REVIEW ARTICLE

Bioremediation of Heavy Metals using the Interaction between Plants and Genetically Engineered Microbes

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Abstract

Excessive levels of heavy metals (HMs) in agricultural soil is a critical concerns for crop production and food safety and pose potential hazards to human and animal health. Anthropogenic sources including agriculture, mining, smelting, electroplating, and other industrial activities have resulted in the deposition of undesirable concentration of metals, such as arsenic (As), cadmium (Cd), chromium (Cr), and lead (Pb) in the soil. Unlike many other pollutants, HMs are difficult to remove from the environment as they cannot be degraded by any method, and are ultimately indestructible. The use of microorganisms and plants for soil remediation of HMs are of great interest because of their high efficiency, ease of use, and cost-effective application. Microorganisms can be used to remediate contaminated soil by detoxification, sequestration, and solubilization of HMs to facilitate their extraction. These microbes may act on HMs by chelation, precipitation, transformation (oxidation-reduction, methylation), biosorption, and accumulation. However, high concentrations of HMs in soil lead to decreased number of soil microbes. These symbiotic rhizospheric microbes depend on plant root exudates for their nutrition, thus to improve the number of microbes, it is also essential to optimize microbial nutrition by optimizing plant-microbe interaction. Different approaches were adopted to address these problems, from enrichment with rhizosphere bacterial consortia resistant to HMs to genetic engineering of plant growth-promoting symbiotic microbes. These genetically engineered (GE) microbes show improved bioremediation potential by enhancing their metal metabolism efficiency as well as increasing soil fertility. In this review, we describe how GE microbes and their association with plants enhance metal tolerance, accumulation, and detoxification in microbes and plants. We also describe the potential of bioremediation using symbiosis between plants and GE microbes.

Keywords: Bioremediation, Heavy Metals, Microbes.

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Introduction

Elevated concentrations of heavy metals (HMs) like arsenic (As), chromium (Cr), cadmium (Cd), lead (Pb), and mercury (Hg) in soil poses a significant risk for the environment, wildlife, and human health (Rahman and Singh, 2019). Soils polluted by HMs represent a significant environmental problem due to the toxic effects of metals, their accumulation throughout the food chain, and the additional risk of groundwater contamination (Tóth *et al*., 2016). Currently, HMs contamination of soil is the dangerous condition and widespread problem (Steffan *et al*., 2018). Increased pollution of HMs in soil occurs from both natural and anthropogenic sources. Human activities like the industrial revolution, agricultural sprays release more and more hazardous HMs in environment and agricultural soils (Govil and Krishna, 2018, Verma *et al*., 2020). Apart from organic pollutants, dangerous HMs are non-breakable, as they cannot be chemically or biologically degraded and stay in grounds for the long term, ultimately reduce the crop yield. The problem is even worse as these HMs concentrated along the food chain and eventually accumulates in human body.

Soil is the primary and essential part of the ecological system and the primary factor for agriculture anchorage and nutrient medium for plants (Saha *et al*., 2017). In recent years, therefore, more consideration has been paid for remediation of contaminated soil; still, particular attention given to the use of plants and microbes to remove hazardous metal ions. Removals of HMs using conventional physicochemical techniques are much expensive and unsuitable in the case of extensive areas (Crini *et al*., 2019). Therefore, biotechnological approaches have received significant importance in recent years. The biotechnological methods use plants, animals, and microbes by improving their efficiency (Adams *et al*., 2015).

Phytoremediation is the process of removing HMs or other pollutants from contaminated sites by plants (Ullah *et al*., 2015). Phytoremediation emerges as a cost-effective, environment friendly biotechnology approach to clean

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local areas affected by contamination (Sarwar *et al*., 2017). Recently to enhance phytoremediation efficiency, genome editing CRISPR-Cas9 technology have been used (Sarma *et al*., 2021). Phytoremediation involves phytovolatilization, phytostabilisation, phytoextraction, and rhizofiltration. Phytoextraction is the use of hyperaccumulator plants, which can tolerate and accumulate a high concentration of metals (Emenike *et al*., 2018). Rhizoremediation involves both plants and their associated rhizosphere microbes to reduce the level of HMs or other pollutants using naturally occurring rhizosphere microbes or by deliberately introducing specific bacteria (Kumar *et al*., 2017). These microbes may be contaminant degraders or plant growth promoter under stress conditions. For an extended period, plant-growth-promoting rhizobacteria (PGPR) used for assisting plants to uptake nutrients from the environment or preventing plant diseases (Etesami and Maheshwari, 2018). Recently, PGPR application extended for the bioremediation of both organic and metal pollutants (Ullah *et al*., 2015).

The rhizobium-legume symbiosis has been proved as a powerful tool for rhizoremediation of HMs in soils (Vaishnav *et al*., 2017; Rai *et al*., 2021). The additional benefit of this naturally occurring symbiotic interaction is the enrichment of soil nitrogen due to dinitrogen fixation in plant root nodules. Legumes are commonly used crops in a wide range of environmental conditions and form symbiosis with nodule forming, nitrogen-fixing bacteria, result in symbiotrophic nitrogen nutrition (Sprent *et al*., 2017). The legumes also form an associative symbiosis with PGPR and endophytic microorganisms exerting multiple effects on plant growth, malnutrition, and disease control. The advanced symbiotrophic capacity of legumes is important for improving soil fertility, soil biota activity, biodiversity, and soil genesis, therefore for sustaining and restoring healthy ecosystems. The ability of legumes to form plant-microbe symbiosis suggests that their growth and nutrition significantly depend on interactions with beneficial microorganisms (Kafle *et al*., 2018). On the other hand, symbiotrophic organisms possess many mechanisms that may improve plant's tolerance to environmental stresses, including those caused by HMs, and may also play an essential role in enhancing phytoremediation technologies. Therefore, in the association with symbiotrophic microorganisms, the usage of legume plants for agriculture and remedial technology should be considered. This review aims to discuss the possibilities for application of plant-microbe systems as a vital tool for phytoremediation of HMs polluted soil and restoration of healthy ecosystems, with emphasis on the use of genetically modified microbes which positively interacted with plants.

Methods for Heavy Metal Contaminated Soil Remediation

Agricultural soil and groundwater are heavily contaminated by HMs. The contaminated soil and water are significant problems for crop production, accumulation of toxic HMs ultimately affect the global environment, including human health. The removal of HMs contamination is complicated by the toxicity and recalcitrance nature of contaminants. Therefore removal of HMs from contaminated sites using conventional technologies such as ion exchange, precipitation-filtration, and reverse osmosis, oxidation-reduction, and membrane separation is expensive, ineffective, and time-consuming. To date, the main four methods were proposed to remediate HMs contaminated soil: 1) chemical or physical remediation, 2) animal remediation such as earthworm, 3) phytoremediation, and 4) microremediation.

Because of many disadvantages, cost-effectiveness, and feasibility problem, wide application of the chemical or physical method is restricted. On the other way, the use of animals for soil remediation is not much effective in case of HMs contamination (Khalid *et al*., 2017). The use of plants and microbes for soil remediation is cost-effective and more feasible to reduce HMs load in the soil. So, firstly we tried to gain knowledge regarding phytoremediation and micoremediation separately, and after that, in the current review, we focussed on plant-microbe systems for HMs remediation.

Phytoremediation

Phytoremediation is a green technology and an eco-friendly approach that is simple, cost-effective, and non-environmentally disruptive. Plants utilized for phytoremediation are hyperaccumulators with a very high HMs accumulation potential, and fast-growing non-hyperaccumulators which possess lesser accumulation capacity, but total biomass yield is substantially higher than hyperaccumulators (Choudhary *et al*., 2016). Numerous processes are used to remove HMs from contaminated soils by some plants are:

(i) Phytoextraction/Phytoaccumulation: Phytoextraction involves the uptake and translocation of HMs from soil to above-ground plant parts through roots, and disposal of plants to remove them from the soil. Hyperaccumulator plants uptake, transport, and accumulate a large amount of HMs in above-ground parts at concentrations from 100 to 1000 times higher than non-hyperaccumulating plants. According to van der Ent *et al*., 2013, in dried foliage of hyperaccumulator plants metal present at concentrations of Cd (100 µg/g), Co, Cu, Cr (300 µg/g), Pb, Ni (1000 µg/g), Zn and Mn (3000 µg/g). These plants possess the following characteristics: (1) greater capacity to take up HMs from the soil; (2) improved root to shoot metal ions translocation; (3) dramatically enhanced ability to detoxify and sequester HMs in shoots; (4) ability to grow fast and plants with a harvestable aboveground stem and leaves which is convenient for subsequent post-processing (Clemens and Ma, 2016).

(ii) Phytofiltration: Phytofiltration is the process of cleanup of the polluted environment using plant roots or seedlings from aqueous wastes. It may use plant roots (rhizofiltration), uses seedlings (blastofiltration), and use of excised plant shoots (caulofiltration) (Rahman *et al*., 2016).

(iii) Phytostimulation: Phytostimulation is the enrichment of microbial activity to remediate contaminants by exudates from plant roots. For example, ethylene produced by PGPR using plant root exudates to degrade metal contaminants (Tak *et al*., 2013).

(iv) Phytostabilization: In phytostabilization, plant roots absorb pollutants or HMs from soil and hold them within the rhizospheric zone, and get separated and stabilized, rendering them harmless and preventing the pollutants from spreading in the environment (Lone *et al*., 2008). The mobility or accessibility of HMs in the environment is prohibited by precipitation around plant roots, metal valence reduction, root sorption, and metal complexation. Phytostabilization is an effective alternative

to the acquisition of metals *in situ* because the pollutants are not translocated into plant tissues and do not spread into the environment. It focuses mostly on HMs sequestration only within the rhizosphere.

(v) Phytovolatilization: It is the process for HMs removal by plants via changing into vapours consequently released into the atmosphere. The volatile forms during phytovolatilization are due to the metabolic potentials of plants associated with rhizospheric microorganisms (Sakakibara *et al*., 2010).

(vi) Rhizofiltration: Rhizofiltration involves plant roots for the elimination of toxic substances or pollutants from groundwater through filtration. The practice of rhizofiltration is based on the mechanism of rhizospheric accumulation of pollutants by plants. Plants that have a higher ability to uptake and resist high concentrations of HMs like hyperaccumulators are appropriate for rhizofiltration. The addition of PGPR to a contaminated site decreases the bioavailability of metals to plants thus reduces toxicity in plants, thereby increased the plant capability to eliminate HMs pollutant and to defend against environmental stress. It was reported that when *Bacillus megaterium* was applied to the root system of *Helianthus annuus*, growing in Pb contaminated soil, the efficiency of rhizofiltration was increased than without treated plants (Pearce *et al*., 2015).

Microremediation

Microremediation involves the use of microorganisms like rhizospheric microbes or free-living microbes to tolerate metal toxicity (Kang *et al*., 2016). The use of microorganisms has been widely studied to precipitate, sequester or change the oxidation state of numerous HMs. If a consortium of bacterial strains is used rather than a single strain culture, the remediation of HMs would be more efficient. A previous study indicated a synergistic effect on bioremediation of Cu, Pb, and Cd mixture from contaminated soils by four strains: *Enterobacter cloacae* KJ-46, *Viridibacillus arenosi* B-21, *Sporosarcina soli* B-22, and *E. cloacae* KJ-47 (Wang and Chen, 2009). They noticed that after 48 hours, remediation efficiencies were 5.6% for Cu, 98.3% for Pb, 85.4% for Cd, the bacterial mixtures had higher efficacy for HMs remediation compared to the use of single strain culture.

Several filamentous fungi have been studied to describe the ability to tolerate HMs stress. Due to presence of negatively charge functional group on fungal cell wall, metal binds to these groups like amine, carboxylic, phosphate, sulfhdryl (Ong *et al*., 2017). Previous report demonstrated the involvement of *Aspergillus niger* var. *tubingensis* Ed8 in Cr(VI) in reduction and sorption process (Coreño-Alonso *et al*., 2014). Another study showed the role of *Trichoderma* sp. in reducing As induced stress in chickpea (Tripathi *et al*., 2017). Arbuscular mycorrhizal (AM) fungi are soil fungi having nonspecific symbiosis with plants roots. The association is beneficial for both symbionts as the host plant provides carbon source to fungi in the exchange with nutrients and water. Similar to PGPR, AM fungi induce several mechanisms for toxic metal tolerance and detoxifications like (a) HMs bound to AM fungi cell wall and accumulate in the vacuoles, (b) metal sequestration with the help of siderophores, (c) HMs bound to MTs or PCs inside the fungal cells and (d) transporters at the tonoplast of fungi catalyze the transport of HMs from cytoplasm (Jan and Parray, 2016). AM fungi alleviate HMs stress of on plant by bioaugmentation. A study displayed that AM fungi alleviate Cd toxicity on plant growth by accumulating 10-20 times more Cd than plant roots (Janouskova *et al*., 2006).

Mechanism of Bioremediation

The following basic mechanisms are used for bioremediation of HMs: (1) sequestration of HMs, (2) modification of biochemical pathways to block metal uptake, (3) conversion of metals to non-toxic forms by enzymes, (4) reduction of intracellular concentration of metals using precise efflux systems (Jan *et al*., 2014).

Metal-binding and Sequestration of Heavy Metals

The process of HMs sequestration occurs both in plants and microbes. Plants can excrete less toxic and biodegradable natural chelators compared with EDTA. Among nature, chelators, phytochelatin (PC), and metallothionein (MT) are well studied by several researchers (Rauser, 1990; Cobbett, 2000; Vašák and Romero-Isart, 2011; Gupta *et al*., 2020). For sequestration, metals bind by intracellular metal-binding proteins and peptides such as MT, PCs, bacterial siderophores, and fungal hydroxamate siderophores.

In plant-metal interaction, MTs, PCs, and certain novel metal-binding peptides play a significant role. MTs bind to (HM) ions and promote their transportation or absorption. Bacteria with a high metal-binding capability of MTs have been commonly used in order to increase the resistance, sequestration, or accumulation of HMs. In *Escherichia coli*, yeast, humans, and plants, MTs from different sources have been expressed intracellularly. MTs are graded into three classes, Class 1 MTs referred to as mammalian-related polypeptides containing 61 amino acids but lack aromatic amino acid or histidines; Class 2 MTs formerly come from yeasts, and *Candida albicans* or cyanobacteria, a natural chelator belonging to this class is *Saccharomyces cerevisiae* MT, contributing to plants high copper tolerance; Class 3 MTs is PCs, which are composed of three amino acids, Glu, Cys, and Gly with glutamine and cysteine residues linked through a γ-carboxamide bond. Sriprang *et al*. (2003) have documented that overexpression of PC synthase in microbes to help accumulate and tolerate metal ions. The increased accumulation of Cd in *Mesorhizobium huakuii* subsp. *E. coli* was observed by the expression of *Arabidopsis thalina* gene encoding PC synthase. Various microbe releases iron-chelating substances called siderophores which enhances mobility and reduces the bioavailability of metals which helps subsequent removal of metals from soil.

Since vacuole is widely regarded as the central storage space for HMs in plant cells, vacuolar compartmentalization regulates the distribution and concentration of metal ions very efficiently (Sharma *et al*., 2016). The vacuole is to "arrest and imprison" toxic metal ions to compartmentalize, constricting them into a restricted site. As a result, such harmful metal ions are not available to other areas of the cell, and protection is, of course, assured. This mechanism is proved to be right in Cd detoxification and tolerance, for instance, Cd induces the synthesis of PCs and then forms Cd-PC molecule which transferred into the vacuole by ATP dependent PC-transporter and Cd/H antiporter.

Microbial cells are also capable of bioaccumulation that is the accumulation of HMs ions in particulates as well as insoluble forms and their by-products. The exopolysaccharide (EPS) are the essential constituent in such bacterial cells that have ion sequestration capability. Exopolysaccharides are mainly composed of complex macromolecules like polysaccharides, along with smaller proportions of protein and uronic acid (Gupta and Diwan, 2017). Exopolysaccharide protects the bacteria from environmental stress such as drought, salinity, and HMs toxicity. Microorganisms such as *Xanthomonas campestris*, *Agrobacterium* spp., *Alcaligenes faecalis, Bacillus* spp., *Zygomonas mobilis, Leuconostoc, Pseudomonas* spp. and *Acetobacter xylinum* are EPS-producing microorganisms with the potential to remove HMs from soil (Donot *et al*., 2012).

Modification of Metal Uptake

In the rhizosphere of hyper-accumulator plants, protons are released by root to acidify soil which mobilizes metal ions and increases metal bioavailability (Ojuederie and Babalola, 2017; Dixit *et al*., 2015). However, due to metal ions charge, the lipophilic cellular membrane would be the first barrier of ion's entrance into cells (Gillet *et al*., 2019). Proteins bind to HMs at their specific binding domain and transport metal ions from extracellular space into cells which indicated that transporter protein plays a crucial role (Das *et al*., 2016). Apart from this, several organic acids (e.g., malate and citrate) have been identified as positive bio-reagents to accelerate the absorption of HMs by root (Suanon *et al*., 2016). In root-shoot transport, this mechanism is even more remarkable. However, with the exception of two points, significant achievements in root-shoot transportation are lacking: one is root-shoot route is closely connected to the transpiration efficiency of plants and another one is chelator ligands which imply that the chelation mechanism also works in the process of xylem transferring. On the molecular level, the accumulation and transport mechanism is partly clarified. Many transporters encoded by specific genes are studied and generally one type of metal ion can be transported by various types of carriers.

Conversion of metals to non-toxic forms

Hazardous HMs exercise a detrimental influence on cells by binding to the vital protein, interfering with cellular activities, and inhibiting the regulation of cells. Plants and microbes have evolved their mechanisms to protect themselves from negative HMs stress (Hall, 2002). Several important mechanisms are explained as follows:

(i) Chelation: Chelation plays a crucial role not only in the accumulation and transportation of HMs but also in the detoxification phase (Hossain *et al*., 2012). In general, chelators have ligands (commonly histidine and citrate) and can bind metal ions. Combined metal ions appear uncharged and inert to react to other substances, by which way HMs damage towards cell is reduced significantly.

(ii) Volatilization: Some plant species prevent the permanent damage caused by accumulation and long stay of HMs by turning metal ions into a volatile form. A representative example is the bioprocess of Hg, which is a volatile global pollutant and accumulates in human bodies. However, not all the plants possess such ability, and even among those innate resistant species, the relatively small amount of accumulation and their spatial distribution have significantly limited their wide cultivation. Researchers have therefore utilized genetic engineering and many transgenic plants which have shown excellent results in converting and volatilizing HMs. For example, transgenic species expressing organomercuriallyase (MerB) have a much higher tolerance to organic Hg complex than wild type by converting into 100 times less toxic form, methylmercury. In addition, both MerA [an enzyme that reduces Hg(2) to Hg(0)] and MerB expressing transgenic plants have demonstrated the highest tolerance to organic Hg. To date, it is reasonably clear how microbes interact with element Hg. In the cells of mercury-resistant bacteria, there is a MerA enzyme, an enzyme that reduces Hg(2) to volatile form Hg(0) (Bizily *et al*., 1999). Another important example is the volatilization of As by arsenic methyltransferase gene of fungus. When this gene is introduced in plants and bacteria, this provides much tolerance by producing volatile tri-methyl arsine which is less toxic than arsenate or arsenite (Verma *et al*., 2016a, 2018). Microbes escape potential adverse effects caused by harmful metal ions by converting metal ions into volatile forms. However, for just a few metals or metalloids, like Hg, As and Se, such a method is feasible. For most other metals that do not have a volatile state under natural conditions, this route is closed.

(iii) Valence transformation mechanism: Metals of different valencies vary in toxicity. Plants skilfully transform dangerous metals to a comparatively less toxic state by excreting specific redox enzymes and reduce HM stress and injury (Chaturvedi *et al*., 2015). For instance, it is commonly studied to reduce Cr(6) to Cr(3), the latter one has less mobility and less toxic. Additionally, Kashiwa has found that *Bacillus sp.* SF-1 was good at reducing the high concentration of Se(6) into elemental Se. The most convincing example of this mechanism is the generation of mercury-resistant bacteria, converting methylmercury to Hg(2) which is a less toxic product.

(iv) Extracellular chemical precipitation mechanism: Quite a several binding substances were excreted by microbes, ranging from organic acid, alcohols to abundant polysaccharides, humic, and fulvic acids. Not only metals but also metal sulfides and oxides can be entrapped and absorbed by an extracellular mixture of polysaccharides, mucopolysaccharides, and proteins (Bramhachari and Nagaraju, 2017). Recent studies have found that peptidoglycan carboxyl groups are the principal cation binding sites for Gram-positive bacteria cell walls during phosphate group for Gram-negative microbes and chitins for fungi. This mechanism is effective in holding harmful metal ions out of the cytoplasm, regardless of whether the precipitation occurs on or off the outer surface of the cell wall.

Reduction of Intracellular Concentration of Metals using Efflux Systems

Microbes possess specific efflux proteins, which actively expel out toxic HMs bind with small peptide glutathione. For example, bacterial ARS operons have ArsA, and ArsB protein actively extrude arsenite from the cell. Similarly, yeast ACR3 posses the capability to extrude $As(GS)_3$ complex outside the cell.

Genetic Engineering of Microbes to Remediate Heavy Metal

The remediation of HMs contaminated soil using indigenous microorganisms is not enough in order to remove intense contamination created by humans. For example, indigenous microbes cannot remove HMs like Hg from the environment. However, recombinant DNA technology plays a significant role in HMs bioremediation as it improves the remediation process (Table 1). The recombinant DNA technology inserts a foreign gene into the microbial genome or extrachromosomal region (plasmid) from an organism of the same or different species to create genetically engineered (GE) microbes. From the early time efforts have been made for the removal of Hg using GE *E. coli* strain M109 and *Pseudomonas putida* containing *merA* gene. Identification and study of the gene involved in metal uptake and detoxification is a prerequisite to generate GE microbes. Advancement of modern techniques and tools in genetics and omics like proteomics, genomics, and metabolomics has enabled researchers to study the catabolism of organic pollutants by microorganisms. Recombinant DNA and RNA technologies have been used for tailoring microbial genes to improve or create new metabolic pathways to enhance bioremediation processes. The use of GE microbes with unique attributes of their metabolic pathways and improved microbial metabolic potential is a safe and cost-effective method for the elimination of contaminants from contaminated sites (Gupta A. *et al*., 2016; Tiwari and Lata, 2018). It has also led to HMs and other recalcitrant compounds detoxification. For example, metal regulatory genes inserted in microbes can help them to convert toxic forms of HMs to less toxic forms (Das *et al*., 2016). GE bacteria are eco-friendly and represent a useful technology for the removal and detoxification of HMs and recalcitrant compounds in contaminated sites (Gupta and Singh, 2017). Nowadays GE bacteria have been used for the removal of HMs such as Cd, Hg, Ni, Cu, As, and Fe (Rojjanateeranaj *et al*., 2017; Hwang and Jho, 2018). New metabolic pathways have been discovered that allow the engineered bacteria to transform toxic HMs into less toxic or harmless forms, thereby improving the processes of bioremediation.

Mercury is a toxic HM that can be released into the environment. GE *E. coli* strain JM109 can remove Hg from contaminated water, soil, or sediment. The *merA* gene containing GE bacteria will eliminate Hg from a polluted site. The expression of *E. coli* mer operon in the bacterium *Deinococcus geothemalis* improves the ability to reduce Hg contamination at high temperatures. In the same way, *Cupriavidus metallidurans* strain MSR33 containing *pTP6* plasmid with genes (*merB* and *merG*) regulate Hg biodegradation, also have organomercurial lyase protein (*merB*) and mercuric reductase (*merA*) help in the reduction of Hg contamination from polluted sites (Rojas *et al*., 2011). Bacteria expressing MTs and polyphosphate kinase also promote practical Hg bioremediation (Giovanella *et al*., 2016).

Chromium is a metal that is extremely carcinogenic and can be found in the industrial wastewater. For the treatment of industrial wastewater, GE bacteria, such as *Ralston metallidurans* may be used to eliminate Cr. Recombinant *Caulobacter* spp. strain JS4022/p723-6H can also remove Cd from industrial wastewater (Benjamin *et al*., 2019). Arsenic is a very toxic metalloid that can be found in nature (Verma *et al*., 2016b). GE bacteria expressing the *ArsM* gene has been demonstrated to remove As through volatilization from contaminated soil (Verma *et al*., 2016c). *E. coli* expressing the *ArsR* gene can promote the bioaccumulation of As when present in contaminated soil (Ke *et al*., 2018).

The genetic system of microbes may be tailored by a different type of genes/ enzyme to make them a powerful tool for metal tolerance. Some essential system modifications include the engineering of HMs uptake, transport, and storage

Table 1: Genetically engineered bacteria involved in heavy metals remediation.

Heavy metals	*GE Bacteria*	*References*
As	*Bacillus Idriensis*	Liu *et al*., 2011
As	*Bacillus subtilis*	Huang *et al*., 2015
As	*Escherichia coli* strain	Kostal *et al*., 2004
As	*Escherichia coli* strain	Yuan *et al*., 2008
As	*Sphingomonas desiccabilis*	Liu *et al*., 2011
Cd	*Bacillus subtilis* BR151 (pT0024)	Ivask *et al*., 2011
Cd	*Caulobacter crescentus* JS4022/ p723-6H	Patel *et al*., 2010
Cd	*Escherichia coli* strain	Freeman *et al*., 2005
Cd	*Escherichia coli* and *Moraxella* sp.	Bae *et al*., 2001, 2003
Cd	*Mesorhizobium huakuii* B3	Sriprang *et al*., 2003
	Mesorhizobium huakuii	Porter *et al*., 2017
Cd	*Ralstonia eutropha* CH34	Valls *et al*., 2000
Cd	*Pseudomonas putida* X3	Zhang *et al*., 2016
Cr	*Deinococcus radiodurans*	Brim *et al*., 2000, 2003, 2006
Cr	*Methylococcus capsulatus*	Al Hasin *et al*., 2010
Cr	*Pseudomonas putida*	Ackerley *et al*., 2004
Hg	*Achromobacter* sp. AO22	Ng *et al*., 2009)
Hg	*Acidithiobacillus ferrooxidans*	Sasaki *et al*., 2005)
Hg	*Acidithiobacillus ferrooxidans*	Valdés *et al*., 2008)
Hg	*Deinococcus geothemalis*	Dixit *et al*., 2015)
Hg	*Deinococcus radiodurans*	Brim *et al*., 2000, 2003, 2006; Gupta D.K. *et al*., 2016
Hg	*Escherichia coli*	Murtaza *et al*., 2002
Hg	*Escherichia coli* JM109	Zhao *et al*., 2005
Hg	*Escherichia coli* and *Moraxella* sp.	Bae *et al*., 2001, 2003
Hg	*Escherichia coli* MC 1061	Bondarenko *et al*., 2008
Hg	*Pseudomonas*	Sone *et al*., 2013a
Hg	*Pseudomonas* K-62	Kiyono and Pan-Hou, 2006; Kiyono *et al*., 2009
Pb	*Bacillus subtilis* BR151, *Staphylococcus aureus* RN4220	Bondarenko *et al*., 2008

system. The most studied uptake and transport system used for genetic modification is channel proteins which facilitate passive diffusion of HMs according to their concentration gradient in an energy-independent manner. Channel proteins such as glycerol facilitators (GlpF) from *E. coli, Corynebacterium diptheriae,* and *Streptomyces coelicolor*, and Fps1 from *Saccharomyces cerevisiae* were expressed to alter arsenite uptake and transport (Garbinski *et al*., 2019). Similarly, MerT/P transporter from *Serratia marcescens* (Deng and Jia, 2011), *Pseudomonas K-62*, and *Pseudomonas K-12* have been used for Hg uptake and transport. Additionally, other importers that can take up Hg are MerA, MerC, MerE, and MerF, although they vary in topology, it is suggested they share the same uptake mechanism (Sone *et al*., 2013b). The rate of this passive uptake is a function of the concentration gradient of targeted HMs. An energy-dependent uptake and storage system is needed if the HM concentration is more in the cell than in the external environment. For example, bioaccumulation of Cd improved by expressing primary active transporters MntA, and cdtB from *Lactobacillus plantarum* (Kang *et al*., 2007) and *TcHMA3* from the flowering plant *Thlaspi caerulescens* have been involved in HM transport (Chang and Shu, 2014). Additionally, ABC transporters are another large class of primary active transporters that are essential in bioaccumulation and detoxification (Remy *et al*., 2013).

Efforts are also made to increase HMs storage in microbes through the production of cytoplasmic metal-binding entities for the sequestration of HMs. These entities are mostly metal-binding proteins, but also include enzymes that produce peptides and other polymers that can also bind to HMs. MTs are the largest group of proteins used as storage systems. Several researchers suggested that the engineered *E. coli* expressing *MT* gene enhanced HM accumulation such as Cd (Kim *et al*., 2005; Deng *et al*., 2007). Singh *et al*., 2008a identified a *MT* gene from As-tolerant marine alga, *Fucus vesiculosus* (*fMT*), and expressed it as a fusion protein in *E. coli*. The overexpression of *fMT* in *E. coli* increased AsV and AsIII accumulation as expressed recombinant protein has a higher binding affinity with As. Further, co-expression of fMT with AsIII transporter GlpF improved AsIII accumulation but also increases AsIII selectivity (Singh *et al*., 2008b). Recently Ma and co-workers find that the oligomeric expression of the *MT* gene significantly enhanced metal tolerance. The *E. coli* expressing *MT* gene accumulate the higher amount of Cu, Cd, or Zn than control cells (Ma *et al*., 2019). Another study suggested that expression of MT protein encoded by *bmtA* gene in *Pseudomonas aeruginosa* N6P6 capable of Pb sequestration under high metal concentration (Kumari and Das, 2019). Due to the high HMs binding capabilities of MTs, GE bacteria overexpressing MTs have great potential as biomaterials for bioremediation.

The second most crucial polymer is PC, composed of a chain of glutathione (GSH) produced from ligating L-cysteine and L-glutamate to form γGlutamylcysteine (γEC), followed by another ligation between L-glycine and γEC. PC biosynthesis occurs when the enzyme, PC synthase (PCS) is triggered by HMs like Cd, As, Cu, Hg, and Pb by transfer of -Glu-Cys from GSH into another GSH (Kumar *et al*., 2014, 2016; Verma *et al*., 2016d).

PCs alone may be adequate for HMs bioaccumulation, but may be become more effective by increasing PC precursor compounds like cysteine, γEC, and GSH by overexpressing *cysE*, *GSHI*, and *GSHII* gene, respectively. Earlier studies reported GSH production as a checkpoint in the PC production pathway, so a feedback inhibition insensitive mutant *GSHI* was used in bioaccumulation studies (Murata *et al*., 1983). Kang *et al*. (2007) overexpressed a variant of *GSHI*, *GSHII* in *E. coli* that results in a significant increase in PC production and a corresponding increase in HMs accumulation. The co-expression of Cd transporter, MntA, has achieved further progress in Cd accumulation, indicating that PC-mediated Cd accumulation can be regulated by rationally controlling the pathways of GSH and PC synthesis (Kang *et al*., 2007). Bae *et al*. (2000) demonstrated that synthetic PCs provide metal-binding ability on host cells and the resulting new bio-adsorbents accumulate a substantially higher amount of Cd than wild-type cells. Another study indicates that *AtPCS* expression enables PC synthesis that increases intracellular metal level significantly when bacterial cells were grown on metal-enriched media (Sauge-Merle *et al*., 2003). These studies pave the way for using enzymes of the PC biosynthetic pathway to enhance metal accumulation in microbes and optimize bioremediation processes of toxic metals. Another component of the storage system uses the production of polyphosphate (polyP) using polyphosphate kinase from *Klebsiella pneumonia*. The polyP was only used to bioaccumulate Hg, but a natural reaction to exposure to AsIII, Cu, and Ni indicated that polyP could be used to store other HMs (Seufferheld *et al*., 2008).

Besides polyP and PCs, various organisms upregulate enzymes to produce organic acids and amino acids for HMs chelation. For example, *Acidithiobacillus ferrooxidans* ATCC 23270 was shown to upregulate expression of its histidine biosynthesis operon, when exposed to 40 mM $CuSO_4$ (Julián-Almárcegui *et al*., 2015) which increase the pool of cytoplasmic histidine to chelate Cu ions to prevent oxidative damage. In the same analysis for enzymes that participate in the cysteine biosynthesis process, a similar upregulation and speculation were noted.

Bioremediation of Contaminated Soil using the Interaction between Plant and Genetically Engineered Microbes

Bioremediation of HMs contaminated soil using GE microbes gives promising results, still, growth and development of microbes can improve by adjusting the pH, as well as levels of nutrients and oxygen. Rhizosphere bacteria obtain nutrients excreted from plant roots, such as organic acids, enzymes, amino acids, and complex carbohydrates. In return, the microbes improve plant nutrient availability (Table 2).

Rhizobium is the most used microbe to remediate contaminated soil. *Rhizobium* is a Gram-negative bacterium that establishes a symbiotic relationship with leguminous plants. Rhizobia grow slowly and form nitrogen-fixing nodules on the root of legumes with up to 108 bacteria progeny (Datta *et al*., 2015). This particular character is useful for biotechnological application for the expression of genes such as MTs that sequesters HMs from contaminated soil (Sarma and Prasad, 2019). After HM uptake, the subsequently, either HM ions accumulated by plants root, transported in the xylem and detoxified through plant system or it will be accumulated in rhizosphere and nodules and thus, provide a less expensive method to remove HMs from soil. For example, GE *Mesorhizobium* sp.

Table 2: Genetically engineered (GE) bacteria which enhanced phytoremediation.

Heavy metals	*Plant*	*GE Microbe*	*Expressed Gene*	*References*
As	*Brassica napus*	*Enterobacter cloacae* CAL2	EC 4.1.99.4	Nie *et al*., 2002
As	*Astragalus sinicus*	*Meshorhizobium huakuii* subsp. *rengei* strain B3	Iron regulated transporter 1 gene from *Arabidopsis thaliana* (*ATIRT1*)	Ike *et al*., 2008
Cd, Hg, Ag	*Triticum aestivum*	*Pseudomonas putida* KT2440	Phytochelatin synthase (*PCS*)	Yong *et al*., 2014
Cd	*Helianthus annuus*	*Pseudomonas putida* 06909	Expression of metal binding peptide (*EC20*)	Wu *et al*., 2006
Cd	*Astragalus sinicus*	*Meshorhizobium huakuii* subsp. *rengei* strain B3	Tetrameric human metallothionein (*MTL4*)	Sriprang *et al*., 2002
Cd	*Astragalus sinicus*	*Meshorhizobium huakuii* subsp. *rengei* strain B3	*PCSAT*	Sriprang *et al*., 2003
Cd	*Astragalus sinicus*	*Meshorhizobium huakuii* subsp. *rengei* strain B3	*MTL4* and *ATPCS*	Ike *et al*., 2007
Cu, Cd, Zn	*Astragalus sinicus*	*Meshorhizobium huakuii* subsp. *rengei* strain B3	Iron regulated transporter 1 gene from *Arabidopsis thaliana* (*ATIRT1*)	Ike *et al*., 2008
Cu	*Medicago truncatula*	*Ensifer medicae* MA11-*copAB*	*copAB*	Delgadillo *et al*., 2015
Cu	*Medicago truncatula* with *mt4a* gene	*Ensifer medicae*	*copAB*	Pérez-Palacios *et al*., 2017

and *M. huakuii* subsp. *rengei* by *AtPCS* gene encoding PC synthase showed increased Cd accumulation in bacterial cells, and inoculation this GE *Mesorhizobium* with *Astragalus sinicus* increased Cd accumulation in root nodules. This symbiotic system was applied for the phytoremediation of paddy soil polluted with Cd. There was an increased Cd accumulation in nodules and roots that contributed to the removal of about 10% Cd from the ground after two months of plant cultivation Gupta *et al*. (2002) generated Cd, Ni, and Cr-resistant mutants of phosphate-solubilizing *Pseudomonas sp.* NBRI 4014, having increased resistance to high concentrations of Cd, Cr, and Ni, on *Glycine max* plants cultivated in metal amended soil.

Mesorhizobium huakuii subsp. rengei strain B3 is bacteria that establish symbiosis with legume *Astra Astragalus sinicus* (Chinese milk vetch), elicit nitrogen-fixing root nodules formation, and used as green manure in Southern China and Japan rice fields. The symbiosis is triggered between leguminous plants and rhizomes when flavonoids and related plant compounds induce the bacteria to produce molecular signals, which stimulate nodule organogenesis. Bacteria enter the developing nodule via infection threads that are taken up by plant host cells in endocytosis like process. The rhizobia undergo differentiation into a distinct cell type called bacteroid, which is capable of fixing atmospheric nitrogen into ammonia and provided to the host plants. Previous studies showed that in Cd contaminated soil, *Lolium multiflorum* Lam. displayed a higher level of Cd phytoextraction and Cd tolerance ability. With higher biomass production, high adaptability, and low management cost, *L. multiflorum* Lam. was regarded as an excellent candidate for HMs phytoremediation (Sabreen and Sugiyama, 2008). However, few studies have investigated the potential effects of rhizobia on plant growth and Cd uptake ability by both leguminous and non-leguminous plants in Cd-contaminated soil.

Another example for the use of genetically modified bacteria was the inoculation of *Rhizobium leguminosarum* with maize and lettuce to increase the growth of plants with enhanced phosphate solubilization (Zaim *et al*., 2017), and sunflowers inoculated with *Rhizobium* sp. exhibited increased nitrogen uptake (Ullah *et al*., 2017). Binding reagents, such as MTs, PCs, and organic acids, would be secreted by the symbiotic system in order to maintain acidification to enhance the solubility, mobility, and bioavailability of HMs. Recently, Verma *et al*. (2019) showed that GE yeast inoculated with rice plants grown in As-contaminated soil, rice plants accumulate less As and showed improved in growth than non-inoculated plants.

Microbial ecologists have been documented the rhizospheric symbiosis between plants and microbes. Apart from this, the introduction of GE microbes or bioaugmentation can contribute to augment the activity of insufficient, indigenous microbes to improve the bioremediation of contaminated sites (Carlos *et al*., 2016). An example is the use of recombinant *P. putida* 06909 with sunflower to remediate Cd toxicity in water (Wu *et al*., 2006). While extracting contaminated water containing soluble forms of metals, the plant roots sustain a stable bacterial population. As a consequence, bacterium benefits from colonization of sunflower roots and induces adequate metabolic activity to generate EC20, which in turn helps seedlings grow at high Cd concentration by absorbing and preventing toxic Cd from being transported into the plant. Another advantage of using rhizosphere bacteria is that Cd bound on the rhizobacterium could be removed by harvesting plants. This self-sustainable rhizobacterial population probably provides both long term growth plant protection and Cd removal. The emphasis on metal phytoremediation by legumes in combination with rhizobium is increasing day by day. In addition to their ability to withstand HMs, legumes interact symbiotically with rhizobia as a source of nitrogen for the biosphere and as a model for studies of plant-microbes interaction (Fig. 1) (Harman and Uphoff, 2019).

Safety Aspects and Survivability of Genetically Engineered Microbes

The GE microbes for bioremediation have the potential to cause adverse effects on the environment and human health.

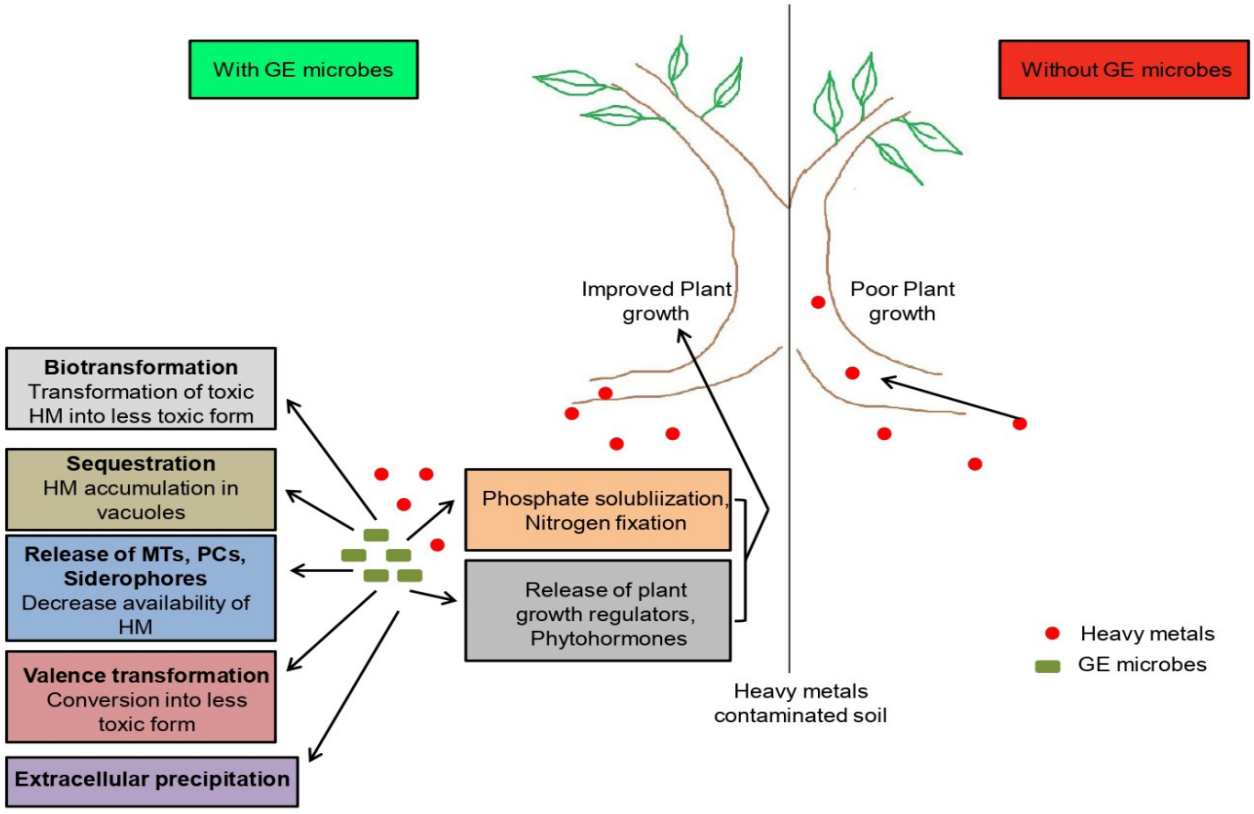

Fig. 1: Mechanism involved in heavy metals contaminated soil by GE microbes-plant interaction.

The harmful effects posed by GE microbes depend on the characteristics of GE microbes and the environment. The degree of application of GE microbes for bioremediation is slow due to its possible risks and low public acceptance. The environmental concerns and regulatory constraints are significant obstacles to the deployment of GE microbes in the field. The most crucial issue in the use of GE microbes is containment. GE microbes could disrupt existing ecological frameworks if they persist in the environment after the pollutant has been depleted. The survivability and horizontal gene transfer of GE microbes are crucial issues concerning the potential impact of their release into the atmosphere for remediation. Researchers should target the application of technical shield in the design of GE microbes for bioremediation of HMs. Every country should have proper regulation for the containment, risk assessment, and monitoring of GE microbes release into the environment. The survivability of GE microbes in the contaminated environment is essential if they perform their bioremediation function to the desired level. The stability of GE bacteria depends on their growth rate and existing environmental conditions, including spatial distribution and the competitiveness between microorganisms and predators. There is a report that GE microbes could stay alive for up to 6 years of co-existence with natural microorganisms. However, competitive situations and adverse environmental conditions hamper the survival of GE microbes which should be considered before the release of GE microbes in the environment for bioremediation.

Conclusion

The critical challenge for the successful application of microorganisms in HMs remediation technologies is the enhancement of their metal tolerance. For the improvement of plant capability to tolerate HMs stress, is the use of beneficial plant-associated microorganisms is undoubtedly advisable. For legume plants, this strategy is of particular importance because they exhibit very high symbiotrophic ability. Various studies demonstrated that the addition of mycorrhiza, nodule bacteria, or PGPR significantly promote plant growth in the presence of high concentrations of HMs in soils. Moreover, positive synergistic and additive effects of different microorganisms on plant growth and nutrition enhanced after the improvement of the genetic material of microbes. This approach supports the perspectives of using GE microbes expressing HM tolerance genes, which have multiple impacts on plants and rhizosphere processes related to the function of microbial community and HMs transformation. More attention should be given to the biodiversity of beneficial microorganisms inhabiting polluted environments, interactions between microbes in the rhizosphere, and selection of metal tolerant strains for genetic improvement and development of efficient symbioses with plants under HMs stress conditions. However, the enhancement of macro-symbiont HM tolerance is of vital importance, as the plant is typically more vulnerable to HM stress and most likely, the plant genotype regulates symbiosis to a greater degree

compared to the microorganism in the presence of HMs toxicity. It is essential to put efforts into understanding limiting steps in the development and efficient functioning of GE microbes and their symbiotic plant-microbe interactions during the HMs stress.

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