Title: Microbial Metal Bioaccumulation

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Article Summary:

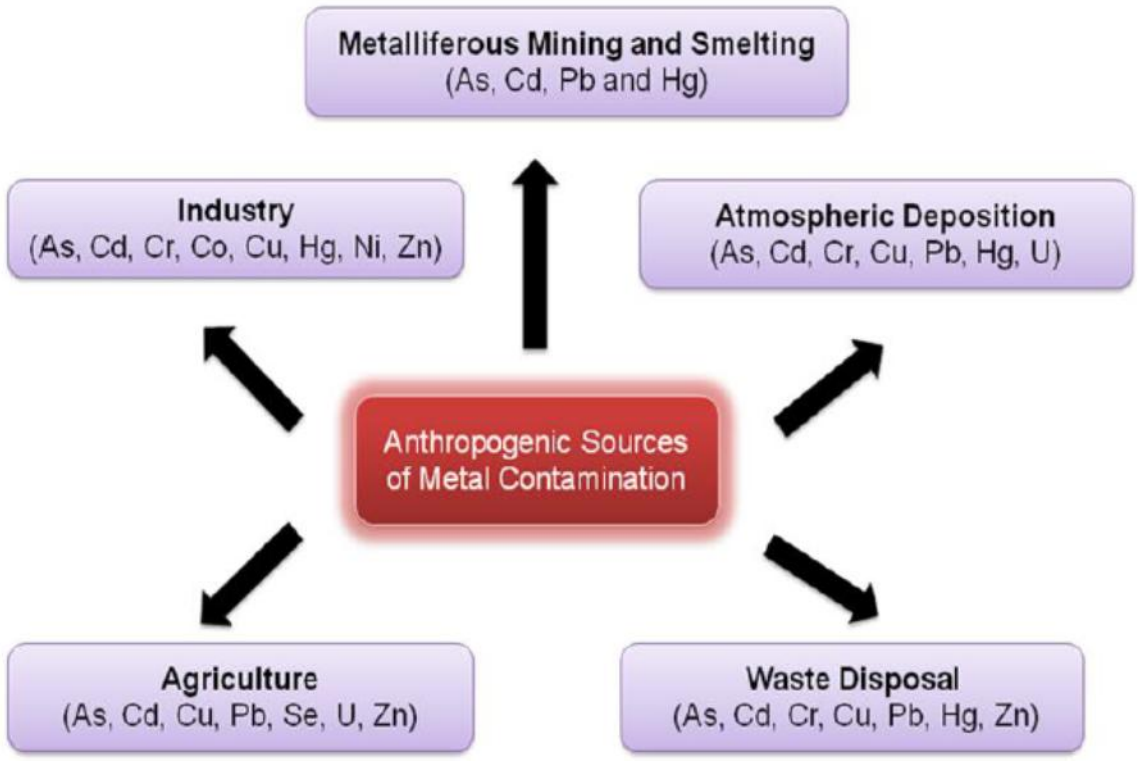
Heavy-metal pollution represents an important environmental problem due to the toxic effects of metals, and their accumulation throughout the food chain leads to serious ecological and health problems. Metal remediation through common physico-chemical techniques is expensive and unsuitable in case of voluminous effluents containing complexing organic matter and low metal contamination. Biotechnological approaches that are designed to cover such niches have, therefore, received great deal of attention in the recent years. Biosorption studies involving low-cost and often dead/pre-treated biomass have dominated the literature and, subsequently, extensive reviews focusing on equilibrium and kinetics of metal Biosorption have also come up. However, the low binding capacity of biomass for certain recalcitrant metals such as Ni and failure to effectively remove metals from real industrial effluents due to presence of organic or inorganic ligands limit this approach. At times, when pure biosorptive metal removal is not feasible, application of a judicious consortium of growing metal-resistant cells can ensure better removal through a combination of bio precipitation, Biosorption and continuous metabolic uptake of metals after physical adsorption. Such approach may lead to simultaneous removal of toxic metals, organic loads and other inorganic impurities, as well as allow optimization through development of resistant species. However, sensitivity of living cells to extremes of pH or high metal concentration and need to furnish metabolic energy are some of the major constraints of employing growing cells for bioremediation. The efforts to meet such challenges via isolation of metal-resistant bacterial/fungal strains and exploitation of organic wastes as carbon substrates have begun. Recent studies show that the strains (bacteria, yeast and fungi) isolated from contaminated sites possesses excellent capability of metal scavenging. Some bacterial strains possess high tolerance to various metals and may be potential candidates for their simultaneous removal from wastes. Evidently, the stage has already been set for the application of metal-resistant growing microbial cells for metal harvesting. This review focuses on the applicability of growing bacterial/fungal/algal cells for metal removal and the efforts directed towards cell/process development to make this option technically/economically viable for the comprehensive treatment of metal-rich effluents.

Article:

Introduction

Industrialization has long been accepted as a hallmark of civilization. However, the fact remains that industrial emanations have been adversely affecting the environment. Industrial effluents containing toxics and heavy metals drain into the river, which is often the source of drinking water for another town downstream. Municipal water treatment facilities in most of the developing countries, at present, are not equipped to remove traces of heavy metals, consequently exposing every consumer to unknown quantities of pollutants in the water they consume. The main sources of heavy-metal pollution are mining, milling and surface finishing industries, discharging a variety of toxic metals such as Cd, Cu, Ni, Co, Zn and Pb into the environment. In the last few decades, concentration of these heavy metals in river water/sediments has been clearly demonstrated. Eventually, build-up of dangerous concentrations of toxic metals in grains and vegetables grown in contaminated soils is most alarming due to harmful effects of metals on human health. It is well known that heavy metals can be extremely toxic as they

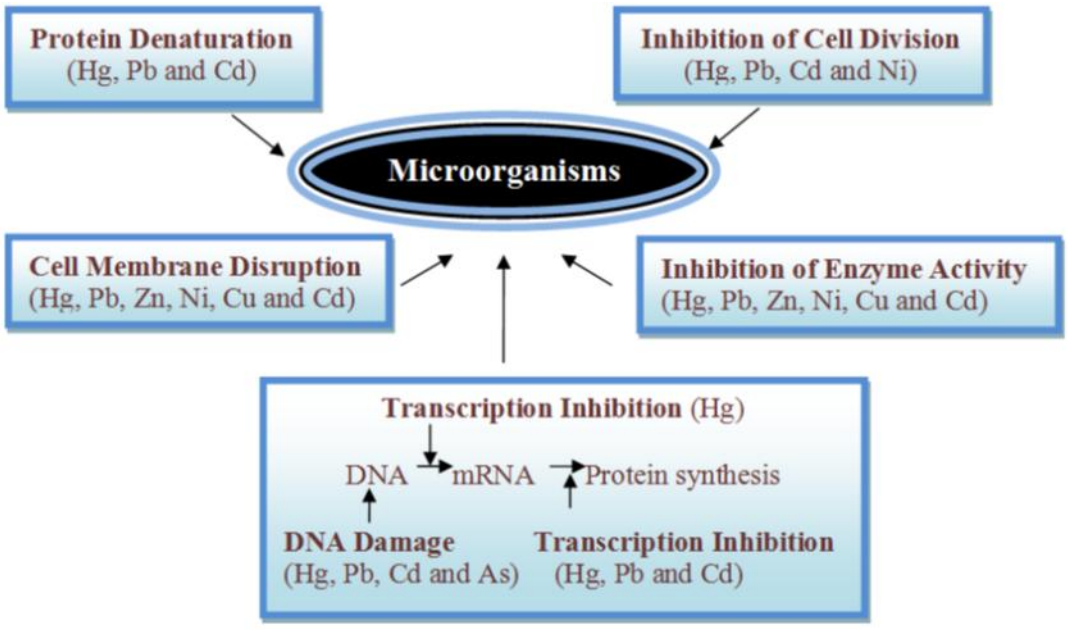
damage nerves, liver and bones, and also block functional groups of vital enzymes (Moore, 1990; Ewan and Pamphlett, 1996).



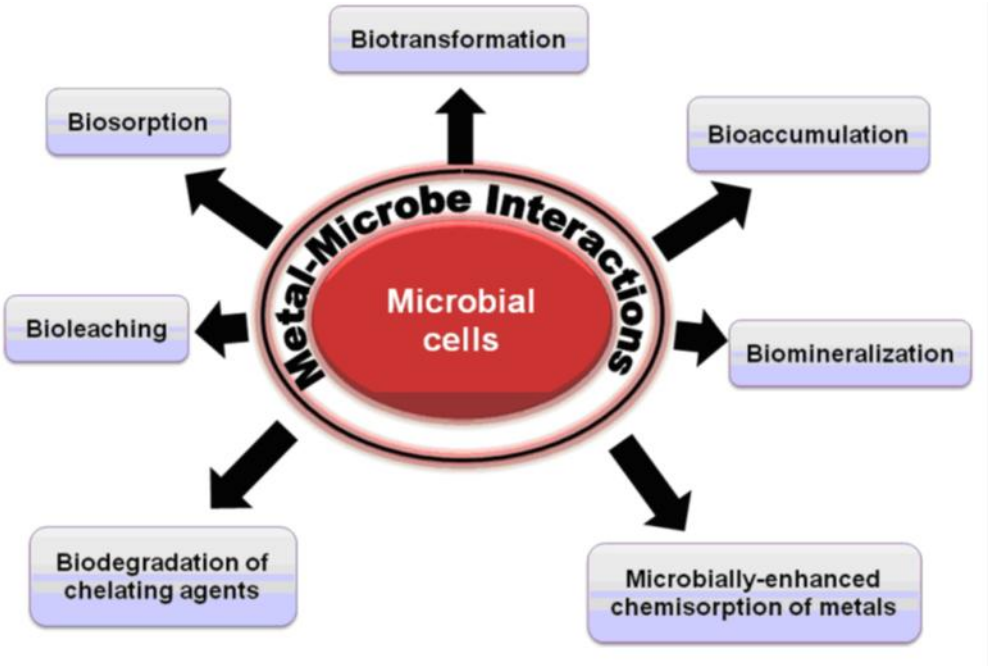
Anthropogenic activities leading to the contamination of soils with heavy metals

Some of the metals like Ni are also listed as a possible human carcinogen (group 2B) and associated with reproductive problems and birth defects. Besides, a range of detrimental effects on fauna and flora are also well documented. Often, these contaminants also inhibit biological remediation processes due to metal sensitivity of the strain and necessitate additional combat strategies for efficient operation (Malik, 2000; Malik et al., 2001). Since these heavy metals are a valuable resource for different industrial applications, their recovery and recycle assumes even greater significance. Further, strict environmental regulations compel industries to shift to cleaner production methods, demanding the development of environmental friendly, low-cost and efficient treatment technique for metal rich effluents. Although the removal of toxic heavy metals from industrial wastewaters has been practiced for several decades, the cost-effectiveness of the most common physico-chemical processes such as oxidation and reduction, chemical precipitation, filtration, electrochemical treatment, evaporation, ion-exchange and reverse osmosis processes is limited. High reagent requirement and unpredictable metal ion removal are some other disadvantages associated with such techniques. Further, strong and contaminating reagents are used for desorption, resulting in toxic sludge and secondary environmental pollution. These disadvantages can become more pronounced and further aggravate the process cost in case of contaminated ground waters, mine tailings effluent and other industrial wastewaters due to voluminous effluents containing complexing organic matter and low metal contamination. Biotechnological approaches can succeed in those areas and are designed to cover such niches. Microorganisms have evolved various measures to respond to heavy-metal stress via processes such as transport across the

cell membrane, biosorption to cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation– reduction reactions (Rai et al., 1981). They have proven capability to take up heavy metals from aqueous solutions, especially when the metal concentrations in the effluent range fromless than 1 to about 20 mg/l (Brierley, 1990). Besides, flexibility to handle the range of physico-chemical parametersin effluents, selectivity to remove only the desiredmetals and the cost-effectiveness are some added advantages of biological metal cleanup techniques. These factors have promoted extensive research on the biological methods of metal removal. High metal-binding capacities of several biological materials have already been identified in part. Among the biosorbents, there are marine algae (Volesky and Holan, 1995), bacteria (Hartmeier and Berends, 1995), yeasts(Sugawara et al., 1997), fungi and waste mycelia from the fermentation (Luef et al., 1991) and food industry (Senthilkumar et al., 2000). Further, the capacities of these microorganisms to accumulate an ample range of metal species have also been described (Volesky, 1994). In the recent past, biosorption studies involving low-cost, easily available, dead or live biomass have dominated the literature (Mogollon et al., 1998). Metal uptake capacity by various biosorbents (algae/fungi/yeasts) has been evaluated using biosorption isotherm curves derived from equilibrium batch sorption experiments and effect of various process parameters such as pH, biomass loading, biomass pretreatments, etc. have been studied extensively (Sag et al., 2000). Further, desorption of adsorbed metals using dilute eluents and cyclic use of regenerated biomass has also been demonstrated (Chang et al., 1997). However, recently, it was reported that live Aspergillus niger cells possessed higher Ni biosorption capacity than dead biomass pretreated with sodium hydroxide, detergents, formaldehyde and dimethyl suphoxide, probably due to intracellular Ni uptake (Kapoor et al., 1999). Similar reports indicating significant metal uptake by growing cells of various microorganisms call for reviewing the performance of such processes in order to carve a niche in the scenario dominated by purely biosorptive processes.



Heavy metal-toxicity mechanisms to microbes



Metal-microbe interactions affecting bioremediation

Growing cells for remediation

Gadd (1988) and Brierley (1990) have described the many ways in which bacteria, fungi and algae can take uptoxic metal ions. Heavy-metal ions can be entrapped in the cellular structure and subsequently biosorbed onto the binding sites present in the cellular structure. This method of uptake is independent of the biological metabolic cycle and is known as ‘‘biosorption’’ or ‘‘passive uptake’’. The heavy metal can also pass into the cell across the cell membrane through the cell metabolic cycle. This mode of metal uptake is referred to as ‘‘active uptake’’. The metal uptake by both active and passive modes can be termed as ‘‘bioaccumulation’’. Most of the studies dealing with microbial metal remediation via growing cells describe the biphasic uptake of metals, i.e., initial rapid phase of biosorption followed by slower, metabolism-dependent active uptake of metals (Donmez and Aksu, 1999). Recent reports employing growing cultures of marine microalgae indicate that intracellular Cd levels are often higher than the biosorbed ones. Mostly during the studies employing harvested biomass (dead/ pretreated), metal is not taken up into the cells; rather, it is just adsorbed at the cell surface and, thus, only a small fraction of bioaccumulation capacity is exploited. Although high biosorptive potential for several types of dead/pretreated microbial biomass has been reported, their strength in field remains to be tested. Most of the metal biosorption studies are conducted using synthetic metalsolutions and when the biosorption potential using real industrial effluent is tested, the efficiency turns out to be very low. Corder and Reeves (1994) found improvement in Ni biosorption (from NiCl2 solution in deionized water) capacity by autoclaving of cyanobacteria biomass. However, none of the three species could bind Ni efficiently in actual effluent sample due to the presence of high concentration of sodium ions. Thus, current methods

of biosorption are sensitive to ambient conditions such as pH, ionic strength and the presence of organic or inorganic ligands. Moreover, Biosorption also lacks specificity in metal binding. Some metals like Ni are more recalcitrant pollutant, and many microorganisms have a relatively low Ni-binding capacity. As per the reports, pure Ni sorption to dead biomass only reaches a loading of 0.05–0.3% at 2–5 mg/l concentration of Ni. This shall result in large volumes of sludge. Reusability of biomass after desorption is possible only if relatively weak chemicals are used for desorption. Thus, the resulting eluents are low in concentration of metals and pose problem in final recovery of metals. Further, biomass needs to be exchanged after a maximum of 5–10 sorptions–desorption cycles. Due to these facts, it seems to be impossible to develop a continuous system based only on biosorptive removal of metals using microbial biomass.

As discussed above, many a times, biosorption alone may not suffice for effective metal remediation. Under such situation, application of active and growing cells might be a better option due to their ability of self-replenishment, continuous metabolic uptake of metals after physical adsorption, and the potential for optimization through development of resistant species and cell surface modification. Further, the metals diffused into the cells during detoxification get bound to intracellular proteins or chelatins before being incorporated into vacuoles and other intracellular sites. These processes are often irreversible and ensure less risk of metal releasing back to the environment. Apart from this, using growing cultures in bioremoval could avoid the need for a separate biomass production process, e.g. cultivation, harvesting, drying, processing and storage prior to the use. In contrast to conventional chemo-physical and biosorptive methods, employment of active microorganisms may allow development of a singlestage process for removal of most of the pollutants present in industrial effluents. Growing cells have unlimited capacities to cleave organo-metallic complexes, degrade organic compounds, as well as take up other inorganic ions such as ammonium, nitrate and phosphate. Further, dissolved and fine-dispersed metallic elements can also be removed via immobilization.

Yet, there are significant practical limitations to biouptake by living cell systems such as sensitivity of the system to extremes of pH, high metal/salt concentration and requirement of external metabolic energy (Donmez and Aksu, 2001). However, such challenges can be met via strain selection and exploitation of organic wastes as carbon substrates. The isolation and selection of metal-resistant strains shall be a crucial aspect to overcome the prime constraint of employing living cell systems. Incidentally, resistant cells are expected to bind substantially more metals, which in turn is a prerequisite for enhanced bioprecipitation/ intracellular accumulation and development of an efficient process. Instead of depending upon single species, a better approach could be towards designing aconsortium of strains having high metal biosorption, bioaccumulation and bioprecipitation capacities. Multispecies consortia can better withstand extreme conditions often encountered with industrial wastewater like spikes of pH or high metal concentration. The rich exopolymer content of the biofilms may also be beneficial for both entrapping dispersed solids and biosorption of dissolved metals. Further, they provide a microenvironment (like alkaline pH, high concentrations of CO2), which could be very beneficial for metal precipitation. The positive interaction between constituent species may also facilitate the survival of sensitive strains. In view of these facts, the applicability of growing cells in suitably configured bioreactor appears to offer promising biotechnology for combating heavy-metal pollution in the environment.

Several studies on application of growing microbial cells for metal scavenging have been reported. However, in toxic metal removal applications, it is important to ensure that the growing cells can maintain a constant removal capacity after multiple bioaccumulations–desorption cycles, and a suitable method is required to optimize the essential operating conditions. The situation demands a multi-prong approach including strain isolation, cell development and process development in order to make the ultimate process technically and economically viable.

These issues form the theme of the present review besides a brief picture of the mechanisms of

accumulation and localization of accumulated metals in living cells. In addition, other integrated microbial processes, which somehow differ from the conventional biosorption, have also been discussed

Mechanisms of four metal-removing-methods

To date, main four methods were proposed by researchers: chemical or physical remediation, animal remediation such as earthworm, phytoremediation and microremediation. Because of the obvious disadvantages and deficiency in feasibility, wide application of the former two methods is restricted. In this part, mechanism of each four methods for removing hazardous heavy metal is explained and compared, and we concerns more about the latter two-phytoremediation and microremediation (bioremediation).

Mechanism of chemical or physical remediation

Chemical or physical method is early used and even endemically commercialized in America. Physical methods (e.g. soil leaching method and absorbent fixation) and chemical methods (e.g. bioreduction and chelate extraction) are used in practice. In these methods the use of chelators cannot be avoided. By adding synthetic chelators such as EDTA (ethylenediamine-tetracetic acid), both the solubility and bioavailability of heavy metals are improved. A chelating reagent's molecule can form several coordinative bonds to a certain metal atom, increasing its concentration in soil aqueous phase and mobility. Considering somebmetal ions strongly bonds to the soil phase and are less bioavailable, powerful chelating reagents are employed such as Na salt of EDTA. However, such approach needs not only expensive chemical reagent and machines but also many technicians. Worse, excessively usage of chemical chelates has been proven to pollute the ground water and negatively affect soil quality, for many necessary ions are also chelated unselectively. For example, elements Fe and Ca are usually lost after the spray of EDTA because their concentration in the soil is much higher than those target heavy metals such as Pb and thus have more access and possibility to chelation. Wenzel et al. conducted an experiment using canola (Brassica napus L) and reported that leaching losses of Cu, Pb and Zn (polluting ground water) far exceeded the amounts of metal taken up by plants after EDTA was applied, which indicated that under some certain circumstance the disadvantage of chelating reagent far outweigh its advantage. Therefore, taking reagent toxicity, unselectively and inefficacy into account, a careful consideration concerning ecology, economy and human health is imperative before chelators are being put into practice.

Mechanism of animal remediation

Animal here mainly refers to earthworm because it is one of the most important soil organisms and plays an indispensable role in improving soil quality. By their feeding, burrowing, excreting and metabolic redox material, both soil texture and nutrition content are improved. Chemical groups such as –COOH and –CO is generated and exuded, which acidify soil and activate heavy metals. Several kinds of gel material are also excreted which facilitate complexion and chelation of metal ions. However, because of the relatively small amount and specific surface area compared with microbes, such improvement is neither notable nor stable. According to Baker et al., after Eisenia foetida earthworm was inoculated, pH of cock manure decreased by 0.7–0.9. However, if the inoculation occurred in an acidic red soil the value drops only by 0.03–0.18; if the inoculation happens in a sandy soil, no obvious decrease of pH is observed. Thus current studies imply that the effectiveness and efficiency of earthworm depend too much on outer conditions and may not be the optimum way of rapidly removing heavy metals. Further investigation in this field is needed.

Mechanism of phytoremediation

Accumulation and transport

In the rhizosphere of hyperaccumulator plants, protons are released by root to acidify the soil, which mobilize metal ions and increase metal bioavailability. This mechanism is supported by Crowley et al. in 1991. However, due to metal ions' charge, lipohilic cellular membrane would be the first barrier of ions' entrance into cells. Fortunately, the following kinds of secretion can facilitate the transportation process. (1) Transporter proteins: Specific binding domain is existed in such proteins, which binds to and transports metal ions from extracellular space into cells. Lasat et al. have found that hyperaccumulator Thlaspi caerulescens had bigger capacity for Zn2+ than its relative T. arvense. And such gap is caused by different amounts of Zn transporter proteins, which indicates that transporter proteins play a crucial role. (2) Nature chelators: As we know chelators such as EDTA can bind to heavy metal ions and make them render uncharged. An uncharged ion is of high mobility and is much easier to get through cellular membrane. In fact, plants can excrete nature chelators, which is much less toxic and more biodegradable as compared to EDTA Among nature chelators, phytochelatin (PC) and Metallothionein (MT) interestmanyscientists and is well studied.MTsare categorized into three classes: Class 1MTs referred to polypeptides related to mammals, which contain 61 amino acids but lack aromatic amino acid or histidines; Class 2 MTs originally come from yeasts, and Candida albicans or cyanobacteria an familiar chelator belonging to this class is Saccharomyces cerevisiae MT, contributing to plants' high copper tolerance; Class 3 MTs is PCs exactly, which are composed of only three amino acid-Glu, Cys and Gly, with Glu and Cys residues linked through a carboxymide bond. In addition, Kagi have found that heavy metals such as Cd, Zn, Hg, Ag and Pb can induce the synthesis of MTs especially in animal and plant species. Arecent study shows that the best activator is Cd followed by Ag, Bi, Pb, Zn, Cu, and Hg and Au Organic acids: Several organic acids (e.g. malic acid and citrate) have been identified as positive bio-reagents to accelerate the absorption of heavy metals by root. Such mechanism is even more notable in the root-shoot transportation. However, substantial achievements are lacked in the root–shoot transportation except two points: one is that root–shoot pathway is closely related to plants' transpiration efficiency; the other is one of chelator ligands (histidine) is found in high levels in the xylem sap of Ni tolerant plant (Alyssum lesbiacum) and the coordination of Ni with histidine is substantiated by Kramer et al. which implies that chelation mechanism also works in the process of xylem transferring. On the molecular level, accumulation and transport mechanism is partly clarified. Many transporters encoded by specific genes are investigated and it is common that one kind of metal ion can be transported by different carriers.

Detoxification

As we know some hazardous heavy metals exercise a detrimental influence on cells by binding to vital protein, interfering with cellular activities and inhibiting regulation of cells. Luckily, hyperaccumulator plants have evolved their ownmechanisms to protect themselves from negative heavy metal stress. Several important detoxification mechanisms are explained as follows:

(1) Chelation: Chelation plays a crucial role not only in the accumulation and transportation of heavy metals but also in the detoxification phase. Usually chelators have ligands (most commonly histidine and citrate) and can bind metal ions. Combined metal ions appear uncharged and inert to react to other substance, by which way heavy metals' damage towards cell is reduced significantly.

(2) Vacuolar compartmentalization: Since vacuole is widely considered as the main storage place of heavy metals in plant cells, vacuolar compartmentalization is quite effective in controlling the distribution and concentration of metal ions. To compartmentalize vacuole is to "arrest and imprison" hazardous metal ions, constricting them into a limited site. Thus other parts of the cell have no access to those dangerous metal ions and safety is of course ensured. This mechanism is proved to be true in the Cd detoxification and tolerance by Salt et al.: Cd induces the synthesis of PCs and then forms a Cd-PC molecule, which will be transferred into the vacuole by a Cd/H antiport and an ATP dependent PC-transporter. Additionally Kramer et al. have reported that by "imprisoning" most of the intracellular Ni

into vacuole, metal tolerance of hyperaccumulator T. goesingense is greatly improved, which confirms the compartmentalization theory, too.
(3) Volatilization: By converting metal ions into volatile state, some plant species avoid the lasting damage caused by accumulation and long-time stay of heavy metals. A representative example is the bioprocess of Hg, which is a worldwide volatile pollutant and which is able to accumulate in human bod ies. However, not all the plants possess such ability and even among those innate Hg-resistant species, the relatively small amount of accumulation and their spatial distribution have greatly limited their wide cultivation. Thus scientists have employed genetic engineering and several transgenic plants have showed satisfactory performance to convert and volatilize metals. Transgenic species expressing organomercurial lyase (MerB) have much higher tolerance to organic Hg complex than wild type and can convert methylmercury to Hg(2), which is 100 times less toxic than the former one. Furthermore, transgenic plants expressing both MerA (enzyme that reduces Hg (2) to Hg (0)) and MerB have shown the highest tolerance to organic Hg (up to 10_M) compared with MerB species' 5_M and wild type's 0.25_M.

Mechanism of microremediation

Metal-binding mechanism

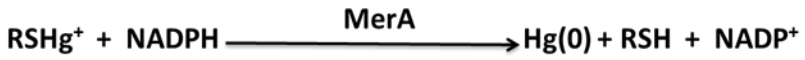
Three substances should be mentioned for this mechanism: MT, PC, and some novel metal-binding peptides. As we know from the former part of this article, MT and PC play a crucial part in plant–metal interaction. In fact, in the microbial world such interplay also exists. By binding to heavy metal ions MTs facilitate microbes' absorption or transportation of metal ions, and so do PCs, which are composed by only three amino acids (Gly, Cys and Gly). That over expression of PC synthase in microbes is effective to the accumulation and tolerance of metal ions has been reported by Sriprang et al.. By expressing the Arabidopsis thalina gene encoding PC synthase, enhanced Cd accumulation is observed in Mesorhizobium huakuii subsp. rengei B3 and Escherichia coli cells. Recent year's novel metal-binding peptides containinghistidines or cysteines have been found and engineered. These peptides are usually of higher affinity, specificity and selectivity for a certain metal ion. Related and in-depth study, however, is scarce.

Valence transformation mechanism

Metals of different valencies vary in toxicity. By excreting special redox enzyme, plants skillfully convert hazard metals to a relatively less toxic state and decrease possible metal stress and damage. For example, reduction of Cr (6) to Cr(3) is widely studied, the latter one of which is both less mobile and less toxic. Additionally, Kashiwa has found that Bacillus sp. SF-1 was good at reducing high concentration of Se (6) into elemental Se. The most persuasive example of this mechanism is the mercury-resistant bacteria, in which organ mercurial lyase (MerB) is produced. As we see from methyl mercury is converted to Hg(2), which is 100-fold less toxic than the former one.

Volatilization mechanism

By turning metal ions into volatile state, microbes escape possible negative effect that dangerous metal ions bring them. However, such approach is feasible for only a few metals such as Hg and metalloid Se. For the majority of most other metals which haveno volatile state at natural conditions, this pathway is closed. To date, the way microbes deal with element Hg is relatively clear. In the cells of mercury-resistant bacteria there is a MerA enzyme, an enzyme that reduces Hg(2) to volatile form Hg(0)



Localization of bioaccumulated metals in cell

In the last few decades, with the advent of sophisticated techniques facilitating deeper insight into the cell structure, many studies have focused on the localization of accumulated metals inside cell. Eventually, one can also predict through these results, whether the uptake is metabolism-dependent or a surface phenomenon shows the chemical nature and site of accumulated Ni in different systems. In case of A. niger, Ni was found to be associated with cell wall as well as inside the cell.The chemical nature was analyzed by X-ray and electron diffraction analysis to conclude that Ni accumulated as nickel oxalate dehydrate crystals. Most of the other studies involving bacterial and fungal strains indicated that Ni was primarily restricted to cell surface or to periplasm and cell membrane. Of the four isolates, Pseudomonas strain H1 and Bacillus strain H9, which were resistant to higher concentration (225 and 275 Ag/ml), appeared to use an intracellular mechanism of Cd sequestration. The resistance mechanisms of these two organisms were linked to plasmid-encoded genes. Although the exact mechanism of intracellular accumulation could not be elucidated, metallothionein production and polyphos phate precipitation could be the two possible explanations. The two other Cd-resistant isolates (20 and 50 Ag/ml), Pseudomonas strain I1a and Arthrobacter strain D9, did not carry plasmids but showed evidence of EPS (extracellular polysaccharides) production upon staining. Transmission electron microscopy (TEM) of these strains showed Cd accumulation external to the cells. Thus, the resistance mechanism and subsequently the location of accumulated metal vary with the strain. P. marginalis, isolated from a soil contaminated with high total (but low soluble) Pb showed higher resistance and extracellular Pb exclusion with high amount of EPS production. On the other hand, B. megaterium isolated from soil containing high soluble Pb showed lower resistance and intracellular accumulation of Pb. This strain produced no discernable EPS as reportedly observed by polarization microscopy.

The studies with growing algal cells confirm that a higher proportion of accumulated Cd is intracellular, suggesting an internal detoxification mechanism. As a common response to Cd, microalgae are considered to synthesize intracellular metal-binding peptides (Class III metallothioneins) as Cd binds to the –SH groups of these molecules during detoxification. Report confirming increased class III metallothioneins in cultures exposed to Cd stress (6 mg/l) and binding of accumulated Cd to this group has also appeared. Using a combination of instrumental techniques, confirmed the role of polyphosphate bodies (PPB) in accumulation of Zn, Pb, Mn and Al in Plectonema boryanum. They observed that living cells with active uptake system are more efficient in sequestering of metals through PPB. Suh et al. (1998), using TEM, observed that the accumulated Pb gradually enters the S. cerevisiae cell, and most of it gets deposited in cytoplasm after 2 h. They inferred a three-step mechanism comprising metabolism in dependent first step (3–5 min) when Pb binds to cell wall, followed by metabolism-dependent second step (5 min–24 h) in which Pb accumulated on cell wall/membrane and, finally, third step of Pb accumulation in cytoplasm (after 24 h). The study could not ascertain whether the third step is metabolism-dependent or -independent. Previously, White and Gadd (1986) also reported about intracellular accumulation of Cd, Co and Cu by trained (adapted) cells of S. cerevisiae, with Co mostly localized to the vacuoles. They observed that the mechanisms for accumulation of different metals were distinct and varied in their stability when the strains were detained in metal-free medium. Thus, it appears that in case of yeast and microalgae as well, most of the metals are accumulated intracellularly. The story is different in case of bacterial strains as both extracellular exclusion and intracellular accumulation are reported depending upon the strain and the metal concerned. Tsezos et al. (1997) observed the biosorption sites of metals using various microbial strains (BP 7/26 Arthrobacter spp., ER 121 Alkaligenes eutrophus and AS302 P. mendocina) and metals (Pd, Ag,

Y and Ni). The localization of the biosorbed metal appeared to be metaldependent rather than strain-dependent. In P. aeruginosa, enhanced accumulation of Cu is linked to higher amount of EPS production (Kazy et al., 2002). Presence of Cu ions in the growth medium caused stimulation of four-fold EPS production in Cu resistant (Cur) strain while such response was not exhibited by the sensitive (Cus) strain. Cu2+ binding capacity of the EPS of Cur was also greater (320 mg/g) than the EPS of Cus (270 mg/g). While studies with B. japonicum showed that the lipololysaccharide (LPS) and not the EPS is responsible for metal (Cd, Cu, Pb, Zn) binding (Oh et al., 2002). LPS mutant (lacking O-polysaccharidepart) bound 50–70% lower concentration of metals than the wild strain, although its EPS composition was unaltered. Thus, it appears that LPS molecules of B. japonicum have properties which effect precipitation of metal-rich mineral phases. Langley and Beveridge (1999), however, proposed that the negatively charged sites locatedin the O-side chains are not directly responsible for the binding of metallic ions in P. aeruginosa. However, the Bband LPS molecule as a whole may contribute to overall cell surface properties, which favor the precipitation of distinct metal-rich mineral phases.
From the reports discussed above, it appears that varied mechanisms of metal accumulation result in different localization of the accumulated product. These variations in the mechanism arise out of the toxicity of the metal concerned as well as the environmental conditions to which the microbial strain was exposed. In general, short Biosorption studies have low possibility to observe and appreciate the delayed intracellular accumulation and hence, most of such studies conclude with the surface adsorption of the metal. However, the studies, which monitor metal removal by growing cells often realize the metabolically linked intracellular accumulation. Recent studies have proved that in spite of low apparent growth, growing cells are able to remove metals continuously through internal detoxification mechanisms. Application of growing cells in bioremediation can well exploit these facts. However, there remains a great challenge to be faced via further development of the strain and the process.

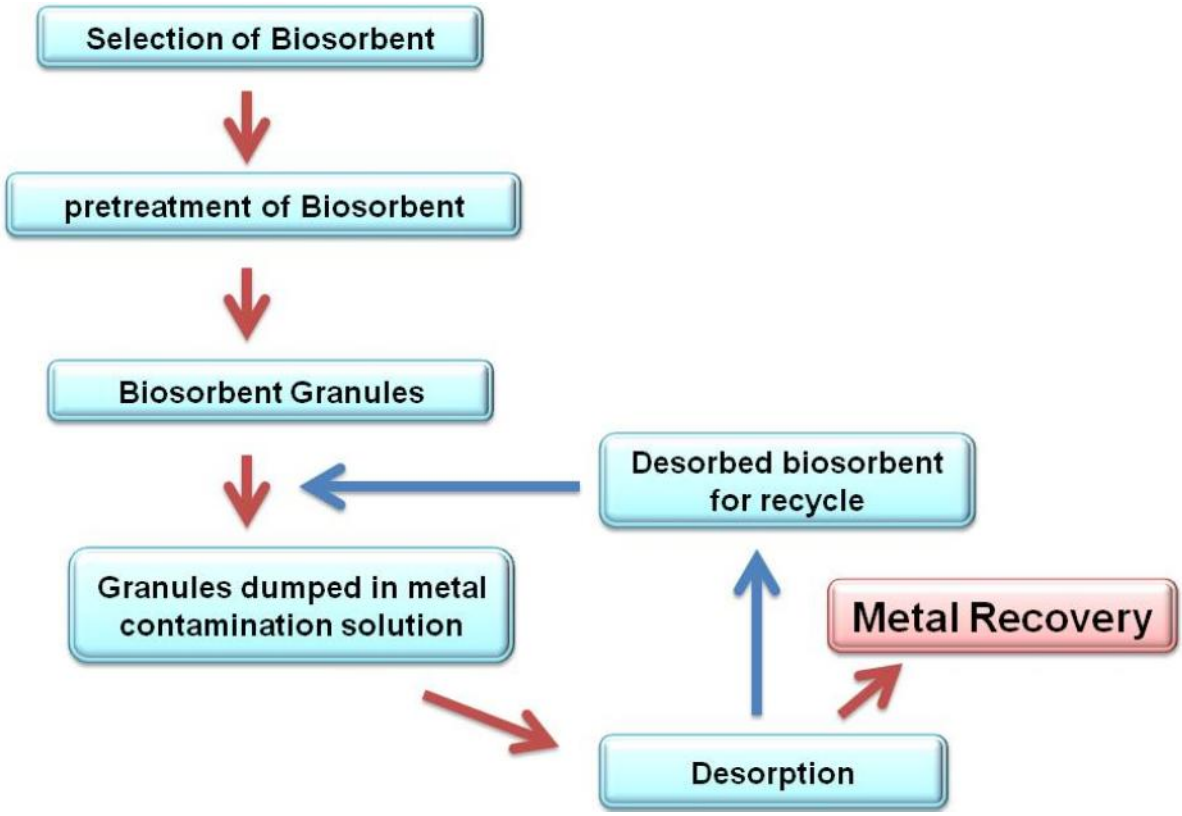
Microorganism (isolation source)	Name of organism	Metal	MRL/Uptake efficiency	Removal (%)	Scale of experiment/metal source (concentration)	Comment
Yeast (sewage)	*Candida* spp. (nonadapted)	Cu	4.7–23 mg/g	22–52 (8 days)	LS (100 ml)/GM (100–1500 mg/l)	Removal depends upon metal concentration and pH
	Candida spp. (adapted)	Cu	5.3–37 mg/g	5–68 (8–13 days)		
Yeast (acquired)	*Saccharomyces cerevisiae*	Cu	2–9 mg/g	13–74 (4 days)	LS (100 ml)/GM (50–700 mg/l)	Removal depends upon metal concentration and pH
	Kluveromyces marxianus	Cu	1.8–11.2 mg/g	10–90 (4 days)		
	Schizosaccharomyces pombe	Cu	0.6–1.3 mg/g	11–25 (4 days)		
	Candida spp.	Cu	1.6–14.8 mg/g	13–73 (4 days)		
Fungi (acquired)	*Aspergillus niger*	Cu	4.9–15.6 mg/g	19–57 (7 days)	LS (100 ml)/GM (25–150 mg/l)	Optimum pH for growth and metal accumulation is metal-dependent
		Pb	5.3–34.4 mg/g	13–88 (7 days)	(25–500 mg/l)	
		Cr	5.1–6.6 mg/g	21–36 (7 days)	(25–75 mg/l)	
Bacteria (wastewater)	*Pseudomonas aeruginosa*	Cr	0.08 mg/ml	46 (2 days)	LS (20 ml)/GM (69 mg/l)	Variable effect on growth, protein synthesis and pigment production
Bacteria (acquired)	*Bacillus thuringiensis*	Cu	0.03 mg/ml	34 (2 days)	(18 mg/l)	
Bacteria (adapted)	*Thiobacillus ferrooxidans*	Cu	0.02 mg/ml (700 mg/g)	25 (15 min)	LS (1.5 l)/metal solution (1000 mg/l)	Live cells more efficient
Yeast (culture collection)	*Sacchaomyces cerevisiae* SN41	Cu	0.32 mg/ml	90 (7 days)	LS (100 ml)/grape must (320 mg/l)	Cu removal from wine
Bacteria	*Pseudomonas aeruginosa* PU21 (Rip64)	Pb	0.5 mg/ml (110 mg/g)	80 (2 days)	LS (100 ml)/metal solution (100 mg/l)	Uptake pH and growth phase-dependent
		Cu	0.3 mg/ml (23 mg/g)	75 (2 days)	(20 mg/l)	

Bioaccumulation of Cu, Pb and Cr by growing/live cells

Biosorption

The biosorption process exhibits two phases. One phase is a solid phase (biomass/ sorbent/ biosorbent/ biological material) and another is liquid phase (solvent, usually water) containing a dissolved species to be sorbed (sorbate/ metal ion). Because of biosorbent affinity for the sorbate, the sorbate is bound with biosorbent with various mechanisms and this process continues till equilibrium is established between the amount of solid-bound sorbate species and its fraction remaining in the solution. The degree of biosorbent affinity for the sorbate determines its distribution between the solid and liquid phases. Principally, Biosorption process, which is metabolism- independent accumulation of metals, is often rapid. On the contrary, bioaccumulation is metabolism-dependent intracellular uptake of metal ions by living microorganisms and is slower process compared to biosorption. Moreover, bioaccumulation process is negatively affected by lower temperature, in the absence of energy source and in the presence of metabolic inhibitors. In fact, the toxicity of some heavy metals to microorganisms is major hindrance in unraveling the underlying mechanisms of bioaccumulation if the metal concentration is above threshold limit. In contrast, the application of dead biomass as biosorbent instead of living microbial cells removes constraint of toxicity of heavy metals. Thus, the biosorption processes are more practically applicable compared to the bioaccumulation processes since living system (i.e. active uptake of heavy metals) commonly needs the nutrients supply to carry out the metabolic activities and consequently, raise biological oxygen demand (BOD) o chemical oxygen demand (COD) of the effluent/

solution. Sometimes, biosorption and bioaccumulation terms are used interchangeably. Figure below shows a generalized schematic process of biosorption for heavy metal removal.



General schematic representation of biosorption process

Selection and Types of Biosorbent

The first major challenge for the biosorption field was to choose the most promising types of biomass/ biosorbent from enormously available and inexpensive biomaterials. Even though several materials of biological origin bind heavy metals, biomaterials with sufficiently high metal-binding capacity and selectivity for heavy metals are appropriate for full-scale biosorption process. A great number of biomass types have been examined for their metal binding capability under various conditions. Biomass may be derived from activated sludge or fermentation wastes from food industries. Microorganisms like, bacteria, fungi, yeast, and algae from their natural habitats are excellent sources of biosorbent. As well, fast growing organisms e.g., crab shells and seaweeds can also be used as biosorbents. In addition to the microbial sources, the agricultural products such as wool, rice, straw, coconut husks, peat moss, exhausted coffee, waste tea, walnut skin, coconut fibre, cork biomass, seeds of Ocimum basilicum, defatted rice bran, rice hulls, soybean hulls and cotton seed hulls, wheat bran, hardwood (Dalbergia sissoo) sawdust, pea pod, cotton and mustard seed cakes, are also proven as good biosorbent sources. One of the most important economic aspects of all remediation technologies is that the biomass used for decontamination of heavy metal pollutant must delineate better performance and should be natural and cheap. Therefore, biosorbents of biological origin particularly various genera of bacteria, algae, yeasts and fungi have received growing interest for heavy metal removal and recovery owing to their superior performance, little cost and large availability, the selective elimination of heavy metals under

wide range of pH and temperature, rapid kinetics of adsorption and desorption. In addition, the high surface to volume ratio of microorganisms and their superior capability to detoxify heavy metals are the main rationale that they are selected as potential alternative to the artificial biosorbents to remediate the heavy metal contaminated sites. Generally, the most important biosorbents of microbial origin can be classified into the following categories:

Bacteria

Bacteria are the most abundant and versatile of microorganisms and constitute a significant portion of an entire living terrestrial biomass of about 1018 g. Previously, bacteria were used as biosorbents on account of their small size, ubiquity, and capability to grow under controlled conditions, and their resistance against a wide range of varying environmental conditions. Further, bacterial biomass is usually produced as a waste by-product in industrial fermentation processes otherwise may be purposely grown in substantial amount. Many bacterial species (e.g. Bacillus, Pseudomonas, Streptomyces, Escherichia, Micrococcus etc), have been tested for metal uptake. The metal uptake capacities of bacteria generally range between 568 to 0.70 mg g-1. In fact, the bacterial cell walls are efficient metal chelating agents. Moreover, bacteria possess polysaccharide slime layers which readily offer amino, carboxyl, phophate and sulphate groups for metals binding. Nevertheless, a lot of variation in heavy metal-uptake capacity is prevalent among different bacterial genera. Heavy metal binding onto the surface of bacterial cell wall is generally, a two-stage process. The first stage involves the interaction between metal ions and reactive groups on cell surface and second stage includes deposition of successive metal species in greater concentrations. In general, the carboxyl groups of glutamic acid of peptidoglycan are the main site of metal deposition. In addition, some bacteria exhibit metabolism-independent biosorption as a major mechanism of heavy metal uptake. Even though biosorption process is metabolism- independent, metabolism-dependent mechanisms may likely augment metal deposition on bacteria cell surface. In a study, Qian et al. reported the concurrent biodegradation of Ni-citrate complexes and removal of Ni from solutions by Pseudomonas alcaliphila. They inferred that addition of an excess amount of citrate to Ni-citrate complexes encouraged the complex degradation as well as Ni removal. They suggested the possible mechanism for this change that the generation of an alkaline pH by the metabolism of an excess citrate resulted into dissociation of citrate from the Ni-citrate complexes thus facilitating the released of Ni. Moreover, bacteria have the biosorption capacity either for many metals or, they may be specific for one metal. Henceforth, microorganisms will likely be modified for a specific metal or a group of heavy metals, by means of recombinant DNA technology

Metal ions	Bacterial species	Biosorption capacity
Pb	*Pseudomonas aeruginosa*	79.5
Pb	*Bacillus firmus*	467
Zn	*Streptomyces rimosus*	30
Zn	*Pseudomonas putida*	6.9
Cu	*Bacillus firmus*	381
Cu	*Sphaerotilus natans*	60
Cd	*Aeromonas caviae*	155.3
Cd	*Enterobacter sp.*	46.2
Cr(VI)	*Aeromonas caviae*	284.4
Cr(VI)	*Staphylococcus xylosus*	143.0
Ni	*Bacillus thuringiensis*	45.9
Pd	*Desulfovibrio desulfuricans*	128.2

Bacterial biomass used for metal removal (mg g^{-1})

Algae

Algae are considered very promising organisms as biosorbents because they have prominent sorption capability and are readily available copiously in seas and oceans. Regrettably, algae have been scarcely used as biosorbent material compared to fungi and bacteria. Of red, green and brown algae, brown algae have been found to have better sorption capacity [42]. Researchers have been working on mostly brown algae to upgrade their sorption ability [41]. Furthermore, Chlamydomonas reinhardtii, Chlorella salina, Chlorococcum sp, Cyclotella cryptica, Lyngbya taylorii, Phaeodactylum tricornutum, Porphyridium purpureum, Scenedesmus quadricauda, Spirulina platensis, Stigeoclonium tenue, Ascophyllum nodosum, Cladophor fascicularis, Codium fragile, Corallina officinalis, Ecklonia sp., Fucus ceranoides, Gracilaria fischeri, Jania rubrens, Laminaria digitata, Padina pavonia, Porphyra columbina, Sargassum asperifolium, Turbinaria conoides and Ulva fascia are some marine micro and macro algal species which are being used as biosorbents for metal recovery. In addition, Chojnacka et al. reported the biosorption of Cr3+, Cd2+ and Cu2+ ions by blue green algae Spirulina sp. These algae were found to be capable of adsorbing one or more heavy metals including K, Mg, Ca, Fe, Sr, Co, Cu, Mn, Ni, V, Zn, As, Cd, Mo, Pb, Se, Al. Similarly, Halimeda tuna, Sargassum vulgare, Pterocladia
capillacea, Hypnea musciformis, Laurencia papillosa delineated the maximum sorption capacity for Cr6+ by 2.3, 33.0, 6.6, 4.7 and 5.3 mg g−1, respectively. In other study by Lodeiro et al., brown seaweeds like Bifurcaria bifurcata, Saccorhiza polyschides, Laminaria ochroleuca and Pelvetia caniculata removed cadmium from aqueous solution between 64 and 95 mg g−1 [45]. Primarily, the potential binding site for heavy metals in algae is cell wall which includes polysaccharides, cellulose, uronic acid and proteins .

Fungi and Yeasts

Metabolism-independent deposition of heavy metal ions also occur on fungal as well as yeast cell walls. In case of these organisms, biosorption process is generally, fast and the substantial concentration of metals can bind. In fact, many industrial fermentation waste biomass products are exceptionally better metal sorbents. Among those products, fungi and yeasts have shown very promising results. Moreover, fungi can also be grown through simple fermentation processes on low-cost media such as, molasses and cheese whey. The sequestering of metallic species by fungal biomass is largely due to the cell wall wherein different polysaccharides are often make complexes with proteins, lipids, and pigments. Furthermore, phosphate and glucouronic acid and chitin-chitosan complex in cell wall bind heavy metals by ion exchange and coordination. However, several types of ionizable sites influence the metal uptake efficiency of fungal cell wall like phosphate and carboxyl groups on uranic acids and proteins, and nitrogen-containing ligands on protein and chitin or chitosan. In yeasts, higher concentration of heavy metals can be accumulated by bioaccumulation process than biosorption. However, general biosorption is responsible for the major uptake of heavy metals for many filamentous fungi. Many species of fungi have been reviewed by Wang and Chen whose biomass adsorbed considerable amount of heavy metals. In a study by Mishra and Malik, the effectiveness of a fungal isolate, Aspergillus lentulus FJ172995) for concurrent removal of heavy metals like chromium, copper and lead from industrial effluent was examined. They inferred that Cr, Cu,

Pb and Ni tolerant A. lentulus accumulated a significant amount of each metal. The removal of metals from synthetic solutions showed the trend like Pb2+ (100%) > Cr3+ (79%) > Cu2+ (78%), > Ni2+ (42%) after five days. When the same fungal strain was used to treat the multiple metal containing electroplating effluent the metal concentrations declined by 71%, 56% and 100% for Cr, Cu and Pb, respectively within eleven days. In other study, Congeevaram et al. [60] concluded that mainly pH was attributable to organism-specific physiology. Batch and tolerance experiments provided information for solid retention time (SRT) design and the lethal tolerance limits for the isolated microorganisms. Their results indicated that expanded solid retention time (stationary phase) can be recommended whilst using the Cr-resistant fungal and bacterial isolates for chromium removal.

Fungal species	Metal ions
Aspergillus niger, *Mucor rouxii*, *Rhizopus arrhizus* (living cells)	Au
Penicillium spp. (living cells)	Ag, Cu, Cd, Pb
Penicillium, *Aspergillus*, *Trichoderma*, *Rhizopus*, *Mucor*, *Saccharomyces*, *Fusarium* (living cells)	Pb, Cu, Cd, Zn
Aspergillus, *Penicillium*, *Rhizopus*, *Saccharomyces*, *Trichoderma*, *Mucor*, *Rhizopus* (living cells)	Th, U, Sr, Cs, La
Phanerochaete chryosporium (living cells)	Cd, Pb, Cu

Biosorption by fungal biomass (mg g^{-1})

Pretreatment of Biomass

The significance of biosorption of a specific metal by a certain biosorbent depends on different factors like, the number, accessibility and chemical state (i.e., availability) of the site and affinity between the site and metals in the biosorbents. To increase the interaction between biosorbent and metal species, biosorbents initially undergo various treatments by different methods. Pretreatment involves heat treatment, washing with detergent/ acids/ alkalies, enzymatic treatment etc. Among these methods, heat treatment and detergent washing increase sorption efficiency by exposing additional metal binding groups on biomass while enzymes destroy unwanted components to facilitate biosorption. Biosorbent

can be pretreated directly if it is larger in size (like, seaweeds). They are sized into fine particles or granules and they are further treated.

Mechanisms of Biosorption

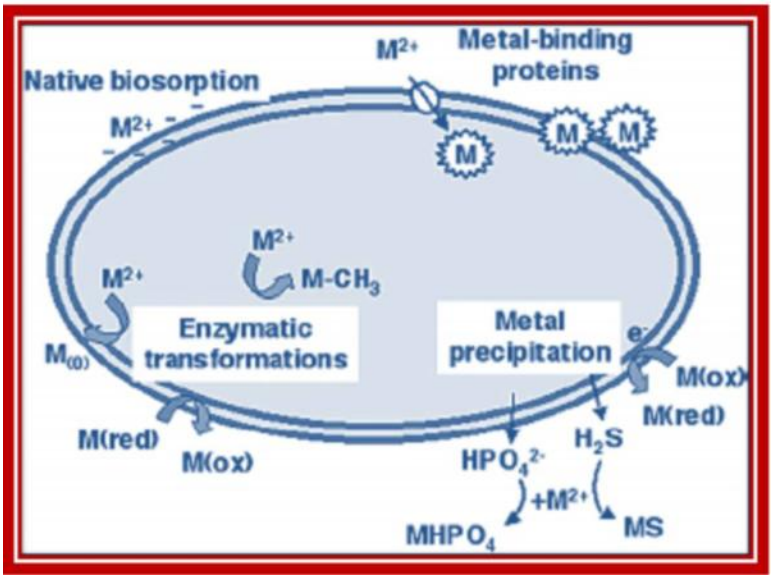
The elucidation of the mechanism of metal uptake is essential to develop technologies related to the metal recovery. In general, microbes mediated metal removal or recovery from contaminated site/ reservoir may involve the following pathways:

a. Metal cations may bind on cell surfaces (biosorption)/ within the cell wall (bioaccumulation) and in turn, metal uptake is augmented through microprecipitation

b. Metal ions may be actively translocated inside the cell through metal binding proteins

c. Metal precipitation may occur when heavy metals react with extracellular polymers or with anions (e.g. sulphide or phosphate) produced by microbes

d. Metal volatilization through enzymes mediated biotransformation



Mechanisms of heavy metal uptake on microbial surface

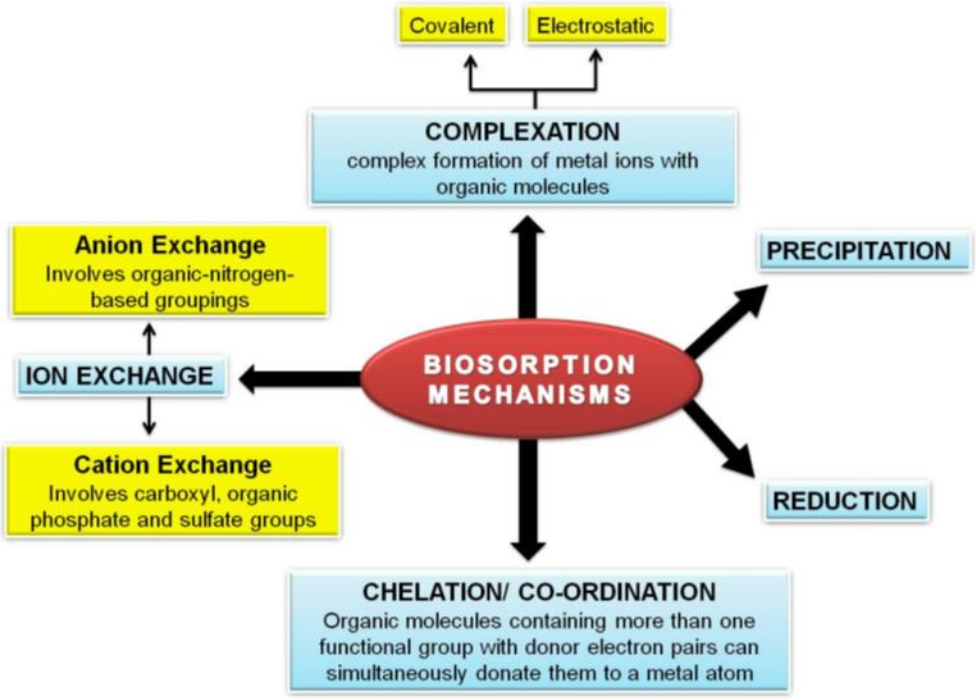
Among the general mechanisms of microbes mediated metal removal, the mechanism of biosorption is very much intricate and therefore, complete account concerning the Biosorption processes is not available. However, extensive literatures regarding the mechanism and modeling of biosorption for specific metal and microbial strain is not available.

However, the key factors which control and characterize these mechanisms are as following:

a. Type of biological ligands accessible for metal binding;

b. Type of the biosorbent (i.e. living /non-living);

c. Chemical, stereo-chemical and co-ordination characteristics of the targeted metals; d. Characteristics of the metal solution (e.g. pH and the competing ions). Based on cell metabolism, mechanisms involved in biosorption can be categorized as metabolism dependent and metabolism independent, while they are classified as extra cellular accumulation/ precipitation, cell surface sorption and intra cellular accumulation on the basis of the location of the sorbate species. Other mechanisms for biosorption are transport across cell membrane, ion exchange and complexation. These biosorption mechanisms can take place simultaneously



The term 'adsorption' is used in a general way and it incorporates numerous passive (non-metabolic) mechanisms

Conclusion

In conclusion, the outstanding features of microbial Biosorption of heavy metals such as, leniency in operation under wide range of physical parameters, possibility of deduction of various kinetic models depending upon the specific metal species, regeneration of biosorbents and the most economical among other bioremediation process, makes the Biosorption process an ideal approach to be used as a promising heavy metal decontaminating technique. Thus, biosorption process of toxic heavy metals by exploiting different microbial genera as biosorbents can be exploited as a promising environment-friendly and an economical tool to decontaminate the metal stressed environment. In addition, this process can be made more relevant by devising the ways to search the cheapest biosorbent in terms of requiring the minimum nutrient resources and compliant to the exceptionally harsh environmental conditions.

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