

IMPLICATIONS OF BACTERIAL RESISTANCE AGAINST HEAVY METALS IN BIOREMEDIATION: A REVIEW

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ABSTRACT

Intensification in industrialization, agricultural practices and various anthropogenic activities add a significant amount of metals in the soils. The continuous magnification in metal concentrations in soil ecosystem beyond the threshold limit leads to the deleterious effect on the microbial communities and their functional activities in soils. Some microorganisms under heavy metals stress may develop resistance against the elevated levels of these toxic metals and evolve various strategies to resist against the metal stress. Therefore, the metal resistant microorganisms including bacteria can be exploited as bioremediation agents. This review deals with bacteria resistance mechanisms against heavy metals (zinc and copper) comprehensively. In addition, biosorption and bioaccumulation processes with reference to the metal resistant bacteria are also explicitly described.

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[I] INTRODUCTION

Environmental contamination owing to the anthropogenic activities and the natural resources is increasing progressively on account of an unabated increment in population, industrialization and urbanization [1, 2]. An enigma for the scientists is how to tackle the contaminants that jeopardize the environmental health. Advancement in science and technologies parallel to industrial revolution has enabled to exploit the inherent traits of natural resources to overcome the pollutants mediated environmental damage. An idyllic process for pollution abatement is 'bioremediation'. The term bioremediation has been introduced to describe the process of exploiting biological agents to eliminate toxic waste from environment. At present, bioremediation is the most effective management tool to manage the polluted environment and recover contaminated environment [3]. In other words, bioremediation is an alternative that offers the possibility to destroy or render harmless various contaminants including heavy metals using natural biological activity. As such, it uses relatively low-cost techniques which generally, have a high public acceptance and can often be carried out on site [4]. Compared to other methods, bioremediation is a more promising and less expensive way for cleaning up contaminated soil and water [3, 5]. Bioremediation uses biological agents, mainly microorganisms, e.g. yeast, fungi or bacteria to clean up contaminated soil and water [6, 7].

Most of the bioremediation systems are generally, operated under aerobic conditions. However, running a system under anaerobic environment may permit microbial organisms to degrade the most recalcitrant pollutants. The most essential parameters

required for bioremediation are the nature of pollutants, soil structure, temperature, pH, moisture content, hydrogeology, the nutritional state, redox-potential, and microbial diversity of the site [8, 9]. In bioremediation processes, microorganisms use the contaminants as nutrient or energy sources [10, 11]. Bioremediation activity through microbe is stimulated by supplementing nutrients (nitrogen and phosphorus), electron acceptors (oxygen), and substrates (methane, phenol, and toluene), or by introducing microorganisms with desired catalytic capabilities [12, 13]. Plant and soil microbes develop a rhizospheric zone (highly complex symbiotic and synergistic relationships) which is also used as a tool for accelerating the rate of degradation or to remove contaminants [2, 14].

Generally, the higher concentration of these metals above threshold levels has deleterious impact on the functional activities of microbial communities in soils. Otherwise, microorganisms exposed to the higher concentrations of toxic heavy metals may develop resistance against the elevated levels of these metals [15]. In addition, microorganisms inhabiting in metal polluted soils have evolved various strategies to resist themselves against metal stress [16]. Such metal resistant microorganisms can be used as successful bioremediation agents [17]. This review highlights the general resistant mechanisms of microbes specifically bacteria against the selective heavy metals (zinc and copper) in detail.

[II] SOURCES OF HEAVY METAL CONTAMINATION IN SOILS

Heavy metals such as lead, arsenic, cadmium, copper, zinc, nickel, and mercury are discharged from industrial operations such as smelting, mining, metal forging, manufacturing of alkaline storage batteries, and combustion of fossil fuel. Moreover, the agricultural activities like application of

agrochemicals, and long-term usage of sewage sludge in agricultural fields also add a significant amount of metals in the soils [18, 19]. Various anthropogenic sources of metal contamination of soils have been shown in **Figure- 1**.

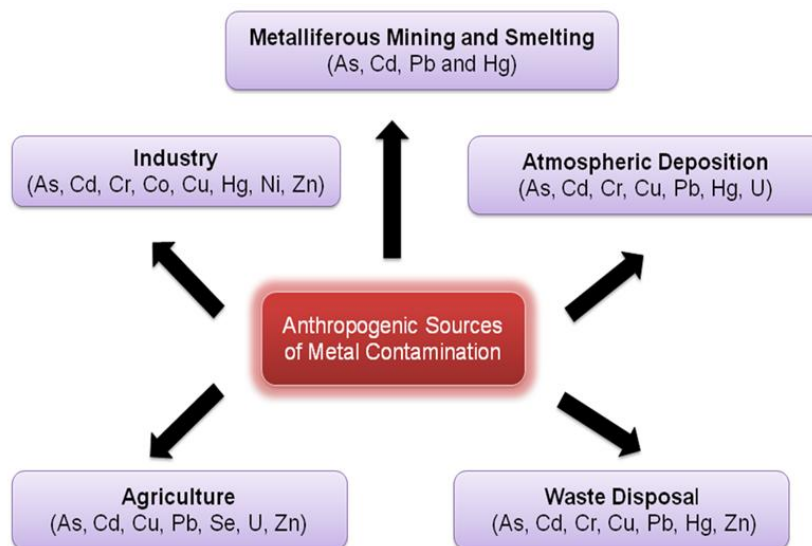


Fig. 1. Anthropogenic activities leading to the contamination of soils with heavy metals

[III] BIOAVAILABILITY OF METALS IN SOILS

Heavy metals exist both in bioavailable and non-bioavailable forms. Their mobility depends on two factors: (i) the metallic element that precipitates as positively charged ions (cations) and (ii) the one, which makes up negatively charged component of salt [20]. Physico-chemical properties of soils, such as cation exchange capacity (CEC), organic matter, clay minerals and hydrous metal oxides, pH and buffering capacity, redox potential and extent of aeration, water content and temperature, together with root exudates and microbial activities determines the metal availability in soils [17, 21]. The toxicity of metals within soils with high CEC is generally low even at high total metal concentrations. Under oxidized and aerobic conditions, metals are usually found in soluble cationic forms while in reduced or anaerobic conditions, as sulphide or carbonate precipitates. At low soil pH, the metal bioavailability increases due to its free ionic species, while at high soil pH it decreases due to insoluble metal mineral phosphate and carbonate formation. The mobility and bioavailability of certain metals in soils is usually in the order: Zn > Cu > Cd > Ni [17, 22]. However, the concentration of heavy metals within all components of the ecosystems varies considerably. Coexistence and persistence of metals in soils as multiple contaminants facilitate the entry and accumulation of these pollutants into food webs and ultimately into the human diets. Contamination of agricultural soils with heavy metals (both

by single or combination of metals) has thus become a global threat to the sustainability of the agro-ecosystems and therefore, is receiving considerable attention from the environmentalists. Therefore, assessment of heavy metal bioavailability helps to evaluate the impact of metals on soil microbes and in predicting the application of bioremediation technologies that could be used to clean up metals from the polluted soils [17].

[IV] GENERAL MECHANISMS OF BACTERIAL RESISTANCE AGAINST HEAVY METAL STRESS

Accumulation of heavy metals in the soil environment and their uptake by plants is a matter of growing environmental concern. Unlike many other pollutants, which can undergo biodegradation and produce less toxic, less mobile and/or less bio-available products, heavy metals are difficult to be removed from contaminated environment [7]. These metals cannot be degraded biologically, and are ultimately indestructible, though the speciation and bioavailability of metals may change with variation in the environmental factors [23]. Some metals such as, zinc, copper, nickel and chromium are essential or beneficial micronutrients for plants, animals and microorganisms [24] while others (e.g., cadmium, mercury and lead) have no known biological and/or physiological functions [25]. However, the higher concentration of these metals has great effects on the microbial communities in soils in several ways- (1)

it may lead to a reduction of total microbial biomass [18] (2) it decreases numbers of specific populations [26] or (3) it may change microbial community structure [27]. Thus, at high concentrations, metal ions can either completely inhibit the microbial population by inhibiting their various metabolic activities [Figure– 2] or organisms can develop resistance or tolerance to the elevated levels of metals. Generally, tolerance may be defined as the ability to cope with pollutant-toxicity by means of intrinsic properties of the microorganisms. In contrast,

resistance is the ability of microbes to survive in higher concentrations of toxic substances by detoxification mechanisms, activated in direct response to the presence of the same pollutant [28]. Toxic heavy metals therefore, need to be either completely removed from the contaminated soil, transformed or to be immobilized, producing much less or non-toxic species. However, in order to survive and proliferate in metal contaminated soils, tolerance has to be present both in microbes and their associative hosts.

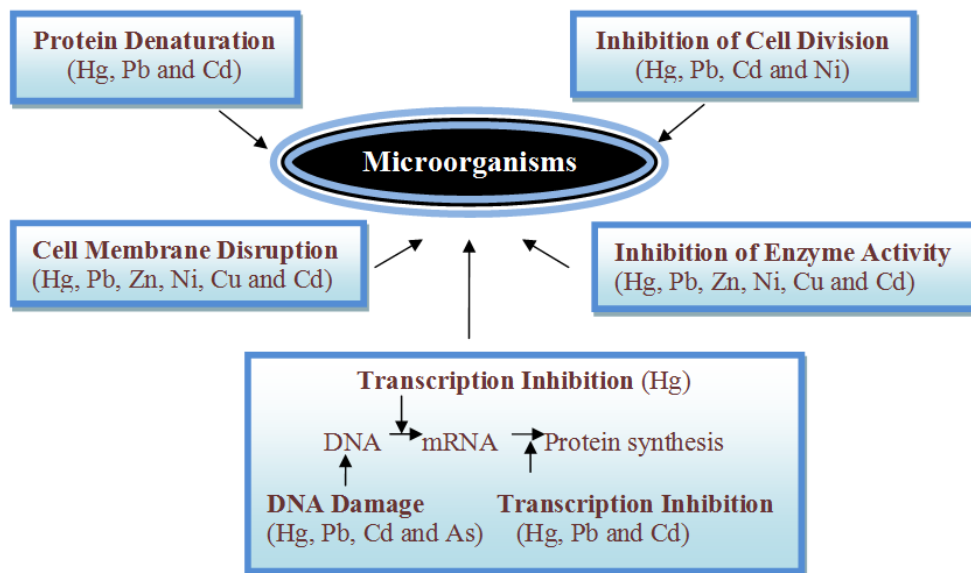


Fig: 2. Heavy metal-toxicity mechanisms to microbes [17]

For survival under metal-stressed environment, bacteria have evolved several mechanisms by which they can immobilize, mobilize or transform metals rendering them inactive to tolerate the uptake of heavy metal ions [29]. These mechanisms include (1) exclusion-the metal ions are kept away from the target sites (2) extrusion-the metals are pushed out of the cell through chromosomal/plasmid mediated events (3) accommodation-metals form complex with the metal binding proteins (e.g. metallothienins, a low molecular weight proteins) [30, 31] or other cell components (4) bio-transformation-toxic metal is reduced to less toxic forms and (5) methylation and demethylation. One or more of these defense mechanisms allows these microorganisms to function metabolically in environment polluted by metals. These mechanisms could be constitutive or inducible. The bacterial resistance mechanisms are encoded generally on plasmids and transposons, and it is probably by gene transfer or spontaneous mutation that bacteria acquire their resistance to heavy metals. For example, in Gram-negative bacteria (e.g. *Ralstonia eutropha*), the *czc* system is responsible for the resistance to cadmium, zinc and cobalt.

The *czc*-genes encode for a cation-proton antiporter (*CzcABC*), which exports these metals [32]. A similar mechanism, called *ncc* system, has been found in *Alcaligenes xylosoxidans* which provides resistance against nickel, cadmium and cobalt. In contrast, the cadmium resistance mechanism in Gram-positive bacteria (e.g. *Staphylococcus*, *Bacillus* or *Listeria*) is through Cd-efflux ATPase. Plasmid encoded energy dependent metal efflux systems involving ATPases and chemiosmotic ion/proton pumps are also reported for arsenic, chromium and cadmium resistance in other bacteria [33]. The exploitation of these bacterial properties for the remediation of heavy metal-contaminated sites has been shown to be a promising bioremediation option [34]. Though, the threshold limit of metal toxicity to soil microorganisms is not conclusive, yet the interaction between heavy metals and microbes do occur in nature. Microorganisms can interact with metals via many mechanisms [Figure– 3], some of which may be used as the basis of potential bioremediation strategies.

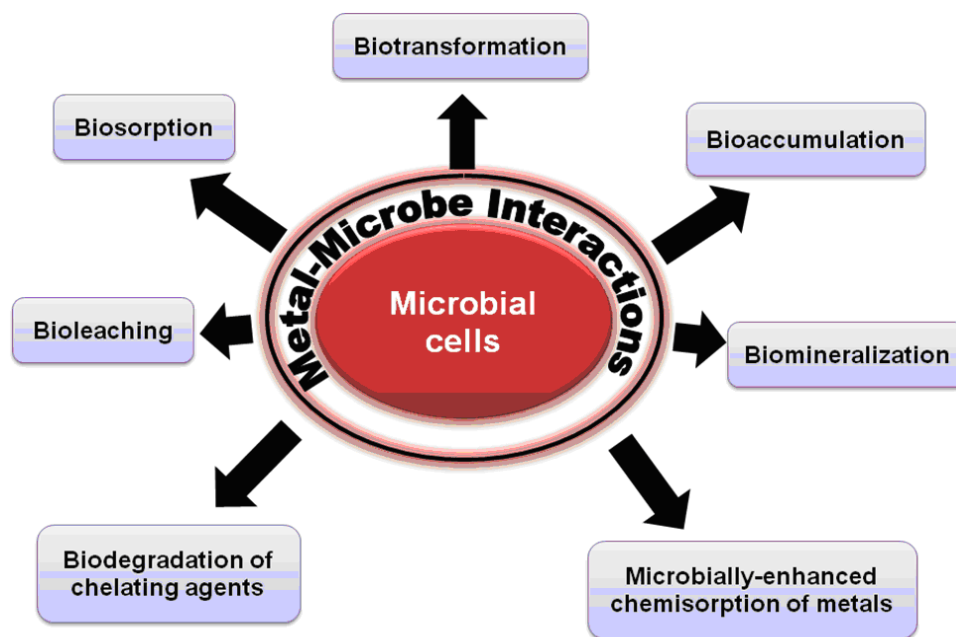


Fig. 3: Metal-microbe interactions affecting bioremediation

[V] BIOSENSORS

Metal resistance trait in microorganisms is regulated by genes which are organized in operons. Generally, the genes for heavy metal resistance are found on extrachromosomal circular DNA i.e. plasmid carried by metal resistant bacteria. The heavy resistance genes are expressed and induced in the presence of specific metals in the vicinity of bacterial niche. Since the regulation of the metal resistant gene expression is specific for each heavy metal and is dependent upon metal species concentration, the promoters and regulatory genes from the bacterial operons responsible for resistance attribute can be used to create metal-specific biosensors (promoter-reporter gene fusions). The metal specific bacterial sensors coupled with tools for chemical analyses can be used to differentiate the bioavailable metal concentration from the total metal concentration of the samples [35].

Various metal-specific sensor strains have been developed and applied in many laboratories. These sensor strains are all based on the same concept: a metal responsive regulation unit regulates the expression of a sensitive reporter gene. Reporter genes include those that code for bioluminescent proteins, such as bacterial luciferase (*luxAB*) and firefly luciferase (*lucFF*) or for β -galactosidase, which can be detected electrochemically or by using chemiluminescent substrates. The light produced can be measured by a variety of instruments, including luminometers, photometers and liquid-scintillation counters [35].

[VI] METAL RESISTANCE MECHANISMS

When the bacterial cells are exposed to the high concentrations of heavy metals, the metals react within cells with various metabolites and form toxic compounds [29, 36]. It is well known that some of heavy metals are essential for functioning of cellular enzymes and for the bacterial growth and metabolism, therefore, mechanisms for uptake of these metal species is present in the bacterial cell through which heavy metals enter the cell. Generally, there are two types of uptake mechanisms for heavy metals: one of them is quick and unspecific which is driven by a chemiosmotic gradient across the cell membrane and consequently, does not require ATP. Although, this mechanism is an energy efficient process, it is responsible for the influx of a number of heavy metals. Obviously, when these metals are present at high concentrations exterior to the bacterial cell, they are more likely to produce noxious impact on bacterial growth metabolisms if entered the cell cytoplasm. In contrast, the second process of metal uptake is comparatively, slower and more substrate-specific and is dependent upon the energy released from ATP hydrolysis [32, 36]. Moreover, most of the bacterial genera have evolved numerous mechanisms to tolerate the uptake of heavy metal ions and to protect their cell homeostasis against the heavy metal induced damage to survive in heavy metal stress. These mechanisms for instance, are the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state [29]. As typical examples, bacterial resistance mechanisms against copper and zinc are discussed as follows:

6.1. Bacterial resistance against copper

Heavy metal, copper is utilized by bacterial cells in small quantities in biosynthesis of metabolic enzymes like, cytochrome c oxidase. However, bacteria in different ecosystems including soil and water, are exposed to very high concentration of this metal as high levels of copper exists in soil ecosystem due to its wide application in mining, industry processes, and agricultural practices [37]. Consequently, bacteria have evolved several types of mechanisms to defend against the high copper concentration and copper induced biotoxicity [36].

Regarding the prevalence of copper resistance in bacteria, Lin and Olson [38] isolated copper resistant bacteria from a copper corroded water distribution system and studied resistance pattern against copper. They observed that 62% of the total isolates exhibited substantial resistance against copper. Among these resistant bacteria, 49% isolates had *cop* or *cop*-like gene systems as well as both compartmentalization and efflux systems [39]. In other study, Cooksey [40] reported that resistance against copper in the plant pathogen *Pseudomonas syringae* was because of the copper accumulation and compartmentalization in the cell's periplasm and the outer membrane and concluded that the protective mechanism against copper in *P. syringae* was due to four types of proteins (CopA, CopB, CopC and CopD). These proteins are encoded by the *cop* operon present on bacterial plasmid and proteins are found in the periplasm (CopA and CopC), the outer membrane (CopB), and the inner membrane and work together to compartmentalize copper away from bacterial cells [36].

In contrast, copper resistance in *E. coli* is dependent upon efflux mechanism to overcome copper stress. The efflux proteins are expressed by plasmid-borne *pco* genes, which are in turn rely upon the expression of chromosomal *cut* genes [39]. Moreover, two *cut* genes (*cutC* and *cutF*) encode a copper binding protein and an outer membrane lipoprotein [41]. Most bacterial species in the metal stressed environment have acquired at least one of the abovementioned protective mechanisms. In addition, the evolution of the bacterial copper resistance occurred through the modification of copper uptake genes found on chromosomes [36].

6.2. Bacterial resistance against zinc

Zinc, an essential trace element is not biologically redox reactive. Hence, it is not used in cellular metabolisms like respiration. However, it is structurally, a vital constituent of several cellular enzymes. Furthermore, it also forms complexes in cells for instance, zinc fingers in DNA [29, 36]. In addition, zinc actually, displays comparatively less toxicity to bacterial cells than other heavy metals and it is generally occurs in higher concentrations within bacterial cells. That is why bacteria in heavy metal polluted environment accumulate zinc by a fast but unspecific uptake mechanism [29]. Generally, uptake of zinc ions by

bacterial cells is coupled with magnesium, and both ions may be transported by similar mechanism [32, 36].

The zinc resistance in bacteria is achieved through the two general efflux mechanisms: (i) mediated by a P-type ATPase efflux system and (ii) mediated by an RND-driven transporter system [36]. As a matter of fact, a P-type ATPase catalyzes the reactions by ATP hydrolysis forming a phosphorylated intermediate [32] whereas, the term RND belongs to a family of proteins involved in the heavy metal transport [29]. The P-type ATPase efflux system transports zinc ions across the cytoplasmic membrane by the energy released from ATP hydrolysis. In this regard, Beard et al. [42] isolated a chromosomal gene, *zntA*, from *E. coli* K-12 and inferred that the gene *zntA* might be accountable for the zinc and other cations transporting ATPase across cell membranes. In contrast to P-type ATPase efflux system, the RND-driven transporter system does not derive energy through ATP hydrolysis to transport zinc within the bacterial cells. As an alternative, it is powered by the proton gradient across the cell wall specifically, in gram-negative bacteria [29, 36].

[VII] BIOSORPTION AND BIOACCUMULATION OF HEAVY METALS

Fundamentally, biosorption of heavy metals by bacterial cells is based on non-enzymatic processes such as, adsorption. Adsorption is characterized by the non-specific binding of metal ions to extracellular/ cell surface associated polysaccharides and proteins [43, 23]. In a nut shell, biosorption is defined as an attribute of the inactive or dead microbial biomass to bind and concentrate heavy metals even from highly dilute solutions [44].

The metal uptake by the microbial biosorbent may be an active or passive process or exhibit both active and passive processes depending upon the microbial species. Moreover, passive uptake is a rapid and reversible process and is independent of cellular metabolisms, physical conditions such as pH and ionic strength. However, the passive process is relatively nonspecific with respect to the metal species. Conversely, the active process is comparatively slow method and depends on the cellular metabolism. In this process, heavy metals form complexes with specific proteins like metallothionins. However, it is affected by metabolic inhibitors, uncouplers and temperature. Both the active and passive mode may occur simultaneously [45].

Generally, many microbial species with high cell wall chitin contents act as an effective biosorbent in addition to the chitosan and glucans. Furthermore, the walls of fungi, yeasts, and algae, are also efficient metal biosorbents. Moreover, the cell walls of the Gram-positive bacteria attach higher concentrations of metals than that of the Gram-negative bacteria [23]. Therefore, bacteria, waste fungal biomass derived from several industrial fermentations are considered the cost-effective and efficient sources of biosorptive materials. These biosorbents loaded with

charged metal species may be regenerated with the treatment of acid or some chelating agents [23].

Bioaccumulation is an active process dependent upon metabolic energy of microorganisms. In other words, bioaccumulation is an energy-dependent heavy metal transport system [46]. Besides, potential bioaccumulation mechanisms of heavy metal influx across the bacterial membranes include ion pumps, ion channels, carrier mediated transport, endocytosis, complex permeation, and lipid permeation. This active mechanism has been reported to be associated with the transport of heavy metals like, mercury, lead, silver, cadmium and nickel. Assessment of heavy metal accumulation in the microbial cells can be done by transmission electron microscopy (TEM). In a study, TEM analysis of *P. putida* 62BN demonstrated intracellular and periplasmic accumulation of cadmium [23]. Similarly, heavy metal transport through bioaccumulation has been reported in many bacterial genera like, *Citrobacter sp.* (lead and cadmium), *Thiobacillus ferrooxidans* (silver), *Bacillus cereus* (cadmium), *Bacillus subtilis* (chromium), *Pseudomonas aeruginosa* (uranium) *Micrococcus luteus* (strontium) *Rhizopus arrhizus* (mercury), *Aspergillus niger* (thorium), *Saccharomyces cerevisiae* (uranium) [23, 47].

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CONFLICT OF INTEREST

Author declares no conflict of interest.

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