The metal microbe interactions have always been a theme of keen interest. The ability of microorganisms to scavenge the metals from their surroundings, either as micronutrients or to alleviate their toxicity is well known. This ability is now harnessed for cleaning up the metal contaminated environment. A wide variety of microorganisms have been found capable of restoring the metal and metalloid polluted waste. However, the mechanism by which the job is accomplished and the efficiency of the microorganisms vary widely from metal to metal.

Detailed results of bioremediation of mercury are presented in the previous chapter. As depicted in those results, microorganisms and physicochemical factors are interdependent as far as their influence on removal is concerned. The main aim here is to draw a clear picture; explaining the mechanism and determining the optimum conditions for bioremediation; so that it could be applied at field level. If the data of individual tables are discussed, it would lead to unnecessary repetition and give an muddled view rather than a clear idea. This necessitate the appropriate grouping of selected tables and graphs for discussion. From the published literature it appears that metabolism independent biosorption is the main mechanism of metal uptake even in living cells. The uptake capacity is significantly affected by external variable factors such as pH, temperature, presence of other cations and anions in the environment, amount of biosorbent, initial metal concentration, etc. Moreover the physicochemical factors showed more variation both qualitatively and quantitatively as compared to the selected biomass for mercury sorption process.

In this context, it was found more logical to discuss the results in the following groups, so it can present a better understanding and furnish information for the selection and use of studied biomass for the remediation purpose with a minimum overlapping during discussion.

5.1 SCREENING

The problem of searching the microorganisms, most suitable to carry out a particular reaction is not an easy task. It is often necessary to test hundreds of microorganisms obtained either from culture collection centres or isolated from different source to locate the appropriate microbe which would remove the metal of choice as per the
requirements.

The results of screening presented in Table 1 to 6 and Graph :1 showed mercury removal in a range of 11.34 to 86.0, 11.32 to 87.5 and 11.6 to 81.6 % amongst Gram negative and Gram positive bacteria and microorganisms other than bacteria. The observed wide variation in mercury removal by different microbes could be due to the inherent difference in genetic make up, cellwall composition and presence or absence of exopolymeric capsules and slime layers. In nature, the microorganisms are surrounded by aqueous solutions containing many different metal ions. The surface layer faces the first exposure to the diffusable components of the surrounding. The microbial cell surface behaves as a bristling forest of molecules that extends in to the environment providing an enormous surface for the interaction with the metal ions. Thus even the slight variation in the surface layer resulted in considerable difference in its mercury removal capacity. Similar variation in metal binding capacity by fungal and bacterial cellmass with respect to metals like Ag, Cd and Cu was reported by Mullen et al (128,129). Several other research findings have also reported the variable ability of algal biomass for metal removal (199,200). The adsorption capacity of particulate matter for adsorbing mercury is dependent upon the surface area of the particle. Microorganisms shows a wide difference in their overall surface area which if arranged in decreasing order, gave following pattern:

 Gram positive cocci
    |
 Gram negative short rods
    |
    Bacillus
    |
    yeast
    |
 fungi

This could be also one of the reason for the observed differences in mercury removal by various microbial isolates.
As the bacterial isolates were more in number during primary screening, they were subjected to further screening for selection. At the end of third screening procedure, 2 and 4 isolates of Gram positive and Gram negative bacteria showing more than 80% mercury removal were chosen. The Gram positive bacteria removed more mercury as compared to the Gram negative bacteria, which could be accounted to their thick peptidoglycan layer in the cell wall. But the difference obtained for mercury removal among Gram positive and Gram negative bacteria does not correlate in terms of the cell wall thickness variation observed between them. This factor suggest the involvement of some other complex mechanism along with surface binding for mercury removal by Gram negative isolates. Similar findings were reported for other metals (201,202).

When various isolates were compared in terms of percent mercury removal range (Table 4); fungi dominated the scene, with 50% isolates grouped under 1st category (>60%). This was due to the less number of fungal isolates tested as compared to bacterial isolates. At the end of final screening, fungal cultures showed less mercury removal as compared to the bacteria. The larger surface area of bacteria in comparison with fungi was one of the reason for this phenomena.

5.1.2. DEMONSTRATION OF MERCURY ACCUMULATION BY BACTERIA GROWN ON SOLID SURFACE

As illustrated by few experimental results, the mercury absorption by the colony itself represented a very useful tool for the isolation and screening of metal accumulating bacteria as well as for the preliminary insight of the possible mechanism for mercury removal. The black colour developed on the plate was due to the reaction between mercury ions and H$_2$S; intensity of which was dependent on amount of mercury. The observed uniform black background was due to even distribution of mercury ions incorporated in to the medium.

The growth of spot inoculated cultures showed different patterns, such as blackening of colony, light or dark zone surrounding the colony. This could be due to the different mechanisms taking part in mercury accumulation.

The most frequently observed pattern was the blackening of the entire colony. This
type of results suggest the adsorption of mercury ions throughout the colony. It also shows equal participation of both young and old cells in mercury accumulation (Photograph : 10a)

The Photograph : 9 showed dark edges of the colony leaving the remaining colony unaffected. This may be due to the adsorption of mercury ions on the periphery of the colony, as diffusion of mercury ions may not be possible through the developed colony.

The darker zone (as compared to background Photograph : 10b) surrounding the colony was the result of exopolymers and extracellular metabolites production which played an important role in metal chelation.

The halo surrounding the black colony was due to the diffusion of mercury ions towards the colony from the immediate vicinity. But the halo observed surrounding the light colour could not be explained. It may be possible that here some kind of intracellular mechanism is active but intracellular deposition of mercury could not be detected as the cells do not allow easy penetration of H₂S gas.

The black crackings/linings observed on the lawn growth of Bacillus were the result of uneven diffusion of mercury ions through the growth. The comparatively distinct black areas could be due to more availability of cell components or metabolites for mercury ions. The similar method was also reported by Pümpel et al (197) for rapid screening of silver accumulating microorganisms.

5.2 MERCURY TOLERANCE STUDY

Metal ions are mainly divided into three classes based upon their toxicity. Mercury belongs to the most toxic group of metals, showing highest toxicity even at low concentration. Hence it was essential to check the minimal inhibitory concentration of mercury for the selected isolates. The findings depicted in Table : 7 indicates Isolate A as the most resistant organisms with 20 ppm of MIC. This resistance may be plasmid mediated or it may be due to enzymatic action where Hg(II) is reduced to Hg(0) which was then volatilized from the system.

The growth response of Isolate A in presence of mercury illustrates the influence of adaptation. The absence of lag phase during the growth cycle was the result of
adaptation. As the mercury concentration was increased adapted culture showed better improvement in terms of generation time (Graph 2 and 3, Table 8 and 9).

The concentration of mercury up to 20 ppm did not prove lethal to the Isolate A as it was its MIC value, but the higher concentration i.e. above 20 µg/ml may result into instant inactivation of the added inoculum; save few resistant cells, which grew slowly giving a long lag phase.

The data is more prominent in Graph 4 where the generation time of the adapted and unadapted culture versus mercury concentration is plotted. Due to initial shock of exposure to mercury concentration, the generation time was almost doubled (as the cells required acquaintance time) as compared to that obtained in absence of mercury. Above 20 µg/ml, up to 40 µg/ml of mercury concentration slow rise in generation time was observed showing the tolerance of the cells towards mercury. But the further rise in mercury concentration above 50 µg/ml proved lethal resulting in sharp increase in generation time due to more time period required for acclimatization of cells to mercury before growth.

As the mercury concentration increases, the influence of adaptation becomes more significant due to the serious toxicity of mercury at higher concentration towards the unadapted cells.

The mercury removal with growing cells of Isolate A showed complete removal of mercury at lower concentrations But above 80 ppm the reduced activity was due to the inhibitory effects of mercury on the organism. After six hours of exposure almost total inoculated population became inactive leading to instant cessation of the process. Comparatively longer time period required for the complete removal suggests the metabolic dependent mercury removal (Table 10).

5.3 PHYSICOCHEMICAL PARAMETERS INFLUENCING THE BIOREMOVAL OF MERCURY

5.3.1. INITIAL MERCURY CONCENTRATION

Influence of initial mercury concentration was studied with respect to bacterial, *Fusarium* and spent mycelial biosorption activity.
Mercury biosorption by all the tested cultures was enhanced with the increase in the initial mercury concentration up to a critical concentration above which the sorption either got constant or showed decreasing trend depending upon the microorganism used and the mechanism involved (Table: 11-14).

Gram positive Isolate 6 showed constant mercury removal above 40 ppm of initial mercury concentration. The removal gets constant because of the saturation of mercury binding sites on the cell surface. Once the sites were occupied no further adsorption could take place. In case of Gram negative isolates a decrease in mercury removal above 40 and 80 ppm of initial concentration suggests inhibition of the active mechanism due to mercury toxicity at higher concentration. The cells or enzyme may be inactivated at higher concentration of mercury giving decreased reaction. This resembles Michaelis-Menton expression. Similar findings are reported for uranium removal by S. cerevisiae and Ps. aeruginosa (203).

The data of Vm and Km values highlights the affinity of the particular isolate for the substrate (Graph 5a - 5d). Isolate 17 gave maximum Vm 310 corresponding to maximum loading capacity of 31.02 μg mercury/mg cellmass. As compared to this, other isolates showed less Km and Vm and subsequent decrease in loading capacity was also noted. This indicates the number of specific sites available for mercury interaction on cell surface and amount of metabolites present. When the concentration of pollution is low, the organisms showing low Km value with almost similar Vm value should be preferred as biosorbent; as they can remove better mercury even from the dilute solutions.

Results of fresh and preserved biomass were compared (Table: 26). The preserved biomass showed a constant increase in mercury removal loading capacity as opposed to fresh biomass where mercury removal was levelled off above 40 ppm. The enhanced activity of preserved biomass could be attributed to the additional availability of sites which were exposed due to autolysis during the preservation. As seen from the Graph: 5a and 6 preserved biomass proved better sorbent and loading rate was increased up to 80 ppm of initial mercury concentration. Further experiments are needed to understand and exploit this increased mercury removal ability of preserved biomass.
When these results were evaluated in terms of mercury accumulation per unit cellmass it showed two phases. Fast initial phase and later slow phase. The fast phase was the result of passive reaction between mercury ions and cell surface. Once the major sites were occupied the rate automatically slowed down. During the secondary phase the free mercury ions has to search for remaining vacant sites. Internalization of this mercury ions is also a possibility which is a time consuming process giving a slower reaction rate. Similar results are reported for Cd, Co and Cu accumulation by \textit{S. cerevisiae} (178, 182, 204).

Data represented in Tables : 28 and 35 and Graph : 16 showed similar trend as obtained with Isolate 6. The spent mycelia I and II were dead biomass and they acted as ion exchange resins suggesting purely physicochemical surface binding. This experiment supported our view of passive mercury uptake by Isolate 6.

5.3.2. CELLMASS CONCENTRATION

The biomass concentration can also have significant effect on mercury adsorption (Table : 21, 22, 27 and 34). The initial increase in mercury removal with increase in cellmass is due to availability of more sites and subsequent optimization of biosorbent: biosorbate ratio. But as the cellmass increased, a decrease in loading capacity was observed as the bound mercury ions shows inverse relationship with cell density at a lower mercury concentration. For maximum removal the optimum metal: cellmass ratio has to be maintained which changes with the change in type of biosorbent. In case of bacterial Isolate 17, 1:15 ratio was found to be optimum for mercury removal, giving decreased activity on its either side. The lowest ratio of 1:30 tested for Isolate A was higher as compared to the required cellmass concentration and hence the initial increase in loading capacity could not be observed. The \textit{Fusarium} biomass showed similar results with 1:80 as optimum ratio. But in case of spent mycelia this trend was not observed. The loading capacity decreased with the increase in cellmass. This result coincide with the findings of other metal sorption by dead biomass (82). The decreasing mercury removal with the increase in cellmass concentration is due to the electrostatic interactions where a large quantity of cations are being adsorbed on the cell surface when the distance between two sites is more. Similar findings were reported
for Ag, Cd and Cu adsorption by fungal biomass (205, 206).

The adsorptive capacity of mercury is highly dependent on available surface area of the particles. Under equilibrium conditions bacterial cellmass have higher surface area per unit cellmass as compared to fungi which was responsible for better bacterial activity at lower mercury cellmass ratio. The chemical composition of bacterial and fungal cell is different. This may also result in better removal by bacteria as compared to fungi. The comparative data of mercury binding between clay and bacterial cellmass was reported where unit weight of bacterial cellmass binds about 20 times more mercury on dry weight basis than the clay where the difference of surface area was 1:3 for clay and bacteria per gram (18).

Our data reports two fold increase in mercury removal by bacteria as compared to spent mycelia when the tested cellmass concentration and obtained removal were analysed.

5.3.3. Reaction pH

The influence of reaction pH on mercury removal is well depicted in Table : 15, 29 and 36. Except for Isolate A, the other biomass studied i.e. Isolate 6, Fusarium and spent mycelia did not show drastic differences in mercury removal at highly acidic or alkaline pH, but the optimum pH range of each biomass was different. This was due to the different isoelectric pH of each organisms. Isoelectric pH is responsible for the net surface charge of microbes which in turn plays an important role in metal mitigation.

Influence of pH is dependent on the characteristics of both the metal involved and the behaviour of biomass at that particular pH. Depending upon the binding characteristics of a metal to the cellmass, the metals are divided as hard and soft acids or bases (122,142,207). Mercury belongs to the soft class along with Ag and Au for which there is no discernible pH dependence for binding. Mercury being soft in nature forms covalent complexes with soft ligands which contain nitrogen and sulphur. The reaction of this metal ion would therefore be expected to be rather pH independent.

In case of Isolate 6 and Fusarium the optimum pH range was between 6-8, well coinciding with the reported data of high mercury removal near pH 8 by Glombitza et
(191). Above neutral pH i.e. at alkaline pH there was drop in mercury removal by *Fusarium* and Isolate 6. This may be due to the competition with Na\(^+\) ions for binding on the cell surface.

The spent mycelia gave 46% removal even at pH as low as 2. Here the threshold pH value which varies with the metal to the cell surface relationship may not have crossed even at pH 2 and hence the broad pH range was obtained for mercury removal by spent mycelia (208, 209).

Mercury removal by Isolate A was found to be pH dependent. This could be explained on the basis of enzyme inhibition at suboptimum pH. Besides at lower pH some biomass show reversibility of charge i.e. the net surface charge which is negative at its isoelectric pH gets positive at lower pH due to proton binding. Under such circumstances mercury may not bind and this could be the reason for the observed drastic decrease in mercury removal by Isolate A.

Overall the mercury sorption was pH independent, but the chemicals used for pH adjustment showed considerable influence on the mercury removal. When the pH was adjusted to 5, with citric acid, buffer and lactic acid, the reduction in the binding was due to the complexing ability of organic acids with the mercury, not allowing easy removal. In case of buffer, along with citrate, presence of Na\(_2\)HPO\(_4\) was additional competition factor as Na\(^+\) ions were found to compete with mercury ions resulting in 9% decrease in mercury removal as compared to pH adjusted with only citric acid. This findings tally with the data reported by Greene et al where the removal of metal ions was decreased in presence of sodium acetate buffer used for precise pH adjustment (142).

The reported findings of Greene et al (166) regarding the inhibitory effects of chloride ions on mercury removal by *Chlorella vulgaris* biomass was not observed. When pH was adjusted with H\(_2\)SO\(_4\) and HNO\(_3\), it gave 38% and 19% less removal as compared to that adjusted with HCl. This difference could be due to the variation in the microbial species used in this study. The presence of anions in the system have varied influence on removal of same metal by different organisms (127,210). As compared to any other reagent, HCl was found to be less detrimental when used to bring down the pH.
The data presented in Graph : 9 and Table : 23 gives further information regarding the presence of various ions. The lowest removal of 57.96% was due to the presence of additional citrate and disodium hydrogen phosphate as compared to that present in buffer used to adjust the pH 7 of the system only little amount of NaOH was required. This system gave maximum removal due to absence of interfering anions and cations.

5.3.4 CONTACT TIME

The influence of contact time on mercury removal using Isolate A, Fusarium and spent mycelia were studied and results are depicted in Table : 25, 30, 37 and Graph : 12,17a, 17 and 20. From these results it is clear that mercury removal is directly proportional to contact time, but it follows two different patterns. In case of Isolate A and spent mycelia, the removal increases with time upto 150 and 40 minutes respectively and above this time period it gets constant; while for Fusarium there was constant increase in mercury removal up to first 45 minutes. During the next 45 minutes, decrease in mercury removal was observed, which again increased above 90 minutes. This was due to desorption and resorption of desorbed mercury ions with respect to equilibrium between the soluble and immobilized metal ions.

Spent mycelia and Fusarium showed 39.6% and 20% more mercury removal than bacteria during first 15 minutes of contact time indicating sole metabolic independent activity. The involvement of metabolic activity in mercury removal by Isolate A become more prominent as it required more time. This was also supported by the results obtained from other experiments with Isolate A.

The high mercury removal rate registered in the initial phase of reaction with Fusarium and spent mycelia were due to the maximum number of available binding sites. As the time period increased, the contact between adsorbing sites and mercury ions also took time as there was a decrease in number of binding sites as well as mercury ions. Hence the reaction rate decreased gradually resulting in a typical hyperbolic curve. Similar findings were reported for various cellmass and different metals (204). A rapid process of biosorption of other metals by fungal biomass is reported which gets completed within few minutes of contact time (205).
The sorption-desorption pattern observed in *Fusarium* with a regular time interval is not reported for any metal or biomass in the literature. The occurrence of the process should be considered for deciding the optimum contact time.

The optimum contact time was also dependent on the biosorbent: biosorbate ratio and amount of pollutant in the system. The observed difference in contact time (Graph :12) between 20:1 and 40:1; mercury : cellmass ratio agrees with the proportion of the pollutant amount present in the system and explains the influence of pollutant concentration in deciding contact time.

**5.3.5 NaCl CONCENTRATION**

Presence of cations interferes with the metal sorption process. During our study presence of 85 mM sodium ions did not affect the biosorption process drastically but showed only 8 to 14% reduction in mercury removal by tested biosorbents (Table : 18). This indicates that the used biomass were selective in uptake of mercury ions. The presence of Na\(^+\) ions at 85mM concentration did not resulted into total inhibition of the sorption process. The obtained results were similar to the findings by Brady & Duncan (204) who have reported 20% inhibition of Cu\(^{2+}\) sorption in presence of 5mM of sodium ions. Similar findings were also reported for Pb sorption from sea water using marine bacteria (211). The Isolate 6 was found to be most selective giving reduction of 14.4% mercury removal even at a concentration as high as 1700 mM sodium chloride. The other two biosorbents showed more reduction, where the observed reduction in case of Isolate A at 1700 mM NaOH was definitely due to the inhibition of enzymatic activity. Spent mycelia showed 40% reduction in presence of 680 mM NaCl. This could be due to the higher Na\(^+\) ions in the solution as compared to mercury ions which were only 0.5mM (Table : 39).

**5.3.6 PRETREATMENT TO BIOMASS**

The biomass in its native form may not prove a good adsorbent. The physicochemical treatments have been reported for the enhancement of the metal removal ability of the cellmass. Our findings of mercury removal with pretreated biomass of Isolate A and 6, *Fusarium* and spent mycelia are reported in Table : 17,31,38 and Graph : 18 and 21. From the reported findings most of the pretreatment were found to be beneficial
for mercury removal except for Isolate A. The negative influence of all the pretreatment in case of Isolate A may be due to the inactivation or inhibition of the cell metabolism and enzymes; which were the major mechanisms of mercury removal by this isolate.

The other bacterial isolate used in the study showed negative influence of acid treatment. This may be due to the change in net surface charge of the cellmass to positive because of the change in its isoelectric pH. The positive charge of the surface is the result of protons bound to it and this prevents interaction of metals with cellmass. Acid treatment to *Fusarium* also resulted in decreased mercury removal, although the decrease was very less. Acid treatment to spent fungal biomass result in increased permeability, which is beneficial for metal sorption (91, 212). The decreased removal with acid treated *Fusarium* biomass may be the net outcome of occupancy of active sites and exposure of new sites.

Spent mycelia being a dead biomass gave good result with all the treatments. Acid treatment here may have exposed new sites by dissolving any kind of nutrients or impurities present on the surface making the biomass more prone to mercury binding.

The KOH treatment also increases the permeability of biomass. In case of fungal biomass, it forms complexes with chitosan-glucan which is an efficient chelating agent for metals (213). This results in increased mercury removal with KOH pretreated biomass. Besides alkali treatment change the surface charge of cellmass giving enhanced mercury removal (122). Other reports have also demonstrated the increased biosorption of metals due to NaOH treatment (162). The reported negative influence of NaOH in our study with *Fusarium* may be the result of solubilization and subsequent loss of biomass components having mercury binding sites. Brierley and Brierley (162) have also reported the unacceptable loss of biomass due to caustic treatment for many metal ions. So treatment with NaOH required strict control so as to prevent loss of biomass.

The treatment with all the organic solvents were found to be beneficial except for formaldehyde and methanol in case of *Fusarium*. The organic solvents forms complexes with cellwall components and increases the cation binding by removing the competing ions such as diaminopimelic acid from the vicinity of carboxyl groups
Besides in case of spent mycelia it forms stable complexes with chitin and chitosans which shows affinity towards metal ions, thus increasing mercury removal. This type of result are also reported by Galun et al (91). The reported decrease in mercury removal by methanol and formaldehyde treated \textit{Fusarium} biomass could not be explained at this juncture due to the complexity of reactions with a wide variety of reactive groups present on the cellwall of laboratory grown \textit{Fusarium}.

Heat treatment was found to be beneficial for all the biomass tested except Isolate A. It could be due to the exposure of new sites. Tobin et al (171) have also observed beneficial effects of certain killing treatments on biosorption ability during their study. Siegal et al (186) have also reported more rapid uptake of lead by \textit{Penicillium} spp. after heat treatment. The uptake capacity of dead biomass was reported to be greater, equivalent or less than live biomass; depending on the killing treatment used and subsequent alterations in the cellwall structure (215, 216).

The comparison of influence of pretreatment on autoclaved and unautoclaved biomass is shown in Graph : 21. Any treatment given to autoclaved biomass resulted in decrease in mercury removal capacity as compared to when treatments were given to unautoclaved biomass. Autoclaving enhanced the bioremediation ability of the biomass to the highest possible level. Similar results were also observed with heat treated (Boiling waterbath for 1 hour) biomass. Both these treatment resulted in maximum improvement; so there was no further scope for additional improvement by the subsequent solvent treatment. The observed decrease could be due to the activity of these solvents on the heat treated surface resulting into the solubilization of some components which may not have solubilized due to these treatments in case of unautoclaved biomass. Among the solvent treatment given to unautoclaved biomass the formaldehyde treatment resulted into least improvement as the obtained spent mycelia was already exposed once to the formaldehyde by the manufacturing industry.

The observed enhancement in the mercury removal by dead biomass after the treatment may prove useful finding as the use of such biomass would be analogous to the use of commercially available ion exchange resins, with an additional advantage of improved biosorption capacity as compared to ion exchange resins. Such application also mean
that it could be used in the existing conventional plant designs which are already in use as an ion exchange process. Similar views are also shared by other workers (89,212,217).

5. 3. 7 CELL AGE

Comparison between two bacterial isolates Unk and 15 showed two opposite trend when studied to check the influence of cell age (Table : 19). Isolate Unk gave better mercury removal with increase in cell age as opposed to Isolate 15 where removal was decreasing with increase in cell age. This could be due to the change in surface layer properties with reference to their age. In case of Isolate 15, sporulation was observed after 24 hours of incubation time which was responsible for significant changes in the cellwall composition. This type of changes were not observed with Gram negative Isolate Unk. The age related changes in the biosorption of Ag and Cd are reported in literature (139,218). The chemical analysis of cell wall of young and old cells are necessary to reveal the fact behind the difference in mercury removal with age amongst the organisms.

5. 3. 8 SUBSTRATES

Influence of substrate components and pH of the medium were also studied. The use of different carbon and nitrogen sources showed wide variation in mercury removing ability of tested culture (Table : 20). This could be due to the change in the cellwall components due to availability and nonavailability of the specific substrates. The cellwall composition plays a decisive role in metal uptake by microorganisms. The observed variation was as high as 8.74% between the minimum and maximum removal recorded with maltose and sucrose as substrates. Our observations during the growth of this organism showed mucoid growth in presence of sucrose as carbon source which was not seen when this organism was grown on other substrates. The produced exopolymers is also one of the factor for the increased metal ion accumulation. The difference in mercury remediation ability was reported when the yeast biomass was cultivated on acetic acid and hydrocarbons (191).

When the pH of the medium was adjusted between 6 to 8, minor decrease in mercury removal was observed on either side of the neutral pH (Table: 24) In case of isolate
used for this study, the enzymatic reactions were mainly responsible for bioremediation of mercury and pH 7 being optimum pH for growth of this organism; it was also responsible for better enzyme formation. Besides pH of the medium also affects the cell surface properties.

5.3.9 TEMPERATURE

Influence of temperature on bioremoval of mercury by *Fusarium* was studied. Increased removal on either side of 30°C temperature was registered (Table : 32) This findings coincide with the reported results of silver removal by Isolate PT 35 (219). The increase in temperature from 30 to 50°C improved the gross mercury removal by 12%, but when the net biological removal was considered throughout the experiment, removal decreased with increase in the temperature. The increase in gross removal was due to the volatilization of 15% mercury which was nil at 15 and 30°C. These findings indicates that if the biosorption is main aim than the temperature should be kept around 30°C. Above 50°C, a slight decrease in mercury removal was observed. This higher temperature affects the chemistry of the metals and increases its solubility as opposed to lower temperature of 15°C, where the reduction in solubility gave nearly 9% increment in mercury removal as compared to 30°C. Low temperature favours physical binding which is very stable. This could also be plausible explanation for better removal at 15°C.

5.4 MERCURY REMOVAL USING CELL COMPONENTS

From the reported data and some of the already discussed parameters, metabolic independent and dependent mechanisms for mercury removal were noticed. The findings reported in Table :16 and 33 showed 46.8% mercury removal by water soluble cell free extract of Isolate A as compared to 5.33% and 3.16 % removal by Isolate 6 and *Fusarium* respectively. These data indicates the participation of active enzymes in mercury removal in case of Isolate A, which was not found for Isolate 6 and *Fusarium* Spp.

These findings were supported by data obtained with influence of pretreatment to biomass. None of these biomass were affected adversely by any of the mild pretreatment, while in case of Isolate A mercury removal was reduced drastically at
low pH and even at high concentration of NaCl, which were responsible for inactivation of enzymes. Besides Isolate A also gave bioremediation of mercury containing organic waste where most probable mechanism was enzyme mediated volatilization of mercury.

Intact cellmass and celldebris showed almost equal removal with no statistical significance indicating the physical binding of mercury to the cell surface. The comparison between the uninoculated broth and spent broth after harvesting the cellmass also resulted in almost equal mercury removal. The observed decrease with spent broth could be due to the exhaustion of some organic ingredients during the growth of bacteria, which resulted in comparatively less availability of the organic complexing components of the medium.

The wide difference observed between the freshly harvested and dried crushed *Fusarium* biomass could be the result of difference in net amount of cellmass present as freshly harvested cellmass may have 60 to 70% water as compared to dried cellmass. The other three components used for biosorption were not having any active biosorbent and hence the recorded low mercury removal.

5.5 FREUNDLICH ADSORPTION ISOTHERMS

The initial mercury concentration influenced the mercury adsorption in a typical manner which has been demonstrated by Freundlich adsorption isotherms as shown in Graph: 14 and 22 for Isolate 6 and spent mycelia. The constant k represents the predicted quantity of mercury removed per unit weight of dry cellmass at an equilibrium concentration.

As can be seen from this data k constant were 56.23 and 4.89 for Isolate 6 and spent mycelia. This indicates that bacterial isolate was more efficient as compared to spent mycelia A good relationship was found for mercury removal from solution as a function of initial mercury concentration from 1 to 10 mg and 1 to 50 mg for Isolate 6 and spent mycelia respectively. The slope of 0.25 and 0.275 for Isolate 6 and spent mycelia respectively indicates the intensity of the reaction.

5.6 MERCURY REMOVAL BY WASTE BIOMASS FROM LABORATORY

Miscellaneous cellmass used in our laboratory were also tested as biosorbent for
mercury removal. As depicted in Table :50, none of the biosorbent used was found efficient as compared to *Micrococcus luteus* which was tested as control along with other sorbents. Nearly 7 to 18 fold reduction in mercury removal was observed with laboratory grown yeast, waste yeast and artificially cultivated activated sludge. This emphasizes the importance of using selected organisms for the process. The loading capacity of *M. luteus* was highest as it had maximum surface area as compared to other biosorbents; as yeast due to its large size and activated sludge due to floc formation gave comparatively less total surface area per unit weight of biosorbent.

### 5.7 IMMOBILIZATION

The results of immobilized biomass are presented in Table: 40 to 46. When alginate was used as immobilizing agent the obtained results were not very encouraging. In all the experiments free cells removed more mercury as compared to immobilized biomass. Besides alginate itself was found to absorb mercury from the system depending upon its concentration used for immobilization. After certain time lapse considerable amount of mercury was desorbed in the system without any definite time bound pattern. The observed decrease in mercury removal as compared to that with free cells was due to entrapment of cells within alginate beads resulting in less available surface area for binding of mercury ions.

Hence attempts were made to immobilize the cellmass on the surface of various materials. Among the used immobilizing agents PVC pieces and sand were found to be inert. But in case of PVC pieces used as support the binding between the cells and support was very weak resulting in the cell wash out during the experiment.

Sand and wood shavings were found to be a better immobilizing supports. Wood shavings facilitated the growth of mercury resistant organisms, which proved to be on additional advantage. The support itself was responsible for mercury absorption but unlike alginate mercury desorption from wood shavings was very difficult, as the adsorbed mercury could not be eluted by mild treatment. On the other hand sand bed was also equally efficient as a support for immobilization. The optimum assorted size combinations were needed for desired percolation and prevention of cell wash out. Sand as a immobilizing support is economically viable and could be used repeatedly due to efficient desorption as compared to wood shavings.
5.8 SCALE UP

The individual experiments with bacteria and fungal biomass as mercury sorbents gave encouraging results during shake flask study with few mg cellmass and limited concentration of pollutant.

To check the viability of the process it is necessary to reproduce the results at larger scale with feasible process design. With this aim scale up experiments were designed using spent mycelia collected from Pune. The selection of biosorbents was due to the easy availability in desired quantity and better loading capacity as compared to spent mycelia.

The main criteria for design selection were economy in terms of energy and simplicity.

Of the first two designs (Table : 47, 48), three funnel assembly gave slightly better loading capacity as compared to heap study. The size of the heap being bigger provided less surface area. Besides in case of three funnel assembly the cellmass was distributed in three stages which provided stepwise exposure to second and third stage where the waste already passed through first funnel resulted in lower concentration of mercury in the solution; facilitating better removal. This type of distribution also reduced chances of channelling which provided better exposure of the entire cell surface area.

Inspite of the nearly 3 fold scale up in terms of biosorbents and adsorbate concentration the loading capacity was reduced by 1 mg only as compared to lower scale. This indicates possibilities of further scale up. The specific design could be selected to give more surface area, equal distribution and percolation of the pollutant. The second set of experiment for scale up study was designed in column so that it could be used directly in the already available plant designs. The airlift percolator used provided continuous recycling and the long retention time of 8 to 16 hours for each batch resulted in enhanced loading capacity (Table : 49).

After the ninth batch, the experiment was ceased for three days and reloaded with the tenth batch. This resulted in nearly 50% reduction in mercury removal efficiency. It could be due to the drying of cellmass and precipitation of soluble impurities over the cell surface forming a barrier between active sites and mercury ions. Some more experiments are needed to check the influence of resting period when field level...
experiments are designed.

The results in Graph : 23 indicates the necessity of recycling to achieve the maximum removal. The removal decreased in each successive batch; as cellmass gets loaded and less active sites are available for succeeding batches. Even in the third batch more than 70% mercury removal was obtained after four cycles. This could be enhanced by providing more recirculation.

5.9 WASTE BIOREMEDATION

5.9.1 INDUSTRIAL WASTE

The results discussed hitherto deals with the remediation of mercury from aqueous HgCl₂ solution without any other major impurity. This type of experiments' though give an good idea about the different factors influencing the process, it however does not present a true picture regarding the technology when applied to the actual waste. Thus it was pertinent to use the developed biomass and the process for mitigation of mercury from industrial waste.

The treatment of waste II with spent mycelia gave 87.34% mercury removal and rise of pH from 2.5 to 4. This could be due to the reaction with the biomass which neutralizes some acidity.

Waste I was subjected to treatment with regard to mercury removal using Isolate A and Fusarium (Table : 51, 52). Both the biosorbent gave almost similar results but the pattern of mercury removal was different in each case. Fusarium was able to remove 80% of mercury within one hour of contact time and remaining 20% was removed during the incubation time of 24 hours. While with Isolate A the picture was reverse. The contact time of 2 hours gave only 23% removal and the remaining mercury was removed as the cells grew during the incubation time of 24 hours. This indicates biosorption as main mechanism of mercury removal by Fusarium biomass where as Isolate A could be giving mercury removal due to its enzymatic activity. Isolate A also showed remarkable resistance to mercury and reports are available indicating that mercury resistant plasmid can code for synthesis of inducible mercury reductase and organomercurial lyase enzymes (21,31).
The use of *Fusarium* biomass was found to be more encouraging for waste treatment as large quantity of mercury was scavenged within one hour of contact time. If the results are compared in terms of mercury removal per unit cellmass Isolate A was found to be superior, but the easy availability of fungal biomass as industrial waste in desired quantity easy immobilization and easy recovery of mercury gives an edge to spent mycelia as biosorbent for treatment of large quantity of waste. If after the mycelial treatment, the residual mercury in the waste is still above permissible limit, it could be treated with actively growing bacterial Isolate A.

The frequent use of MEMC, HgCl₂, PMA, ethyl mercuric phosphates and other mercuric compounds in agriculture as fungicides and seed dressings is main cause of mercury pollution of soil (120). Hence one set of experiment was conducted with MEMC and results are depicted in Table : 53. The adaptation of culture to MEMC substrate enhanced bioremediation ability by 11.1%. Here the involvement of possible enzymes could be organomercurial lyase and mercuric reductase. Organomercurial lyase break C-Hg bond releasing Hg²⁺ which then reduced to Hg⁰ (volatile form) by mercuric reductase or picked up by cell components and ultimately removed from the system. Reports are also available for induction of mercury reductase and organomercurial lyase in *Beijerinckia*, *Azatobacter* and *Thiobacillus* (120, 161).

The developed fungal and bacterial biomass were found to useful as they gave mercury removal from industrial waste with good efficiency. They could be further optimized by incorporating the selected pretreatment to biomass.

5.9.2 LABORATORY WASTE

Spectrophotometric method used for mercury estimation extracted mercury-iodide-malachite green complex in benzene. The colour intensity of benzene depended on the concentration of Hg-I-malachite green complex which in turn was directly proportionate to the amount of mercury present in the sample. As can be seen from photographs : 18 and Graph : 25 the used Isolate A reacted with the complex and brought about the decolourization of waste due to the removal of Hg-I-malachite green complex. Here the mercury could have been volatilized and some complex mechanism may be responsible for decolourization. The observed gradation in colour and residual mercury (Photograph : 17) was due to time bound activity of the culture.
and respective enzyme.

On the basis of preliminary success and importance of the process optimization was carried out. As can be seen from Tables: 53 to 55 and Graphs: 26 and 27, the mercury remediation process was slow and gradual as compared to treatment of inorganic waste with dead biomass suggesting involvement of enzymes in the reaction. Twenty minutes of contact time was required for 60% mercury removal even with the highest cell density tested. The amount of mercury removal with respect to increase in the cellmass at any time interval was more than stoichiometric removal due to physicochemical binding. The observed difference in decreased removal rate with increase in contact time and cellmass concentration also supports the participation of actively metabolizing cells. The obtained 15µg mercury removal in absence inoculum was due to aeration giving auto conversion of Hg²⁺ to more volatile Hg⁰.

Loading capacity and loading rate showed same increasing trend upto 6mg cellmass above which both the response declined. This was due to the excess of cellmass in the system and amount of the pollutant.

The observed variation in mercury removal rate with respect to contact time could be explained on the basis of enzyme substrate ratio as well as the exerted inhibitory effect due to presence of high amount of mercury. Also the cellmass required time to acclimatise itself to the inhibitor which again depended upon the concentration of inhibitor. This factor may be responsible for the variation in mercury removal rate with respect to time.

The results reported in Table: 56 and 57 could be explained on the basis of typical enzyme kinetic reactions. In none of the system, the substrate was limiting during first 10 minutes, but as the time passed it became limiting in first two systems i.e. 10 and 20 ml substrate.

The difference in removal obtained with increasing waste volume could be due to the difference in the available reactor volume ratio. (This parameter is discussed in detail in the later text).

The influence of mercury concentration on its removal presented in Table: 58 and 59
and Graph: 31 follows the substrate and enzyme kinetics. When the data are presented in terms of time profile reaction, it follows a typical growth phase pattern. The initial logarithmic phase of reaction supports involvement of only enzymes rather than other metabolites, as it did not show any lag phase. The reduction in reaction rate increases up to 20 ppm of mercury above which it gets constant giving overlapping of graph.

The results of organometallic waste were analysed to understand the kinetics of the reaction.

Graph: 29 and 32 represents the highest rate of reaction and maximum removal versus substrate concentration. Both the graphs followed similar pattern giving the removal rate of 70 μg mercury/minute. When the overall mercury removal versus substrate was plotted, it gave maximum 18.5 μg/ml removal. As the volume of system was kept variable the rate was considered for Graph: 29 instead of μg/ml removal. The rate of reaction was found constant when mercury concentration was kept above 20 ppm and waste volume above 40 μg/ml. This indicates that above this mercury amount; either in terms of varying waste volume or concentration, it is not limiting and so does not control the reaction rate.

When the removal rate/minute was considered (Graph: 33), the data showed constant increase between 4 to 12 μg/ml mercury concentration. The sharp increase between 12 to 16 μg/ml mercury may be due to optimum substrate enzyme ratio. Above 16 μg/ml mercury, the amount of enzyme in the system may not be sufficient to accelerate the reaction with corresponding increase in the substrate concentration. During 10 minutes time interval the difference in rate of reaction between 16 and 20 μg/ml became less prominent. As during first 5 minutes some of the mercury gets removed, the available substrate at 6th minute is less giving less variation in reaction rate as compared to that in first five minutes.

The line weaver Burk plot for reaction rate with respect to waste volume variation and amount of mercury in a fixed volume represented almost identical pattern. The Km value for 1/V versus 1/S plot; where substrate was in terms of waste volume was 200 μg as compared to 58.8 μg observed with variation in mercury concentration in a fixed volume. The reaction rate was almost constant for first 10 minutes of reaction.
giving some Km value of 100 μg as opposed to the Vm value of 250 μg/minute and 200 μg/minute with 5 and 10 minutes respectively.

The Vm value was further reduced to 100 μg/minute when overall reaction rate was considered. This indicates the influence of total contact time and substrate enzyme ratio on the overall process. The Km value gives the optimum substrate concentration to obtain the desired activity. All the four graphs gave almost similar Km: Vm ratio which was 1:2.

When the experiment was conducted with varying volume of waste, the reaction rate was significantly influenced by change in reactor: waste volume ratio. The data are represented in Table : 60 and Graph : 37 to 39. As the reactor: waste volume ratio was raised, the reaction rate increased due to better aeration, agitation and diffusion of air into the medium. The larger the ratio better the activity was observed. Both mercury removal rate and loading rate were also affected equally with the change in the vessel: reactant volume ratio. The ratio of maximum: overall removal rate followed inverse pattern, where the larger vessel volume enhanced the overall rate but maximum reaction rate did not show increase in similar proportion as the reaction of bioremediation of laboratory waste was regulated by the enzyme activity.

When the agar immobilized cellmass was used for decolourization of waste the reaction was completed in 120 minutes but the continuous agitation resulted in breaking of agar gel giving increased optical density. This indicated unsuitability of immobilizing agent.

5.10 DESORPTION STUDY

It is desirable to recover the absorbed mercury from the biomass particularly if the biomass source is not easily renewable. Incineration is not always the best choice as it destroys the biosorbent. Hence different eluting agents were used to recover mercury from mercury loaded biomass of Fusarium and spent mycelia as depicted in Table : 61 to 64.

In case of Fusarium both HCl and HNO₃ failed as they resulted in only 12 and 14% efficiency. HNO₃ was more successful in eluting mercury from spent mycelia with 39.49% efficiency as compared to Fusarium. This may be due to internal
compartmentation of mercury in case of *Fusarium* biomass apart from only surface binding. HCl as eluant is reported for other metals. Mattuschka et al (185) reported HCl for desorption of copper. Mercury sorption process by most of the biomass was pH independent, hence in this case acids proved poor eluants. Of all the tested eluants EDTA proved better not only in terms of efficiency, but it also caused minimum damage to the cell surface. The EDTA treated biomass of spent mycelia when reused gave almost equal loading capacity. Use of EDTA is reported for Zn recovery from dead biomass (147).

During our study only 53.29% mercury could be recovered which was considerably less than the reported data for other metals (70). This was due to the lack of efficiency of used eluants.