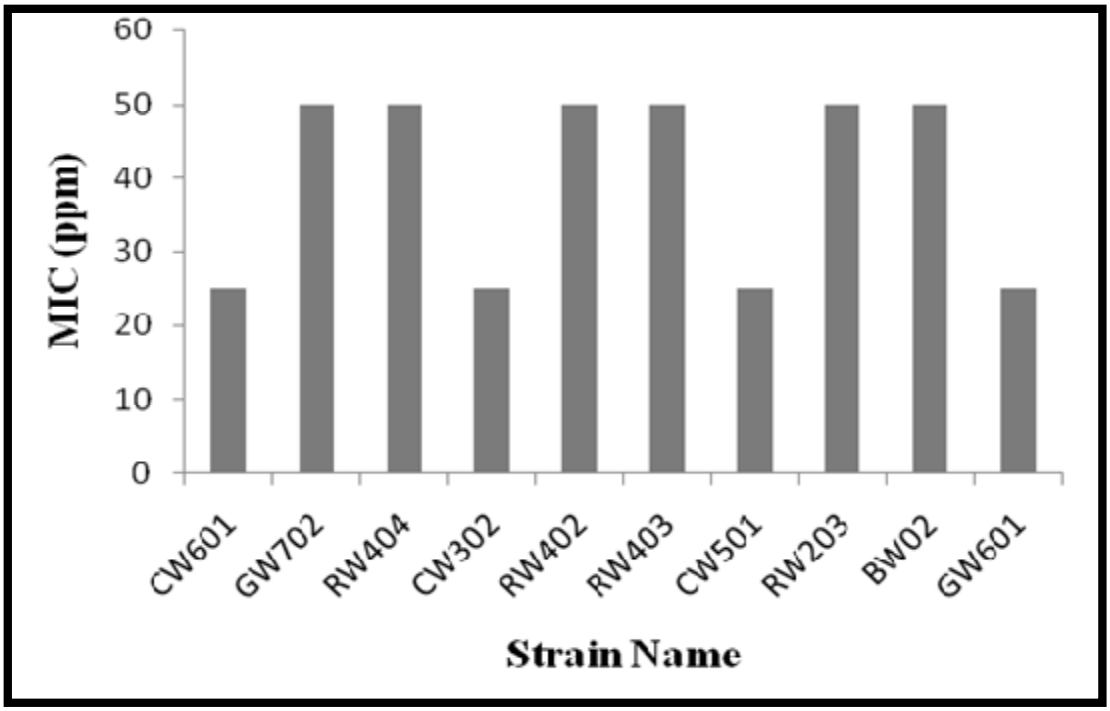


Fig 10. MIC results for bacterial isolates

5.5 Amplification of *merA* gene in the resistant isolates

Non *mer* mediated genes were not amplified in the gel run showing absence of *mer* A gene in the genome of the bacterial isolates. PW05 is the positive control and thus a thick single band of 431 bp was seen in the lane 15 fig. 11.

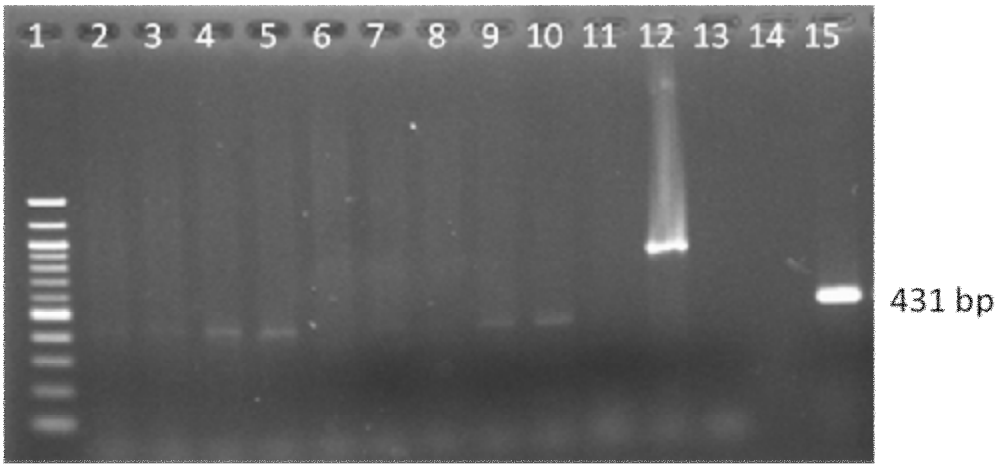


Fig 11. Gel photograph showing *merA* amplification in the isolated strains. Lane 1-100bp ladder, lane2-Cw-601, lane3-GW-702, lane4-RW-404, lane5-CW-302, lane6- RW-402, lane7- RW-403, lane8- CW-501, lane9- RW-203, lane10- BW-02, lane11- GW-601, lane13-RW-401, lane14- -ve control and lane15 PW-05 are +ve control.

5.6 Mercury bioaccumulation by H_2S assay of the *mer* negative isolates

The 10 isolates showing MIC more than 10ppm were selected to study the bioaccumulation of mercury in the samples by H_2S assay. Mercury has high affinity for sulphur and thus accumulation of mercury was confirmed by exposing the cell mass to Hydrogen sulphide gas. One by one each sample was exposed to H_2S for 10 min and corresponding readings were noted. Mercury bioaccumulation by H_2S assay of the *mer* negative isolates is shown in Table 4 and fig 12, 13 and 14. PW-05, another isolate which is harboring *merA* gene has been taken as positive control and the most potent isolates which showed black colony in relatively lesser amount of time i.e., GW-702, RW-404 and BW-02 were characterized further.

Table 4. H_2S Assay Result of Bacterial Isolates

Sl.No.	Strain Name	MIC (ppm)	Time (min)	Interpretation
01	**CW601**	25	10	-ve
02	**GW702**	50	5	+ve
03	**RW404**	50	4	+ve
04	**CW302**	25	10	-ve
05	**RW402**	50	10	+ve
06	**RW403**	50	10	+ve
07	**CW501**	25	10	-ve
08	**RW203**	50	10	-ve
09	**BW02**	50	2	+ve
10	**GW601**	25	10	-ve

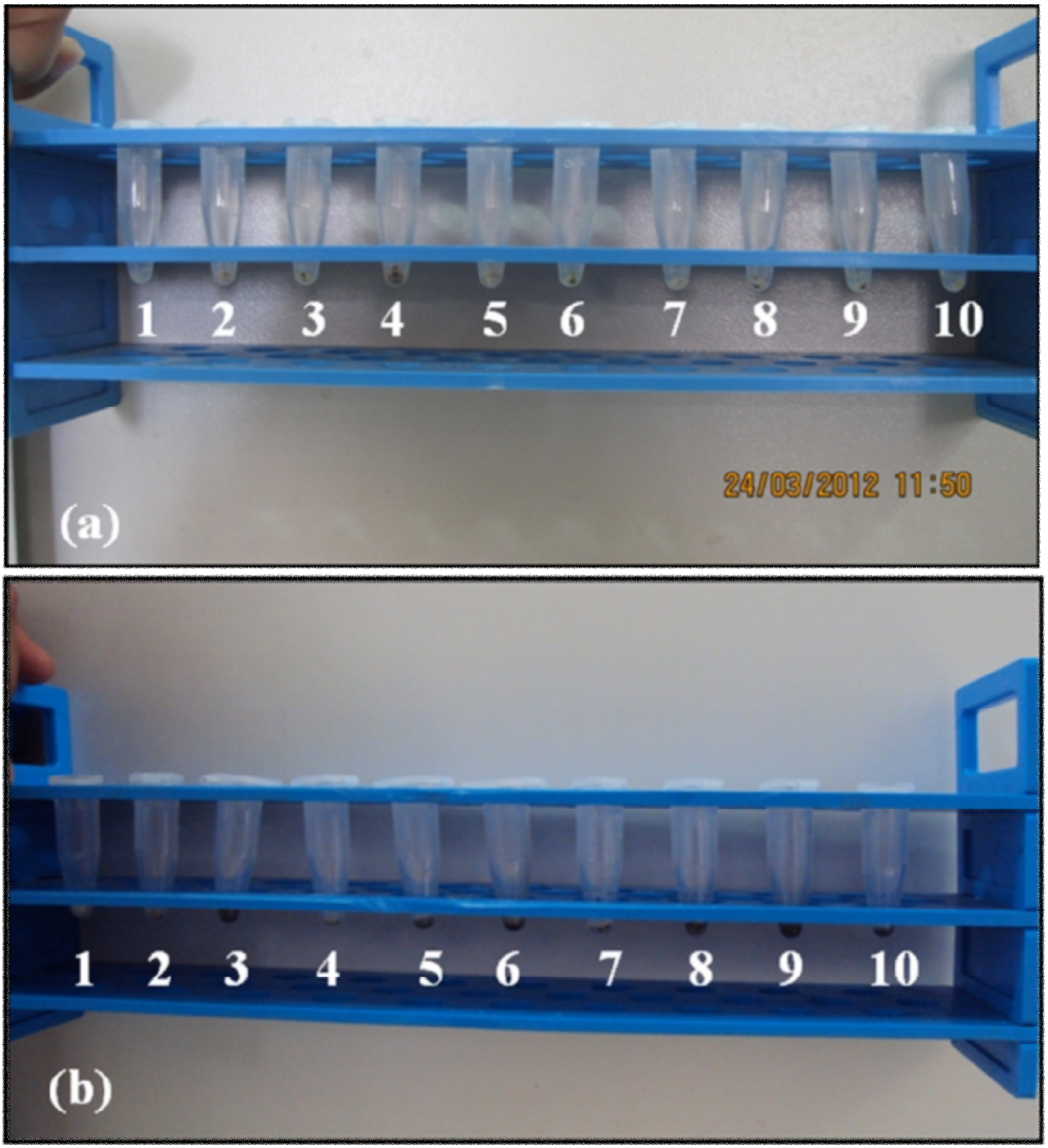
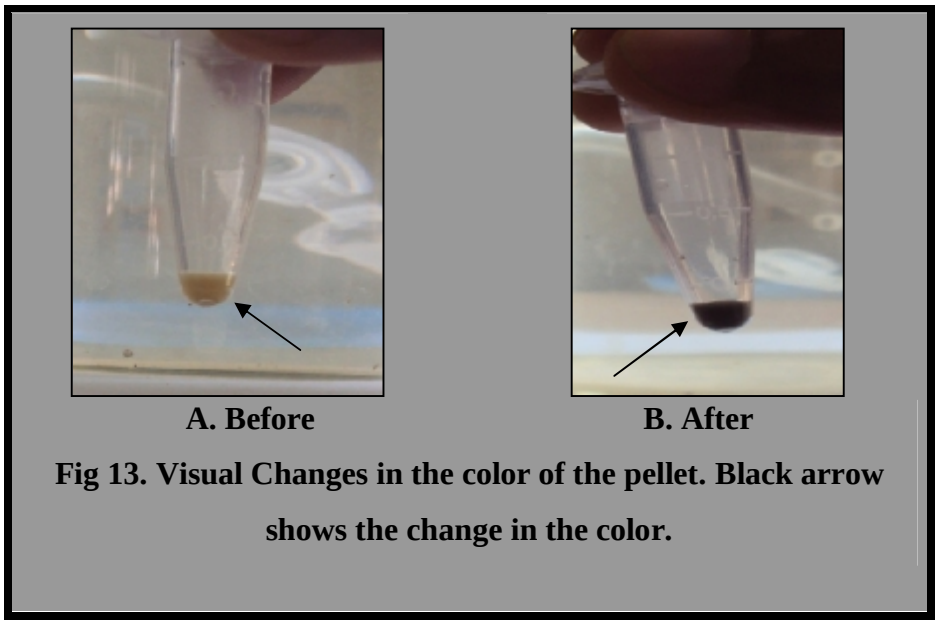


Fig 12. H_2S assay result of the bacterial isolates when exposed to H_2S gas [(a) before; (b) after]



A. Before **B. After**

Fig 13. Visual Changes in the color of the pellet. Black arrow shows the change in the color.

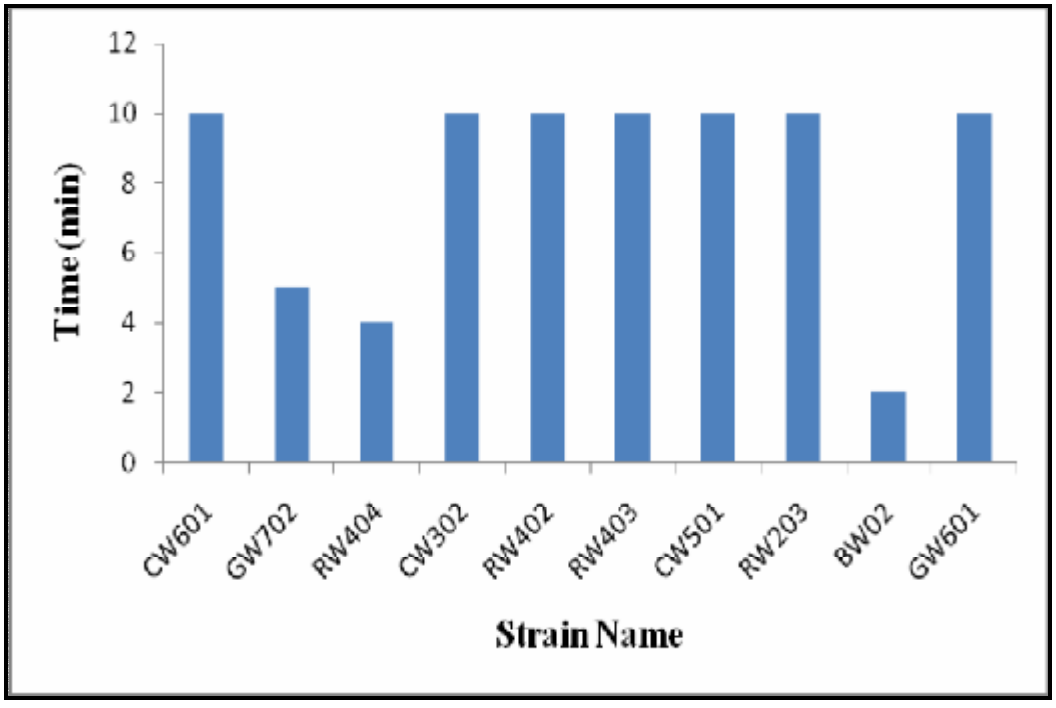


Fig 14. H_2S assay of bacterial isolates

5.7 Biochemical Characterization of the isolates

Different biochemical tests were performed to characterize the mercury resistant bacterial strains and observations are collectively given in Table 5 and fig. 15.

Table 5. Biochemical Test Results

Sl.No.	Tests Conducted	BW02	GW702	RW404
01	Malonate	-	-	-
02	Voges Proskauer's	-	-	-
03	Citrate	+	+	+
04	ONPG	+	+	-
05	Nitrate Reduction	-	-	+
06	Catalase	+	-	+
07	Arginine	+	+	+
08	Sucrose	+	+	+
09	Mannitol	+	+	+
10	Glucose	+	+	-
11	Arabinose	-	-	-
12	Trehalose	+	+	+

Fig 15. Himedia Rapid Biochemical Identification kit, *Bacillus* Identification Kit [KB013 Hi25®]

[(A) = Strain BW02; (B) = Strain GW702; (C) = Strain RW404]

[From left to right; Test name: Malonate, Voges Proskauer's, Citrate, ONPG, Nitrate Reduction, Catalase, Arginine, Sucrose, Mannitol, Glucose, Arabinose and Trehalose]

5.8 Antibiotic Susceptibility Test

Antibiotic susceptibility test of the bacterial strains is shown in Table 6 and fig. 16 and fig. 17 (a,b).

Table 6. Antibiotic Susceptibility Test Result (Mean±SD, n=3)

CW601:

Sl. No.	Antibiotic	Mean ± SD (mm)	Interpretation
1.	Amoxycillin (Am^{30})	25±0	AM^S
2.	Chloramphenicol (C^{30})	18.6±2.51	C^S
3.	Gentamicin (GEN^{10})	22.3±0.57	GEN^S
4.	Kanamycin (K^{30})	20.6±1.15	K^S
5.	Neomycin (N^{30})	20±0	N^S
6.	Tetracycline (T^{30})	25±0	T^S

GW702:

Sl. No.	Antibiotic	Mean ± SD(mm)	Interpretation
1.	Amoxycillin (Am^{30})	17±0.1	Intermediate
2.	Chloramphenicol (C^{30})	29.3±1.15	C^S
3.	Gentamicin (GEN^{10})	20.6±0.5	GEN^S
4.	Kanamycin (K^{30})	17.6±0.5	K^S
5.	Neomycin (N^{30})	20.3±0.5	N^S
6.	Tetracycline (T^{30})	21±1	T^S

RW404

Sl. No.	Antibiotic	Mean ± SD(mm)	Interpretation
1.	Amoxycillin (Am^{30})	15.3±0.5	Intermediate
2.	Chloramphenicol (C^{30})	30±0	C^S
3.	Gentamicin (GEN^{10})	30.6±0.5	GEN^S
4.	Kanamycin (K^{30})	20.3±0.5	K^S
5.	Neomycin (N^{30})	27.6±1.15	N^S
6.	Tetracycline (T^{30})	20.6±0.5	T^S

CW302

Sl. No.	Antibiotic	Mean ± SD(mm)	Interpretation
1.	Amoxycillin (Am^{30})	12±0	AM^S
2.	Chloramphenicol (C^{30})	32.6±0.5	C^S
3.	Gentamicin (GEN^{10})	29.6±0.5	GEN^S
4.	Kanamycin (K^{30})	26.6±0.5	K^S
5.	Neomycin (N^{30})	28.6±0.5	N^S
6.	Tetracycline (T^{30})	31±1	T^S

RW402

Sl. No.	Antibiotic	Mean ± SD(mm)	Interpretation
1.	Amoxycillin (Am^{30})	17.6±0.5	Intermediate
2.	Chloramphenicol (C^{30})	31±1	C^S

3.	Gentamicin (GEN^{10})	30.6±1.15	GEN^{S}
4.	Kanamycin (K^{30})	25.3±0.5	K^{S}
5.	Neomycin (N^{30})	29±1	N^{S}
6.	Tetracycline (T^{30})	31±0	T^{S}

CW501

Sl. No.	Antibiotic	Mean ± SD(mm)	Interpretation
1.	Amoxycillin (Am^{30})	22±1	AM^{S}
2.	Chloramphenicol (C^{30})	22.6±0.5	C^{S}
3.	Gentamicin (GEN^{10})	27.6±0.5	GEN^{S}
4.	Kanamycin (K^{30})	25.3±0.5	K^{S}
5.	Neomycin (N^{30})	25±0	N^{S}
6.	Tetracycline (T^{30})	23.6±0.5	T^{S}

RW203

Sl. No.	Antibiotic	Mean ± SD(mm)	Interpretation
1.	Amoxycillin (Am^{30})	19±0	Intermediate
2.	Chloramphenicol (C^{30})	22±0	C^{S}
3.	Gentamicin (GEN^{10})	28.3±0.5	GEN^{S}
4.	Kanamycin (K^{30})	23.6±0.5	K^{S}
5.	Neomycin (N^{30})	26±0	N^{S}
6.	Tetracycline (T^{30})	27.3±0.5	T^{S}

BW02

Sl. No.	Antibiotic	Mean ± SD(mm)	Interpretation
1.	Amoxycillin (Am^{30})	18.6±0.5	Intermediate
2.	Chloramphenicol (C^{30})	33.3±1.15	C^{S}
3.	Gentamicin (GEN^{10})	33.3±0.5	GEN^{S}
4.	Kanamycin (K^{30})	29.3±1.15	K^{S}
5.	Neomycin (N^{30})	31.3±0.5	N^{S}
6.	Tetracycline (T^{30})	34.6±0.5	T^{S}

GW601

Sl. No.	Antibiotic	Mean ± SD(mm)	Interpretation
1.	Amoxycillin (Am^{30})	19.6±0.5	Intermediate
2.	Chloramphenicol (C^{30})	18.6±1.15	C^{S}
3.	Gentamicin (GEN^{10})	29.6±0.5	GEN^{S}
4.	Kanamycin (K^{30})	22.3±0.5	K^{S}
5.	Neomycin (N^{30})	26.3±0.5	N^{S}
6.	Tetracycline (T^{30})	22±1	T^{S}

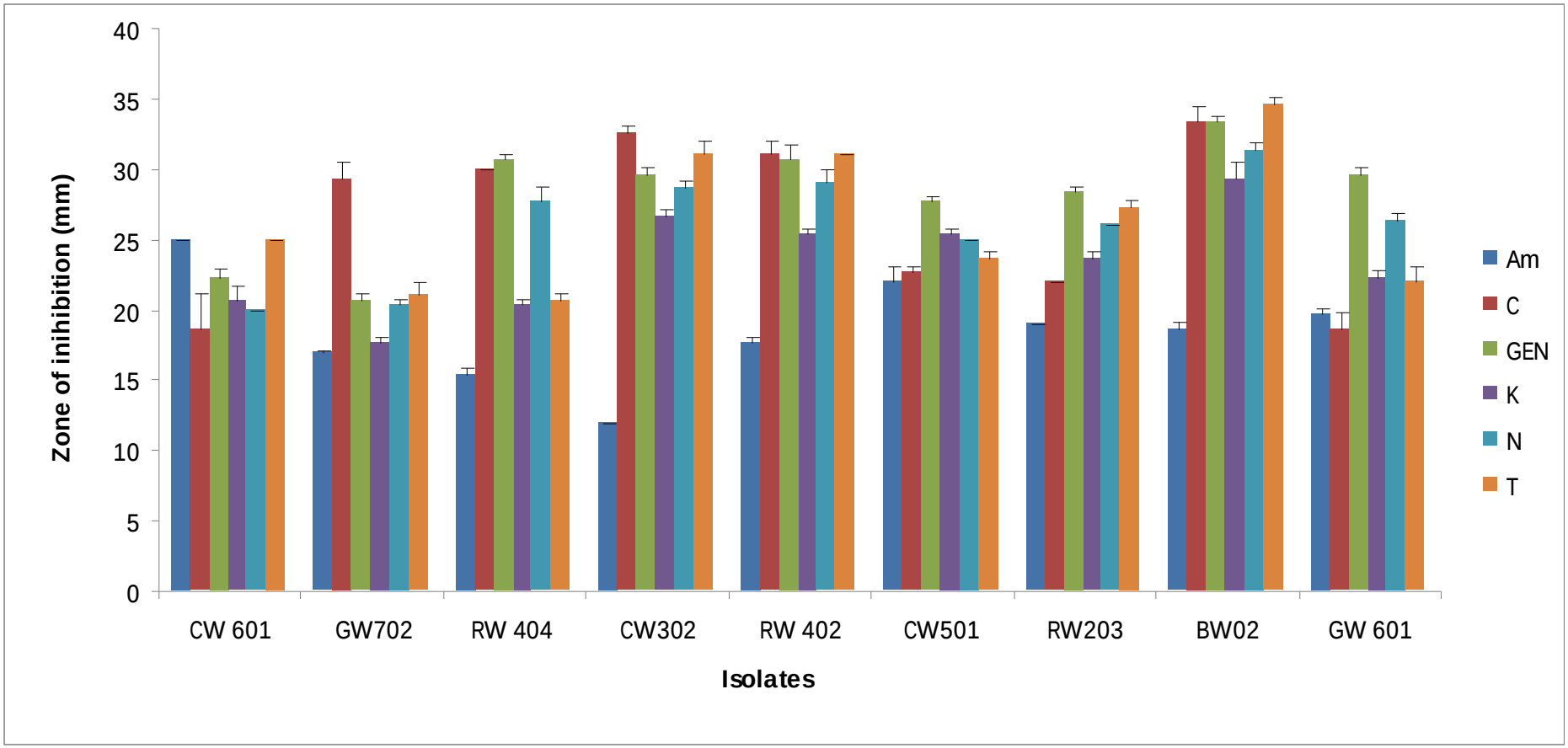


Fig 16. Antibiotic Susceptibility Test Results [Zone of inhibition in mm (mean±SD), n=3]

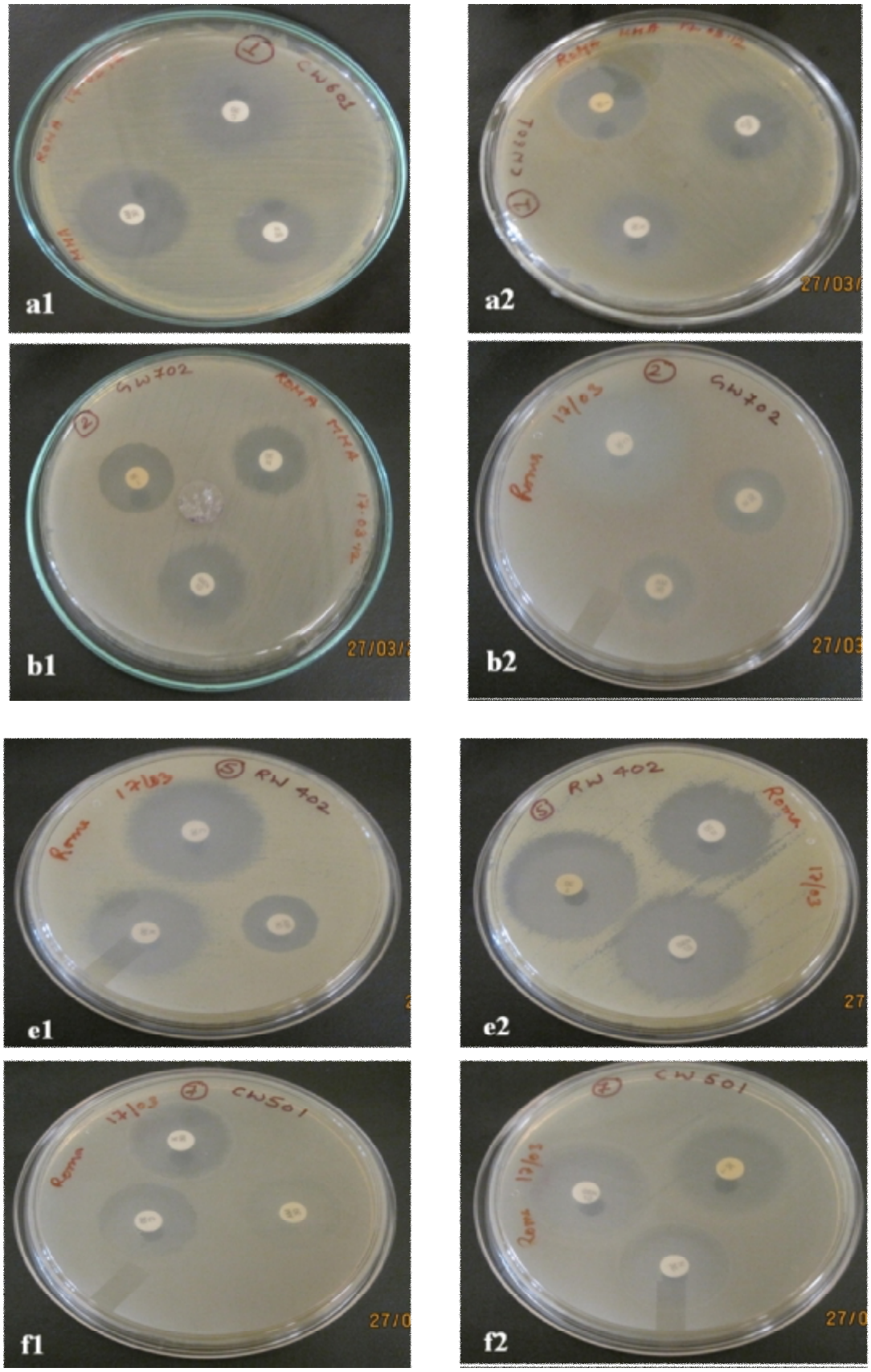


Fig 17 (a). Antibiotic Susceptibility Test Results

[a1,a2: CW601; b1,b2: GW702; c1,c2: RW 404; d1,d2: CW 302]

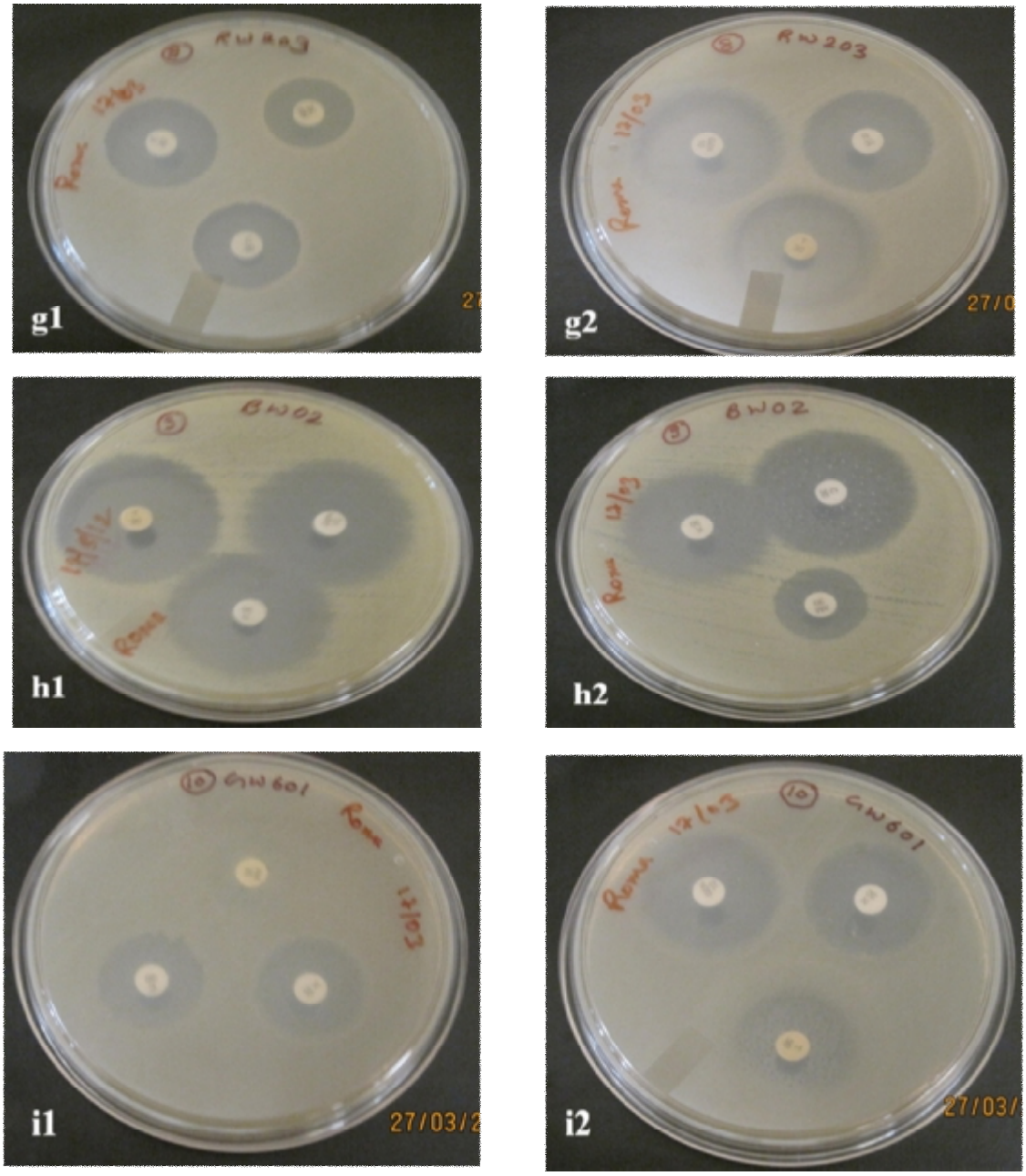


Fig 17 (b). Antibiotic Susceptibility Test Results

[e1,e2: RW 402; f1,f2: CW 501; g1,g2: RW 203; h1,h2: BW 02; i1,i2: GW 601]

5.9 Quantification of mercury accumulation by Atomic Absorption Spectroscopy

Bacterial strains showing maximum affinity to H_2S gas were selected and amount of mercury accumulated in the bacterial strains (quantification) was done by Atomic Absorption Spectroscopy. Atomic Absorption Spectroscopy results of the bacterial strains are shown in Table 7 and fig. 18.

Table 7 Atomic Absorption Spectroscopy Results

Initial Hg Supplemented (ppm)	Final Hg (Supernatant) ±SD(ppm)	Final Hg (Pellet) ±SD(ppm)	% Hg Bioaccumulated
	PW-05		
50	0.66±1.15	1.96±1.45	3.93
	GW-702		
50	1.73±0.75	23.66±2.08	47.33
	RW-404		
50	1.76±0.25	28.16±1.04	56.33
	BW-02		
50	1.53±0.30	36.16±0.76	72.33

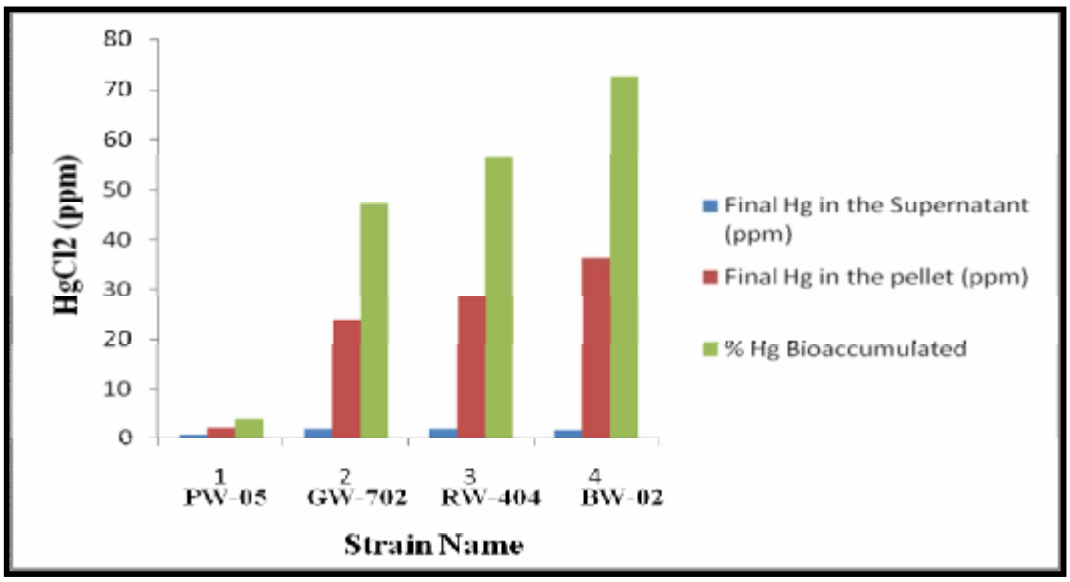


Fig 18. Atomic Absorption Spectroscopy result showing bioaccumulation of mercury in the isolates.

5.10 Confirmation of bioaccumulation by Fluorescent Microscopy and Scanning Electron Microscopy (SEM)

Mercury accumulations in the bacterial strains were future confirmed by Fluorescent Microscopy and Scanning Electron Microscopy (SEM). Fig 19 and fig. 20 shows the photographs of the potent mercury resistant isolates at different magnifications.

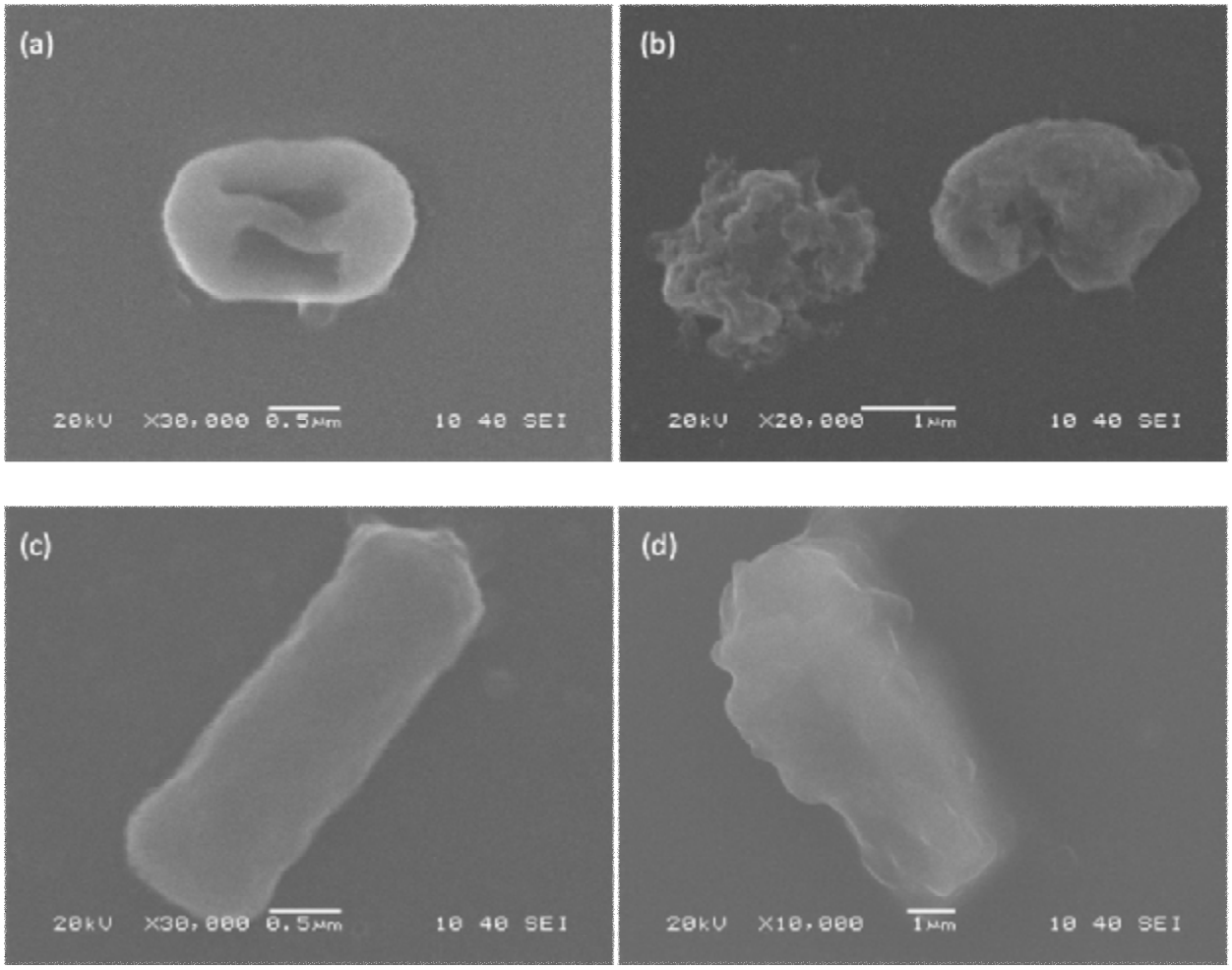


Fig 19. SEM Photograph of the potent mercury resistant isolates when grown under mercury stress (a) BW-02, (b) GW-702, (c) PW-05, (d) RW-404

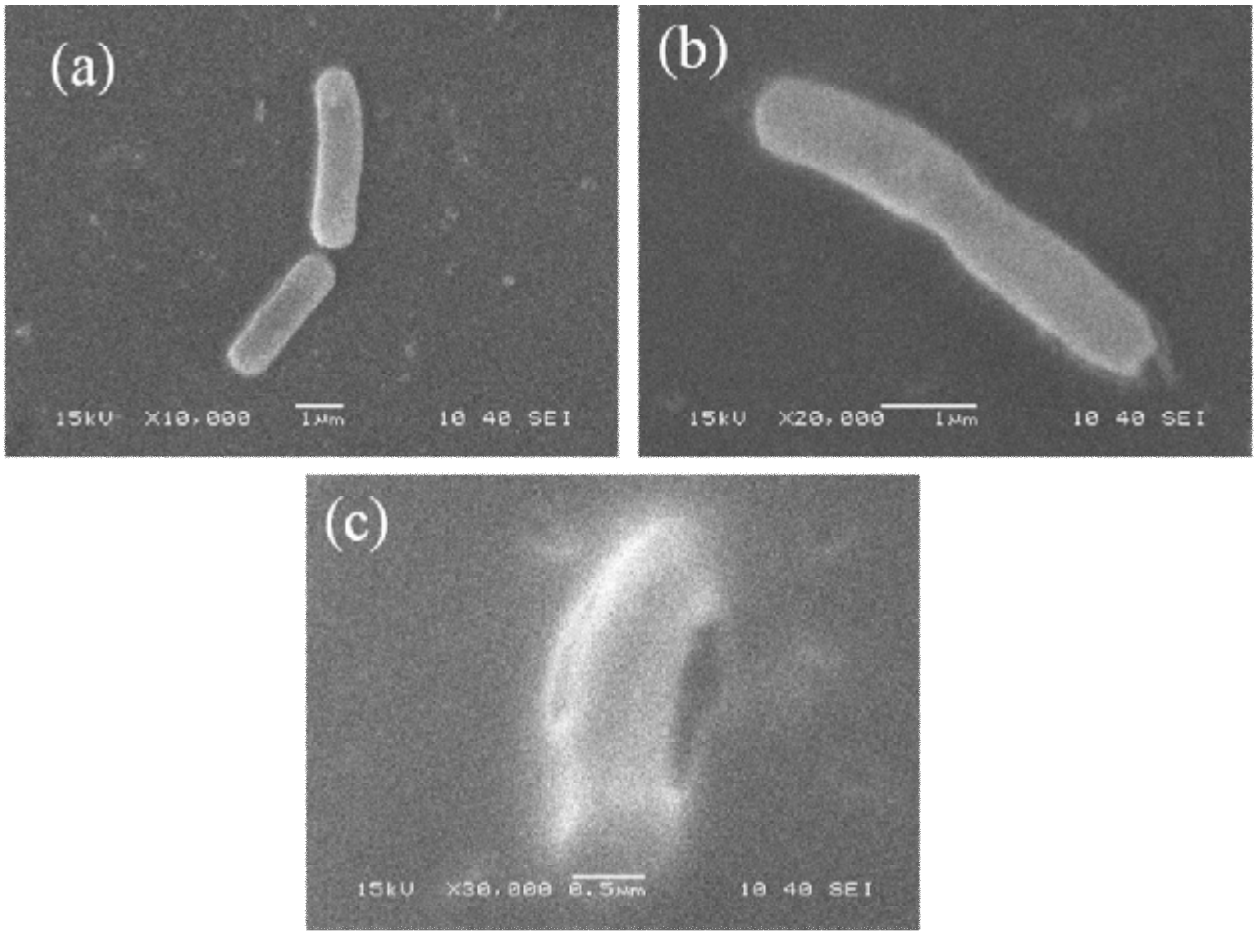


Fig. 20 SEM Photograph of the potent mercury resistant isolates when grown without mercury stress (a) BW-02, (b) PW-05, (c) RW-404

6. Discussion

A total of 10 mercury resistant bacteria from four different sites of Bay of Bengal along the Odisha coast were isolated on Sea Water Nutrient Agar (SWNA) supplemented with 10 ppm of $HgCl_2$. These 10 strains were tested for minimum inhibitory concentration (MIC) and all these strains showed MIC above 25 ppm and were further amplified by PCR to show the absence of *merA* gene in the isolates taking PW05 as positive control having *merA* gene. All the 10 isolates showed negative result to PCR amplification and thus absence of *merA* gene in the isolates were confirmed.

The *mer* negative isolates were exposed to H_2S gas to study mercury bioaccumulation and to deduce the alternative mechanism by which these isolates are able to resist mercury inside them. All the 10 strains were exposed to H_2S gas one by one for 10 mins each. The change in the color of cell mass from white to black was interpreted as accumulation of mercury within the cell as mercury has high affinity for sulphur. Out of the 10 isolates, 50% of the isolates showed positive results i.e GW-702, RW-404, RW-402, RW-403 and BW-02. Out of those 5 strains, GW-702, RW-404 and BW-02 were selected as they showed change in color of the cell mass within 5 mins. The quantity of mercury accumulation was studied by Atomic Absorption Spectroscopy, which suggested that a percentage of mercury bioaccumulation in the three strains BW-02, GW-702 and RW-404 were 72.33%, 47.33% and 56.33% respectively. Out of those three strains BW-02 has the highest percentage of mercury accumulation and thus Bhitarkanika mangrove ecosystem bacterial communities have highest potential of mercury bioaccumulation was confirmed. The morphological and colonial characteristics were also studied. Approximately all the strains were whitish yellow, round and gram negative. Except RW-404 which is filamentous in shape, rest all were rods or coccus. Characterization of the isolated strains was done by biochemical analysis which showed that strains are positive for Citrate, Arginine, Sucrose, Mannitol and Trehalose and negative for Malonate, VP and Arabinose. The isolated strains were subjected to Antibiotics susceptibility test viz. Amoxycillin, Chloramphenicol, Gentamicin, Kanamycin, Neomycin and Tetracycline to understand their antibiotic resistant strength. Out of the ten isolates, GW-702, Rw-404, RW-402, RW-203, BW-02 and GW-601 were resistant to Amoxycillin and rest all the isolates were sensitive to all the other antibiotics (Kotrba et al., 1999).

The potent mercury resistant isolates when grown under mercury stress showed change in the morphology of the cell due to accumulation of mercury. Mercury accumulations in the

bacterial strains were thus confirmed by Scanning Electron Microscopy (SEM) which showed clear picture of mercury bioaccumulation in the cells. There was no change in the shape of the PW-05 positive control strain, which suggested that the potent bacterial cells have changed due to accumulation of mercury (El-Hendawy et al., 2009; Ahemad and Malik, 2012). Quantification of mercury accumulation and biochemical characterization tests has shown that BW-02 is highly potent among all the strains.

7. Conclusion

Ten strains were isolated from four different sites of Odisha coast out of which six strains viz. GW 702, RW 404, RW 402, RW 403, RW 203 and BW 02 showed highest minimum inhibitory concentration (MIC) of 50ppm of $HgCl_2$ and rest four strains viz. CW 601, CW 302, CW 501 and GW 601 showed MIC of 25 ppm respectively. Absence of *mer*A gene in the genome of these isolated strains was confirmed by PCR amplification and thus there is an alternative mechanism of mercury resistance i.e., non *mer* mediated which was established in these isolates. The proposed alternative mechanism i.e., bioaccumulation of mercury was further confirmed by H_2S assay and Scanning Electron Microscopy. Change in the color of the cell mass after exposure to hydrogen sulphide gas showed bioaccumulation of mercury in the isolates and the change in the shape of the bacterial cell showed that there is accumulation of mercury inside the cell and thus the bacterial cells can accumulate mercury inside the cytoplasm and help in bioremediation. Bacterial mobile genetic elements such as plasmids or transposons, carry multiple genes encoding metal and antibiotic resistance. Mercury resistant marine bacteria (MRMB) isolated from contaminated environments is extremely potential to remove mercury from contaminated sites. So, it is suggested that mercury elimination ability of these bacteria should be evaluated. Moreover these isolates can be genetically engineered to reach better results in removal of mercury. However, before exploiting the strain as an efficient biotechnological tool for mercury detoxification further investigation needs to be carried out in laboratory scale and in-situ metal reduction potential of the genus has to be assessed.

The following conclusions can be withdrawn from the present investigation:

i. Chilika, Bhitarakanika, Gopalpur and Rushikulya are mercury polluted sites in the Odisha coast.
ii. Bacterial community play a vital role in bioremediation by reducing the toxic form of mercury to non toxic form either by converting toxic form to non toxic form or by accumulating mercury within them.
iii. Ten mercury resistant bacteria were isolated that helped in detoxification of mercury in the environment.

7.1 Future perspectives:

1. Future investigation on mercury-resistant bacteria may lead to new and better understanding of the existing concept. For instance, absence of *merA* gene in the genome of marine bacteria but still having potential for bioremediation.
2. Future studies on bioaccumulation potentials of bacteria may lead to better and healthy mercury contaminated free environment.
3. Future studies including on-site experiments will be useful in developing practical means for environmental cleanup.

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Appendix

A. Media:

The media used and their compositions are given below:

Table 1: Details of media used and their used and their composition

1. Sea Water Nutrient Agar (SWNA):

Components	Quantity (Gram's/Litre)
Peptone	5.0g
Yeast Extract	3.0g
NaCl	15g
Agar Powder	15g
Milli Q	1000ml
pH (at 37^0C)	7.5±0.1

2. Mueller Hinton Agar (MHA):

Components	Quantity (Grams/Litre)
Beef infusion solids	4.0
Starch	1.5
Casein hydrolysate	17.5
Agar Powder	17.5
pH (at 37^0C)	7.4±0.2

3. Mueller Hinton Broth (MHB):

Components	Quantity (Grams/Litre)
Beef infusion solids	4.0

Starch	1.5
Casein hydrolysate	17.5
pH (at 37^0C)	7.4±0.2

4. Luria Bertani Media:

Components	Quantity
Tryptone	2.00
NaCl	1.00
Yeast Extract	0.5%
pH (at 25^0C)	7.0

B. Stains:

Bacterial isolates were stained by using Gram's staining methods:

TABLE 2: COMPOSITION OF GRAM'S STAIN:

Ingredients	Uses
Crystal violet	Primary Staining Agent
Safranin	Secondary Staining Agent
Lugol's Iodine	Mordant
Acetone	Decolourising Agent

i) $HgCl_2$ Solution:

HgCl2	15gm
Conc. HCl	2.5gm