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Engineering Bacteria for Bioremediation

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1. Introduction

Bioremediation is a process that uses microorganisms or their enzymes to promote degradation and/or removal of contaminants from the environment. The use of microbial metabolic ability for degradation/removal of environmental pollutants provides an economic and safe alternative compared to other physicochemical methodologies. However, although highly diverse and specialized microbial communities present in the environment do efficiently remove many pollutants, this process is usually quite slow, which leads to a tendency for pollutants to accumulate in the environment and this accumulation can potentially be hazardous. This is especially true for heavy metals. Heavy metal contamination is one of the most significant environmental issues, since metals are highly toxic to biota, as they decrease metabolic activity and diversity, and they affect the qualitative and quantitative structure of microbial communities. For treating heavy metal contaminated tailings and soils, bioremediation is still the most cost-effective method, although various heavy metals are beyond the bioaccumulation capabilities of microorganisms. Perhaps, because of the toxicity of these compounds, microorganisms have not evolved appropriate pathways to bioaccumulate them; populations of microorganisms responsible for this bioaccumulation are not large or active enough to remove these compounds completely, or complex mixtures of pollutants resist removal by existing pathways. The pathway used to accumulate these compounds is adsorption, where metals are taken up by microbial cells (biosorption). Biosorption mechanisms are numerous and are not yet fully understood. However, biosorption capacity often varies with test conditions, such as initial metal concentration, solution pH, contact time, biomass dosage, processing method, and so on. Accordingly, populations of microorganisms that are able to promote metal adsorption and accumulate them are not large or active enough to support these compounds by existing pathways. Furthermore, there are several strategies that optimize the bioremediation process of pollutants. One approach to enhance populations of microorganisms capable of pollutant removal is the addition of exogenous microorganisms in order to expand indigenous populations. This process, commonly known as bioaugmentation, can be performed either by adding microorganisms that naturally contain catabolic genes or those that have been genetically modified (GMOs). This strategy can also result in the transfer of plasmids containing the necessary genetic material between the different populations. Recent advances in the molecular biology field have been applied to microorganisms in order to produce novel strains with desirable properties for the

bioremediation processes. These include the construction or adaptation of catabolic pathways; redirection of carbon flux to prevent the formation of harmful intermediates; modification of catabolic enzyme affinity and specificity; improvement of the genetic stability of catabolic activities; increasing the bioavailability of pollutants; and enhancement of the monitoring, yield, control, and efficiency of processes. Despite the many advantages of GMOs with regards to bioremediation, their use is still limited in the environment because of the instability of the inserted genetic material. There are two major reasons for this: first, the efficiency of GMOs is dependent on their ability to carry the genetic material in a stable manner; second, the transfer of genetic material to the indigenous organisms is perceived to be a negative attribute, despite the fact that this transfer is a common phenomenon among native organisms. These factors have incentivized the study of survival, competition and persistence of GMOs in the environment, as well as the potential risks involved in their use. Besides the significant advances that have already been made with regards to the development and utilization of GMOs for bioremediation of contaminants in the environment, many more challenges still remain. In this chapter, we will detail how genetic engineering may improve bioremediation through the engineering of bacteria. Several genetic approaches have been developed and used to optimize enzymes, metabolic pathways and organisms that are relevant to biodegradation. New information on metabolic routes is still being accumulated, thus the available toolbox is continuously being expanded. With molecular methods enabling the characterization of microbial community structure, metabolic pathways and enzyme activities, the performance of microorganisms under *in situ* conditions can be improved by making heavy metal bioremediation a much more efficient process. The present review also highlights the current situation pertaining to biosorbents, their mechanisms and advantages and disadvantages. Thus, this article reviews the achievements and current status of biosorption technology, which exploits natural biodiversity and molecular tools, in order to engineer microorganisms and provide new information about this research frontier.

1.1 Heavy metals and toxicicity

Heavy metals are considered to be chemical elements with an atomic mass greater than 22 and a density greater than 5g/mL. This definition includes 69 elements, of which 16 are synthetic. Some of these elements are extremely toxic to human beings, even at very low concentrations (Roane & Pepper, 2000; Wang & Chen, 2006). The main heavy metals associated with environmental contamination, and which offer potential danger to the ecosystem, are copper (Cu), zinc (Zn), silver (Ag), lead (Pb), mercury (Hg), arsenic (As), cadmium (Cd), chromium (Cr), strontium (Sr), cesium (Cs), cobalt (Co), nickel (Ni), thallium (Tl), tin (Sn) and vanadium (V) (Wang & Chen, 2006). In general, metal ions can be classified as: 1) Essential and important for metabolism (Na, K, Mg, Ca, V, Mn, Fe, Co, Ni, Cu, Zn, Mo and W); 2) Toxic heavy metals (Hg, Cr, Pb, Cd, As, Sr, Ag, Si, Al, Tl), which have no biological function (in ecotoxicology terms, hexavalent forms of Hg, Cr, Pb and Cd ions are the most dangerous); 3) Radionuclides (U, Rn, Th, Ra, Am, Tc), which are radioactive isotopes and, although toxic to cells, they are nonetheless important in nuclear medicine procedures; 4) Semi-metals or metalloids (B, Si, Ge, As, Sb, Te, Po, At, Se), which exert distinct biological effects on metals. However, metals are predominantly present in the environment in cationic and anionic forms in semimetals, and As is often classified as heavy metal (Roane & Pepper, 2000; Ahluwalia & Goyal, 2007). In the environment, metals can be

divided into two categories: 1) bioavailable (soluble, non-absorbed, mobile); and 2) non-bioavailable (precipited, complexed, sorbed, non-mobile). The ionic form (speciation) of a metal determines its bioavailability and its destination. Most heavy metals are cations and this determines their sorption to negatively charged functional groups that are present in: cell surfaces, which are generally anionic at a pH of between 4 and 8; surfaces with residual hydroxides (OH^-) or thiol (SH^-) and anionic salts, such as PO_4^- and SO_4^-, humic acid, and clay minerals (Roane & Pepper, 2000). Heavy metal ions possess great electrostatic attraction and high binding affinities to the same sites that essential metal ions normally bind to various cellular structures, causing destabilization of the structures and biomolecules (cell-wall enzymes, DNA and RNA), thus inducing replication defects and consequent mutagenesis, hereditary genetic disorders and cancer. This occurs, for example, with arsenate, which competes with phosphate, and cadmium, which competes with zinc. By employing microarray technology, Kawata *et. al.* (2007), found that six heavy metals (arsenic, cadmium, nickel, antimony, mercury and chromium) induce gene expression patterns that are very similar to the pattern induced by DMNQ (2.3-dimethoxy-1, 4-naphthoquinone), the reactive oxygen species (ROS) chemical generating agent, which causes "oxidative stress", leading to deleterious effects (membrane damage or other cellular lipid structures, modification of proteins, fragmentation and cross-links, changes in DNA that can induce mutations or be repaired by repair mechanisms). Therefore, the ions of heavy metals cause oxidative damage, both directly, by producing ROS, and indirectly, by inactivating the cellular antioxidant system, thus leading to cell damage (Mannazzu *et. al.*, 2000; Liu *et. al.*, 2005).

1.2 Heavy metals and the environment

Among the different contaminants, heavy metals have received special attention due to their strength and persistence in accumulating in ecosystems, where they cause damage by moving up the food chain to finally accrue in human beings, who are at the top of this chain (Figure 1) (Volesky, 2001; Ahluwalia & Goyal, 2007; Machado *et. al.*, 2008).
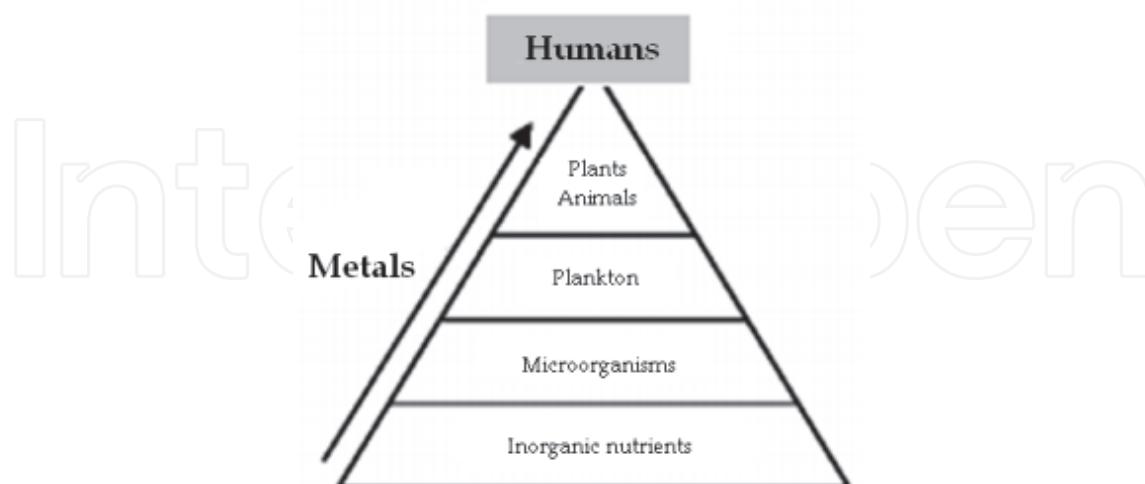

Fig. 1. The destiny of heavy metals released into the environment and their accumulation throughout the chain food. Adapted from Volesky (2001).

The toxic potential of heavy metals with regards to the human body is diverse and, because of their toxicity and persistence in nature, the levels of heavy metals in the environment

need to be controlled by mandating waste treatment at the sources of pollution. The development of new treatment technologies is required at these sources; however, even though there is awareness of this problem, sustainable solutions are not easily accessible. In general, the conventional treatment methods used to remove metals from wastewater are inefficient and cost-prohibitive.

1.3 Conventional technologies for treating environments contaminated by heavy metals

Environments contaminated by heavy metals are treated by means of conventional technologies based on physicochemical principles, which are considered inefficient and uneconomic. One method often employed to remove metals from aqueous solutions involves the addition of reagents that increase pH, thus converting metals from a soluble form into an insoluble form (hydroxides), which results in their precipitation. This procedure produces large quantities of mud in the final wastewater with concentrations of metals in the order of mg/L, which is difficult to dispose of. More complex processes of this type can involve single or multiple steps: 1) precipitation with hydroxides, carbonates or sulfides; 2) redox chemistry; 3) sorption (adsorption with activated carbon/ion exchange); 4) use of membranes (ultrafiltration, electrodialysis and reverse osmosis-RO); 5) electrolytic recovery; 6) evaporation; 7) liquid-liquid extraction; 8) electrodeposition. On the other hand, bioremediation is increasingly gaining importance as an alternative technology, due the advantages it offers: simplicity, efficiency and low cost (Goyal *et. al.*, 2003; Tabak *et. al.*, 2005; Hameed, 2006; Machado *et. al.*, 2008; Wang & Chen, 2009).

1.4 Types of bioremediation

Bioremediation involves the use of plants or microorganisms, viable or not, natural or genetically engineered to treat environments contaminated with organic molecules that are difficult to break down (xenobiotics) and to mitigate toxic heavy metals, by transforming them into substances with little or no toxicity, hence forming innocuous products (Dobson & Burgess, 2007; Li & Li, 2011). With the objective of improving the process of bioremediation, different strategies can be employed, depending of the state of the contaminated environment. One of these strategies, biostimulation, involves promoting the growth of autochthonous microorganisms at the contaminated site, in order to introduce pH-correction substances, nutrients, surfactants and oxygen. As a consequence, the rate of biodegradation/bioremediation can be increased. Another strategy, bioaugmentation or bioaddition, is the addition of microbial populations to indigenous, alien or genetically modified organisms (GMO), in places where there is an insufficiency of indigenous microorganisms with ecophysiological characteristics compatible with the habitat conditions that are conducive to the promotion of bioremediation (Vidali, 2001; Silva *et. al.*, 2004; Gaylard *et. al.*, 2005; Li & Li, 2011). Bioremediation is a versatile process that can be applied *in-situ*, at the contaminated site, or *ex-situ*, involving removal of contaminated material to be treated elsewhere. *In-situ* bioremediation technologies are more economical and release fewer pollutants into the environment; however, they may require longer treatment timeframes than the *ex-situ* techniques (Vidali, 2001; Tabak *et. al.*, 2005; Costa *et. al.*, 2007). Currently, there is wide variety of microorganisms (bacteria, fungi, yeasts and algae) that are being studied for use in bioremediation processes, and some of these have already been employed as biosorbents of heavy metals (Roane & Pepper, 2000; Machado *et. al.*, 2008;

Bogacka, 2011). The main advantages of biosorption over conventional treatment methods include: low cost; high efficiency; minimization of chemical and biological sludge; selectivity to specific metals; no additional nutrient requirement; regeneration of the biosorbent; and the possibility of metal recovery (Kratochvil & Volesky, 1998). Most studies on the bioremediation of heavy metals present the following as a biphasic biosorption process: 1) rapid initial phase (adsorption), not dependent on metabolism or temperature, which is generally reversible; and, 2) slower phase (intracellular accumulation) metabolism-dependent, influenced by environmental factors such: temperature, which decreases the biosorption capacity due to damage to the target sites (in general, the temperature considered "ideal" is between 25-35ºC); and metabolic inhibitors (Roane & Pepper, 2000; Malik, 2004; Tabak, 2005). Desorption is a very important process in the success of the applicability of bioremediation for the treatment of wastewater, because it allows for the recovery of adsorbed metal ions, as well as the recycling and reuse of biomass for a new cycle of metal recovery. There is obviously great interest in the development of a procedure that enables the recovery of removed metal ions, as well as for the cellular integrity of the biosorbents to be maintained, thus allowing for their regeneration and reuse in successive cycles of sorption-desorption. This results in the simultaneous acquisition of two valuable products: treated water and low-cost recovery of metal (Aldor *et. al.*, 1995; Volesky, 2001; Yu & Kaewsarn, 2001).

2. Exploitation of the natural biodiversity

2.1 Microorganisms as biosorbents of heavy metals

Several studies have shown that many organisms, prokaryotes and eukaryotes, have different natural capacities to biosorb toxic heavy metal ions (Table 1), giving them different degrees of intrinsic resistance, particularly in diluted solutions (between 10 to 20 mg/L^{-1}), due to their mobility, as well as the solubility and bioavailability capacities of these metal ions (Malik, 2004; Tabak *et. al.*, 2005; Kim *et. al.*, 2007; Chen & Wang, 2008).

The manner in which microorganisms interact with heavy metal ions depends partly on whether they are eukaryotes or prokaryotes. Eukaryotes are more sensitive to metal toxicity than bacteria. In the presence of toxic concentrations, several resistance mechanisms are activated, for example: the production of peptides of the family of metal binding proteins, such as metallothioneins (MTs); the regulation of the intracellular concentration of metals, with expression of protein transporters of ligand-metal complexes from the cytoplasm to the inside of vacuoles, and efflux of metal ions by ion channels present in the cell wall. In bacteria, these tolerance mechanisms are often encoded by plasmids, which facilitate their dispersion from cell to cell (Valls & Lorenzo, 2002). For the bioremediation of heavy metals on an industrial scale, it is important to use low-cost biomaterial, which can be a byproduct or waste material with high removal capacity, since the low cost of this biomass is crucial for the process to be economically viable (Volesky & Holan, 1995; Zouboulis *et. al.*, 2001). Several studies have been conducted with the purpose of improving the resistance and/or the ability of microorganisms to accumulate heavy metal ions, including a number of studies that follow parameters: pH (Naeem *et. al.*, 2006); temperature; different metal concentrations and biomass (Soares *et. al.*, 2003; Kim *et. al.*, 2005), competitiveness of ions of different elements; microorganism-metal contact time (Kotrba & Ruml, 2000), composition of the culture medium (Ghosh *et. al.*, 2006); bioaugmentation/biostimulation (Roane &

Pepper, 2001; Silva *et. al.*, 2004), resistance to toxicity of heavy metals of Gram positive/Gram-negative bacteria (Samuelson *et. al.*, 2002); intracellular/extracellular bioaccumulation; viable /non-viable cells, free /immobilized cells, and biological processes by aerobic/anaerobic microorganisms (Dias *et. al.*, 2002; Liu *et. al.*, 2005; Tabak *et. al.*, 2005; Wang & Chen, 2006).

Organisms	Genus/species	Reference
Bacteria	*Arthrobacter*	Roanne & Pepper, 2001
	Bacillus sp	Gupta *et. al.*, 2000; Dias *et. al.*, 2002 ; Kim *et. al.*, 2007
	Citrobacter	Renninger *et. al.*, 2001
	Cupriavidus metallidurans	Roanne & Pepper, 2001 ; Grass *et. al.*, 2005
	Cyanobacteria	Gupta *et. al.*, 2000
	Enterobacter cloacae	Hernandes *et. al.*, 1998; Gupta *et. al.*, 2000
	Pseudomonas aeruginosa	Dias *et. al.*, 2002; Zhang *et. al.*, 2005
	Streptomyces sp	Dias *et. al.*, 2002
	Zoogloea ramigera	Gupta *et. al.*, 2000
Archea	Filo *Crenarchaeota*	Sandaa *et. al.*, 1999
	Phanerochaete chrysosporium	Wu & Yu, 2007
Fungi	*Aspergillus tereus*	Kumar *et. al.*, 2008
	Penicillium chrysogenum	Dias *et. al.*, 2002
Yeast	*Candida utilis*	Kujan *et. al.*, 2006
	Hansenula anomala	Breierová *et. al.*, 2002
	Rhodotorula mucilaginosa	Dias *et. al.*, 2002
	Rhodotorula rubra GVa5	Ghosh *et. al.*, 2006
	Saccharomyces cerevisiae	Gupta *et. al.*, 2000; Dias *et. al.*, 2002; Ghosh *et. al.*, 2006

Table 1. Examples of microorganisms studied and strategically treated for bioremediation of heavy metals.

The search for new technologies for the removal of toxic metals from contaminated sites has focused on biosorption, which is based on the metal binding capacities of various biological materials. Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physicochemical uptake pathways (Fourest & Roux, 1992). Algae, bacteria, fungi and yeasts have all proven to be potential metal biosorbents (Volesky, 1987). Many indigenous organisms isolated from sites contaminated with heavy metals have tolerance to heavy metal toxicity and these microbial activities have always been the natural starting point for all biotechnological applications. It is therefore necessary to isolate bacterial strains with novel metabolic capabilities and to establish degradation pathways both biochemically and genetically. Potent metal biosorbents in the bacteria class include the genus *Bacillus* (Nakajima & Tsuruta, 2004; Tunali *et. al.*, 2006), *Pseudomonas* (Chang *et. al.*, 1997; Uslu & Tanyol, 2006) and *Streptomyces* (Mameri *et. al.*, 1999; Selatnia *et. al.*, 2004a). The biosorption process involves a solid phase

(sorbent or biosorbent; biological material) and a liquid phase (solvent, normally water) containing a dissolved species to be sorbed (sorbate, metal ions). Due to higher affinity of the sorbent for the sorbate species, the latter is attracted and bound there by different mechanisms. The process continues until equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbent affinity to the sorbate determines its distribution between solid and liquid phases.

Biosorbent material: Strong biosorbent behaviour of certain microorganisms towards metallic ions is a function of the chemical make-up of the microbial cells. This type of biosorbent consists of dead and metabolically inactive cells. Some types of biosorbents probably have a broad range, binding and collecting the majority of heavy metals with no specific preference, while others are specific to certain metals. Some laboratories have used easily available biomass whereas others have isolated specific strains of microorganisms and some have also processed existing raw biomass to a certain degree to improve its biosorption properties. Recent biosorption experiments have focused attention on waste materials, which are by-products or residues from large-scale industrial operations. E.g., mycelia waste available from fermentation processes, solid waste from olive oil manufacturing plants (Pagnanelli *et. al.,* 2002), activated sludge from sewage treatment plants (Hammaini *et. al.,* 2003), biosolids (Norton *et. al.,* 2004) and aquatic macrophytes (Keskinkan *et. al.,* 2003), etc. The biosorption mechanism is complex, including mainly ion exchange, chelation, adsorption by physical forces, entrapment in intra and interfibrilliar capillaries and spaces of the structural polysaccharide network as a result of the concentration gradient and diffusion through cell walls and membranes. There are several chemical groups that are thought to attract and sequester metals in biomass: acetamido groups of chitin, structural polysaccharides of fungi, amino and phosphate groups in nucleic acids, amido, amino, sulphydryl and carboxyl groups in proteins, hydroxyls in polysaccharide and mainly carboxyls and sulphates in polysaccharides of marine algae that belong to the divisions Phaeophyta, Rhodophyta and Chlorophyta. However, this does not necessarily mean that the presence of a particular functional group guarantees biosorption, perhaps due to steric, conformational or other barriers. The choice of metal for biosorption process: The appropriate selection of metals for biosorption studies is dependent on the angle of interest and the impact of different metals. Accordingly, they can be divided into four major categories: (i) toxic heavy metals (ii) strategic metals (iii) precious metals and (iv) radionuclides. In terms of environmental threats, categories (i) and (iv) are the ones of most concern with regards to their removal from the environment and/or from point source effluent discharges. Apart from toxicological criteria, the interest in specific metals may also be based on how representative their behavior is in terms of an eventual generalization of results regarding their biosorbent uptake. The toxicity and interesting solution chemistry of elements such as chromium, arsenic and selenium make them good candidates for study. Although not environmentally threatening, strategic and precious metals are important to consider from the standpoint of their recovery value. Research activities related to biosorption were initiated in the 1980s (Volesky & Holan, 1995; Volesky, 2001). Previously, research on this topic involved the use of biorremediation involving microorganisms alone to degrade organic compounds (Lovley & Coates, 1997). Since then, there has been an intensive effort to investigate the binding properties of heavy metals to different types of biomass (Chen & Wang, 2008). In general, biosorption is physicochemical interactions of metal ions with specific groups of the cell surface of microorganisms, which may enhance or inhibit intracellular transport or influence the processes of transformation and extracellular

precipitation (biomineralization) (Davis *et. al.*, 2003; Gavrilescu, 2004). Biosorption is based on passive (metabolism-independent) or active (metabolism-dependent) accumulation processes (Wang & Chen, 2006), in combinations that differ qualitatively and quantitatively, depending on the type of biomass, its origin, feasibility, and type of processing (Veglio & Beolchini, 1997). A classification of the mechanisms of metal accumulation can be made based on the dependence of cellular metabolism or according to the location of the metal within the cell.

2.2 Biosorption mechanisms
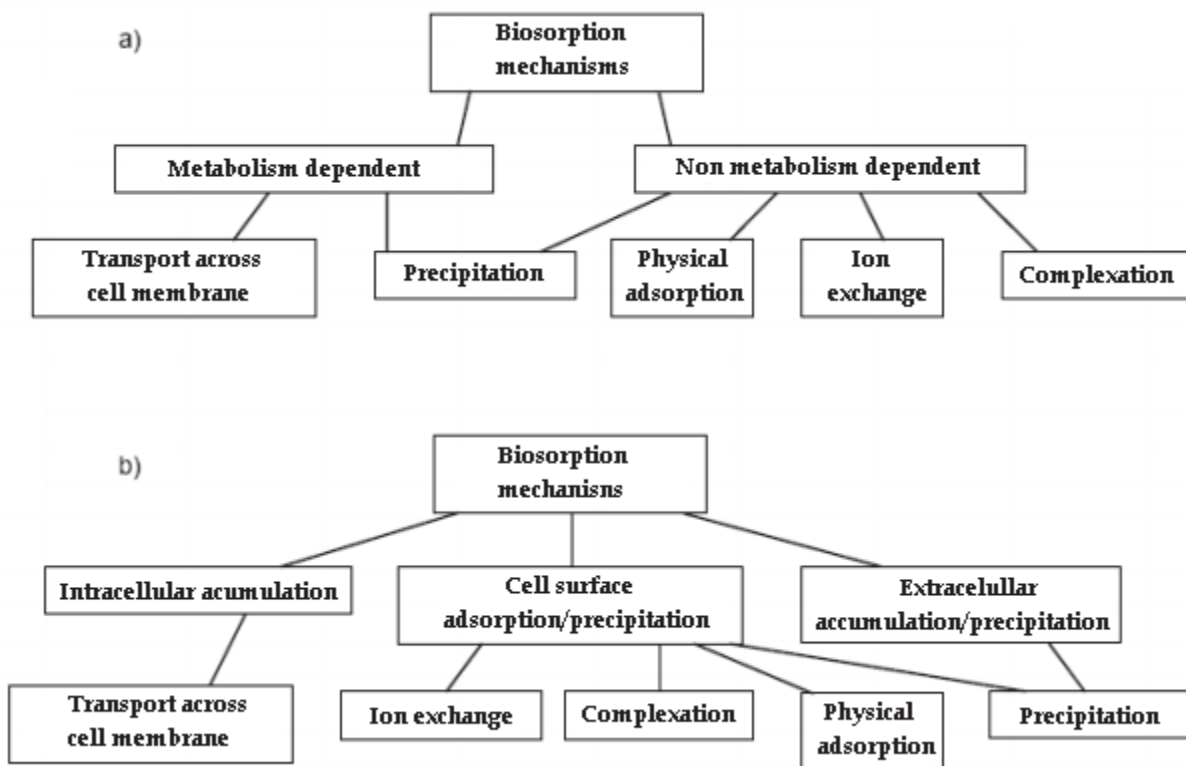
Fig. 2. Mechanisms of biosorption, a) classification according to dependence on cell metabolism, b) classification according to the location within the cell and the metal removed (Veglio & Beolchini, 1997).

The complex structure of microorganisms implies that there are many ways for the metal to be taken up by the microbial cell. There are various biosorption mechanisms and they are not yet fully understood. They may be classified according to various criteria (Figure 2).

The transportation of metal across the cell membrane yields intracellular accumulation, which is dependent on the metabolism of the cell. This means that this kind of biosorption may only take place within viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of a toxic metal. During non-metabolism dependent biosorption, metal uptake occurs by means of physicochemical interaction between the metal and the functional groups present on the microbial cell surface. This is based on physical adsorption, ion exchange and chemical sorption, which is not dependent on cell metabolism. Cell walls of microbial biomass, which consists mostly of polysaccharides, proteins and lipids, have abundant metal binding groups such as carboxyl,

sulphate, phosphate and amino groups. This type of biosorption, i.e., non-metabolism dependent, is relatively rapid and can be reversible (Kuyucak & Volesky, 1988).

In the case of precipitation, metal uptake may take place both in the solution and on the cell surface (Ercole *et. al.,* 1994). Furthermore, it may be dependent on cell metabolism if, in the presence of toxic metals, the microorganism produces compounds that favor the precipitation process. Precipitation may not be dependent on cell metabolism, if it occurs after a chemical interaction between the metal and the cell surface.

Due to the complexity of biomaterial, it is possible for several of these mechanisms to occur simultaneously, in degrees that depend on the biosorbent and environmental conditions (Kefala *et. al.,* 1999; Volesky, 2001; Gavrilescu, 2004). Studies on biosorption by Wang & Chen (2006) have focused on selecting the most promising types of biomass and its future success by sorption will depend on interaction between three knowledge areas: biology, chemistry and engineering (Kefala *et. al.,* 1999). Biosorption capacity is affected mainly by three factors: 1) characteristics of the metal ion (atomic weight, ionic ray, valence), 2) environmental conditions (pH, temperature, ionic strength, contact time, biomass concentration), and 3) the nature of the biosorbent, which may determine differences in selectivity and affinity to metal ions, since the type and species of microorganisms, conditions of growth, physiological state and cell age may all affect the binding mechanism to heavy metals (Wang & Chen, 2006; Chen & Wang, 2008).

Different strategies have been developed using recombinant DNA technology to produce genetically improved strains for use in the biosorption process, and many of these strategies "equip" the bacterial cell wall with metal ion-binding polypeptides to act as anchors. One of these studies was on the fusion protein: the β-domain of IgA protease of N. *gonorrhoeae* with metallothionein (MT) from rats (Valls *et. al.,* 2000), and lpp-ompA-various sizes of peptides (EC20) (Bae *et. al.,* 2000). The following items are the main biosorption mechanisms, which can also be considered mechanisms of resistance or tolerance to heavy metals developed by microorganisms.

Transport across cell membranes: Heavy metal transportation across microbial cell membranes may be mediated by the same mechanism used to convey metabolically important ions such as potassium, magnesium and sodium. The metal transport systems may become confused by the presence of heavy metal ions of the same charge and ionic radius associated with essential ions. This kind of mechanism is not associated with metabolic activity. Basically, biosorption by living organisms comprises of two steps: first, an independent binding metabolism where metals are bound to the cell walls; and second, metabolism-dependent intracellular uptake, whereby metal ions are transported across the cell membrane (Costa *et. al.,* 1990; Gadd *et. al.,* 1988; Huang *et. al.,* 1990; Nourbaksh *et. al.,* 1994).

Physical adsorption: In this category, physical adsorption takes place with the help of van der Waals forces. Kuyucak & Volesky (1988), hypothesized that uranium, cadmium, zinc, copper and cobalt biosorption by dead biomasses of algae, fungi and yeasts takes place through electrostatic interactions between the metal ions in solutions and the cell walls of microbial cells. Electrostatic interactions have been proven to be responsible for copper biosorption by the *Zoogloea ramigera* bacterium and the *Chiarella vulgaris* alga (Aksu *et. al.,* 1992), and for chromium biosorption by the *Ganoderma lucidum* and *Aspergillus niger* fungi.

Ion Exchange: Cell walls of microorganisms contain polysaccharides and bivalent metal ions exchange with the counter ions of the polysaccharides. For example, the alginates of marine algae occur as salts of K^+, Na^+, Ca^{2+}, and Mg^{2+}. These ions can exchange with

counter ions such as CO^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+}, resulting in the biosorptive uptake of heavy metals (Kuyucak & Volesky, 1988). The biosorption of copper by *Ganoderma lucidium* (Muraleedharan & Venkobachr, 1990) and *Aspergillus niger* fungi was also taken up by the ion exchange mechanism.

Complexation: An extracellular complexation or coordination is the result of electrostatic attraction between a metallic ion chelating agent and a polymer that can be excreted by a microorganism that is viable or not. It can be caused by: biosurfactants, polysaccharides, proteins and nucleic acids. These chelating agents contain pairs of electrons that present electrostatic attraction and if they cling to the metallic ions, there is no electron transfer. The final structure has the electric charge of the sum of individual charges of the participants of the complex (Veglio & Beolchini, 1997; Davis *et. al.*, 2003). When this detoxification/ complexation system is overloaded, "oxidative stress" of the cell occurs (Gavrilescu, 2004).

Metal removal from the solution may also take place by complex formation on the cell surface after interaction between the metal and the active groups. Aksu *et. al.* (1992) hypothesized that biosorption of copper by C. *vulgaris* and Z. *ramigera* takes place through both adsorption and the formation of coordination bonds between metals and amino and carboxyl groups of cell wall polysaccharides. Complexation was found to be the only mechanism responsible for the accumulation of calcium, magnesium, cadmium, zinc, copper and mercury by *Pseudomonas syringae.* Microorganisms may also produce organic acids (e.g., citric, oxalic, gluonic, fumaric, lactic and malic acids), which may chelate toxic metals, thus resulting in the formation of metallo-organic molecules. These organic acids help in the solubilization of metal compounds and leaching from their surfaces. Metals may be biosorbed or complexed by carboxyl groups found in microbial polysaccharides and other polymers.

Precipitation: Precipitation may be either dependent on the cellular metabolism or independent of it. In the former case, metal removal from solutions is often associated with the active defense system of the microorganisms. They react in the presence of toxic metal-producing compounds, which favor the precipitation process. In the case of precipitation that is not dependent on cellular metabolism, it may be a consequence of the chemical interaction between the metal and the cell surface. The various biosorption mechanisms mentioned above can take place simultaneously.

Adsorption: Physical adsorption is a process where the metal ion in solutions binds onto polyelectrolytes present in microbial cell wall through electrostatic interactions, Van der Waals forces, covalent bonding, redox interaction and biomineralization to achieve electroneutrality. This process is independent of metabolism, and it is reversible and very promising, as it presents many advantages, especially for treating large volumes of wastewater with low concentrations of contaminants (Ahluwalia & Goyal, 2007; Kuroda & Ueda, 2010; Nishitani *et. al.*, 2010). In physical adsorption, the metal ions are attracted by the potential negative of the cell wall, and both are dependent on pH. The influence of pH on metal accumulation by yeasts, algae and bacteria are very similar; for example, in yeast, pH < 2 the accumulation of metals is practically zero, because in low pH the active sites of the cell wall are associated with protons, restricting the approach of metal cations, and thus resulting in a repulsive force. Therefore, as the pH increases, an increasing number of sites (acetamide chitin, structural polysaccharides of fungi, phosphate and amino groups of nucleic acids, amino and carboxyl groups of proteins and hydroxyl groups of polysaccharides) are replaced by negative charges, increasing the attraction of metallic cations and adsorption on the cell surface. Accordingly, the solubility of metallic ions

decreases and, consequently, their bioavailability is reduced, and precipitation occurs (Esposito *et. al.*, 2002; Chen & Wang, 2008; Nishitani *et. al.*, 2010; Kuroda & Ueda, 2011). Thus, a pH range of between 4 and 8 is generally accepted as "good" for the biosorption of heavy metals for almost all types of biomass (Borroka & Fein, 200; Wang & Chen, 2006; Machado *et. al.,* 2010). Also, has been studied the role of extracellular polymeric substances (EPS) excreted by bacteria in the removal of heavy metal ions by adsorption process (Gupta *et. al.*, 2004).

Siderophores: Like heavy metal chelating agents, some types of microorganisms have low molecular weight, and these are called siderophores. When microorganisms are grown in an iron deficient medium, they produce specific iron chelators, so-called siderophores, in the medium. They play an important role in the complexation of toxic metals and radionuclides, by increasing their solubility. Siderophores have low molecular weights, and have compounds of the catecholate, phenolate or hydroxamate as their binding groups. Many bacteria, such as *Actinomycetes Azotobacter* and those of the genus *Pseudomonas*, synthesize these substances to capture the iron ions they require for their metabolic activity, or for biosorption (Pattus & Abdallah, 2000; Gazsó, 2001).

Biosurfactants: Most surfactants used in bioremediation are produced industrially from petroleum, but they can also be synthesized by microorganisms. Biosurfactants are natural surfactants synthesized by plants: (saponins), microorganisms (glycolipids) and the bodies of organisms (bile salts), and they have several advantages over industrially-produced surfactants, such as lower toxicity to degrading microorganisms and less recalcitrance in the environment, greater diversity of chemical structures; performance over a broader range of conditions at different temperatures and pHs (Bognolo, 1999). Biosurfactants of microbial origin (mainly aerobic) are the result of the metabolic byproducts of bacteria, fungi and yeasts, which are released into the medium. The hydrophilic portion may consist of amino acids, peptides or saccharides, and the hydrophobic portion is usually formed by saturated or unsaturated fatty acids. Biosurfactants are able to form various structures, such as micelles, vesicles and spherical or irregular lamellar structures, among others (Figure 3) (Champion *et. al.*, 1995; Mulligan, 2005; Li & Li, 2011).
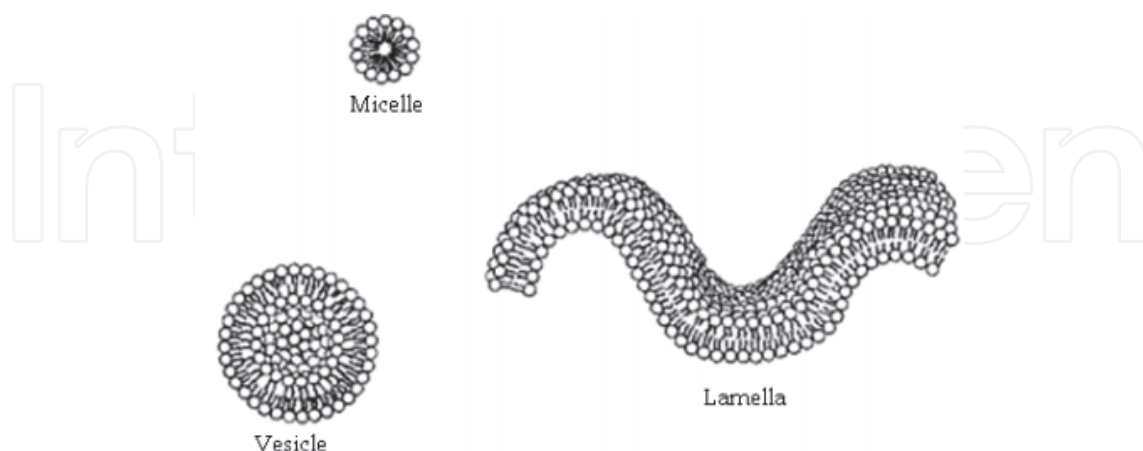

Fig. 3. Basic structures formed by biosurfactants (Champion *et. al.*, 1995).

Microorganisms naturally overcome their limitations when faced with insoluble organic and inorganic pollutants (hydrocarbons, oil, pesticides, heavy metals - uranium, cadmium, lead, etc.), performing the excretion of these structures to the culture medium, associated their cell

walls. This facilitates the transport and translocation of insoluble substrates, in turn facilitating biosorption. In the process of heavy metal ion bioremediation, a complex non-toxic biosurfactant/metallic ion structure is formed, resulting in emulsification and solubilization of the ions, and, consequently, their physical sequestration. One of the most widely used natural biosurfactants in bioremediation is rhamnolipids, produced by *Pseudomonas aeruginosa* (Bognolo, 1999; Champion *et. al.*, 1995; Mulligan, 2005; Tabak *et. al.*, 2005; Zhang *et. al.*, 2005).

Oxidation-reduction (redox): Microorganisms can mobilize or immobilize metal ions, metalloid and organometal compounds, thus promoting redox processes. Only prokaryotes are capable of oxidizing metals such as Mn^{2+}, Fe^{2+}, Co^{2+}, Cu, AsO_2^-, Se^0 or SeO_3^{2-}, or reducing Mn^{4+}, Fe^{3+}, Co^{3+}, AsO_4^{2-}, SeO_4^{2-} or SeO_3^2, whilst obtaining energy from these reactions (Gavrilescu, 2004). When, for example, the Fe^{3+} ion is reduced to Fe^{2+}, or the Mn^{4+} ion is reduced to Mn^{2+}, there is an increase in the solubility of these metals. Microbial species can efficiently immobilize heavy metals through their ability to reduce heavy metal ions, reducing them to a lower oxidation state, and giving rise to metallic elements (load zero) which are less bioactive (Valls & Lorenzo, 2002; Gadd, 2004).

Biomethylation: Microorganisms can transform metal ions from a more toxic to a less toxic form through the biomethylation mechanism. Hg, As, Cd, Se, Sn, Te and Pb ions can be methylated by a variety of bacteria, filamentous fungi and yeasts, under both aerobic and anaerobic conditions, which results in increased mobility, and also in their suitability for involvement in processes that result in a decrease in their toxicities. This enzymatic mechanism involves a transfer from the methyl group (CH_3) to metals and metalloids. The methylated compounds formed differ in their solubility, volatility and toxicity (Roane & Pepper, 2000; Gadd, 2004). For example, methyl and dimethyl mercury are more toxic than inorganic Hg ions, however, these are intermediate forms of processing for Hg^0. Inorganic forms of As are more toxic than methylated species (acids and methyl-As dimethyl-As), the methylated and inorganic forms of Se and Cd are highly toxic (Roane & Pepper, 2000; Tabak, 2005).

Metal-binding cysteine-rich peptides: When cells are exposed to heavy metals in toxic concentrations an induction of expression of peptides rich in cysteine residues, metallothioneins (MTs), glutathione (GSH) or phytochelatin (PCs) occurs. These are non-enzymatic molecules, with low molecular weight, which are resistant to thermo-coagulation and acid precipitation. The main feature of these peptides is to form complexes with divalent metals and metal-thiols, which consist of important metabolites to combat ROS (Bae *et. al.*, 2000; 2001).

Metallothioneins (MTs): Metallothioneins (MTs) are a group of well-preserved structures of proteins that act as antioxidants, and they are distributed among all living organisms. They have low molecular weight and they are rich in cysteine residues. The presence of thiol groups (SH) in the cysteine chemical structure enables the capture of metal ions such as Cd^{2+}, Fe^{2+}, Hg^{2+}, Cu^{2+} and Zn^{2+}. MTs are composed of two separate domains, the β domain in the N-terminal region, and the α domain in the C-terminal region. The β domain possesses nine cysteine residues linking three divalent ions, the α domain already has eleven cysteine residues and binds four ions, thus a total of seven ions per molecule are bound (Cobbett & Goldsbrough, 2002; Thirumoorthy *et. al.*, 2007). The MTs have several functions, including the detoxification of heavy metals and protection against the presence of ROS. Thus, MTs are responsible for reducing the effect of oxidative stress caused by these ions, but they are also responsible for maintaining homeostatic cellular redox balance.

According to these characteristics, protein synthesis is only induced by metals. (Cobbett & Goldsbrough, 2002; Smith *et. al.*, 2007).

Glutathione (GSH): Glutathione (GSH), L-Glutamyl-L-cysteinyl-glycine (Figure 4), is a soluble antioxidant, recognized as the most important non-protein thiol present in all living organisms. It consists of three amino acids (Glu-Cys-Gly), and it is the cysteine thiol group of the active site that is responsible for its biochemical properties (Bae & Mehra, 1997; Penninckx, 2000; 2002; Mendonza-cózatl *et. al.*, 2005).
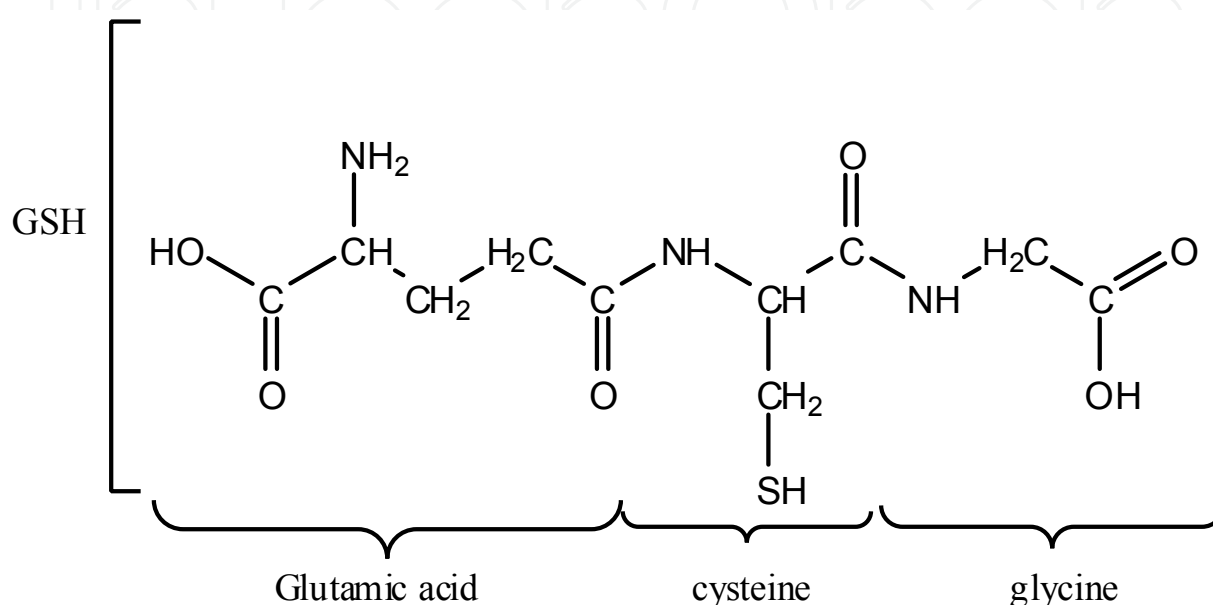
Fig. 4. Chemical structure of glutathione (GSH) (Bae & Mehra, 1997).

The GSH controls its own synthesis and participates in multiple processes: regulation of the intracellular redox state, inactivation of ROS, transport of GSH-conjugated amino acid and other molecules, stock of the sulfur and cysteine. In mammals, it is present in greater quantities in the liver. Its biosynthesis is similar in plants, yeast and protists. The mitochondria and the nucleus have their own GSH reservation, which is critical or instrumental in protecting these structures against ROS action (Penninckx, 2002; Inouhe, 2005; Mendoza -Cózatl *et. al.*, 2005). In yeast, the cell protection system against the toxicity of Cd^{2+} occurs through the formation of a GSH- Cd^{2+} complex, because this causes a decrease in the levels of lipid peroxidation of the cell membrane and allows for the transportation of GSH-Cd^{2+} conjugate to the vacuole. This results in a decreased concentration of toxic metals in the cytosol, thus promoting a reduction in the levels of oxidative stress (Penninckx, 2000; Adamis *et. al.*, 2004; Kobayashi *et. al.*, 2005; Preveral *et. al.*, 2006).

Natural phytochelatins (PCs) and synthetic phytochelatin (EC20): PCs are small cysteine-rich peptides with the general structure (Glu-Cys) nGly (n = 2-11) (Figure 5-A) (Grill et. al., 1985; Cobbett, 2000). PCs are synthesized from glutathione (GSH) in steps catalyzed by PC synthase (Grill et. al., 1985; Gupta et. al., 2004). They enable ions to bind to various heavy metal ions through thiol residues and carboxyl (Kobayashi et. al., 2005; Inouhe, 2005). These PCs are present in plants, fungi, nematodes, parasites and algae, including cyanobacteria. Despite being classified as MT-III, the PCs have a greater capacity for binding heavy metal ions (1 atom per cysteine basis) than MTs. The pioneering work that targeted the expression of recombinant PCs in *E.coli* were faced with the difficulty imposed by the chemical bonds

of type γ present between Glu-Cys units, which are the result of multi-enzyme processes. These bindings are different for type α among the present amino acid chains of all proteins (Bae *et. al.*, 2001; Cobbett, 2000; Penninckx, 2000; Gupta *et. al.*, 2004; Inouhe, 2005; Hirata, 2005; Mendoza-Cózatl, 2005; Wu *et. al.*, 2006). An alternative was to synthesize an *in vitro* gene that codes for proteins similar to PCs, whose general structure corresponded to (Glu-Cys) nGly (ECs) with all amino acids linked chemically by type α (Figure 5-B). Thus, the synthetic phytochelatin EC20 contains 20 units of repeated Glu-Cys (EC). The synthetic phytochelatin EC20 has greater capacity for binding to heavy metal ions than natural PCs. Recombinant strains of bacteria are currently being constructed (Bae *et. al.*, 2000; 2001; Xu *et. al.*, 2002; Lee *et. al.*, 2006; Wu *et. al.*, 2006), and though few studies have been developed for yeast, we can cite (Schmitt *et. al.*, 2006) expressing EC20 with the aim of identifying microorganisms with increased capacity for binding heavy metal ions for use in bioremediation processes.
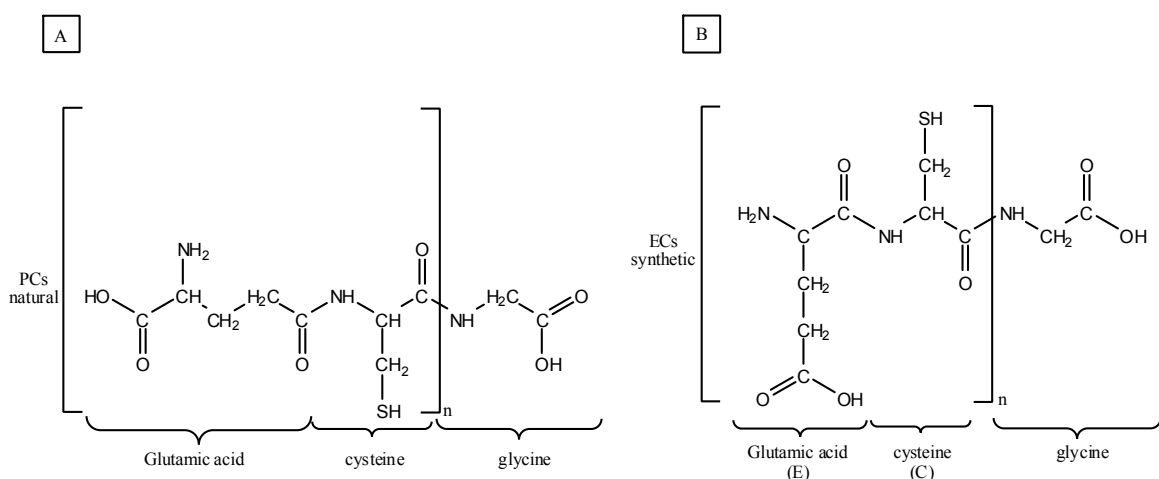

Fig. 5. Chemical structures of molecules binding heavy metals: A) natural phytochelatin (PC) and B) synthetic phytochelatin (EC) - glutamic acid (glu-E) and cysteine (Cys-C) (Bae *et. al.*, 2000).

Among these genetic engineering techniques for the construction of new recombinant microbial strains (Deng *et. al.*, 2003; Merle *et. al.*, 2003; Kim *et. al.*, 2005; Nishitani *et al.*, 2010), MTS, PCs and ECs were also explored so that these proteins remain anchored to the outer surface cell ("cell-surface display"). The aim was to increase the capacity these special microorganisms in adsorption processes involving heavy metal ions in comparison with non-recombinant microorganisms (Bae *et. al.*, 2000; 2001; Kuroda *et. al.*, 2002; Kuroda & Ueda, 2003; Jiang *et. al.*, 2007).

The "cell-surface display" system: Cell surface proteins constitute an important class of biomolecules because they are situated at the interface between the cell and the environment. The cells have systems for anchoring specific proteins to the surface and confining them to certain areas. A great many systems are being used in bacteria and in *S. cerevisiae* (Kondo & Ueda, 2004). The expression of heterologous peptides on the cell surface ("cell-surface display ") is a powerful technique widely used in the biotechnology area in the following processes: production of recombinant vaccines, antigens, antibodies, enzymes and library peptides (Kuroda *et. al.*, 2001; Chen & Georgiou, 2002; Samuelson *et. al.*, 2002; Rutherford & Moures, 2006; Wang *et. al.*, 2007; Kuroda & Ueda, 2010; Nishitani *et. al.*, 2010; Kuroda & Ueda, 2011).

2.3 Factors affecting biosorption

The investigation of the efficacy of metal uptake by microbial biomass is essential for the industrial application of biosorption, as it yields information about the equilibrium of the process that is necessary to design the equipment to be employed.

Metal uptake is usually measured by the parameter 'q', which indicates the milligrams of metal accumulated per gram of biosorbent material, while 'qH' is reported as a function of the metal accumulated, the sorbent material used and the operating conditions.

The following factors affect the biosorption process:

1) temperature does not seem to influence biosorption performance in the 20-35 ^{0}C range (Aksu *et. al.*, 1992); 2) pH seems to be the most important parameter in the biosorption process: it affects the solution chemistry of the metals, the activity of the functional groups in the biomass and competition between metallic ions (Machado *et. al.*, 2010); 3) biomass concentration in solution seems to influence specific uptake: for lower values of biomass concentrations, there is an increase in the specific uptake (Fourest & Roux, 1992; Gadd *et. al.*, 1988). Gadd *et. al.* (1988) suggested that an increase in biomass concentration leads to interference among the binding sites. Fourest & Roux (1992) invalidated this hypothesis by attributing the responsibility of the decrease of specific uptake to a shortage of metal concentration in the solution. Hence this factor needs to be taken into consideration in any application of microbial biomass as a biosorbent; 4) biosorption is mainly used to treat wastewater where more than one type of metal ion are probably present; the removal of one metal ion may be influenced by the presence of other metal ions. For example: Uranium uptake by bacterium, fungus and yeast biomass was not affected by the presence of manganese, cobalt, copper, cadmium, mercury and lead in solution (Sakaguchi & Nakajima, 1991). In contrast, the presence of Fe^{2+} and Zn^{2+} was found to influence uranium uptake by *Rhizopus arrhizus* (Tsezos & Volesky, 1982) and cobalt uptake by different microorganisms seemed to be completely inhibited by the presence of uranium, lead, mercury and copper (Sakaguchi & Nakajima, 1991).

3. New developments in organisms capable of enhanced bioremediation

3.1 The use of recombinant bacteria for metal removal

The use of recombinant bacteria to remove specific metals from contaminated water is currently being investigated. For example a genetically engineered *E.coli,* which expresses the Hg^{2+} transport system and metallothionein (a metal binding protein), was able to selectively accumulate 8 µmole Hg^{2+}/g cell dry weight. The presence of the chelating agents Na^+, Mg^{2+} and Ca^{2+} did not affect bioaccumulation.

3.2 Genetically modified biosorbents

Genetic engineering has the potential to improve or redesign microorganisms, where biological metal-sequestering systems will have a higher intrinsic capability as well as specificity and greater resistance to environmental conditions (Bae *et. al.*, 2000; Majare & Bulow, 2001). It is well known that virgin biosorbents usually lack specificity in metal-binding, which may cause difficulties in the recovery and recycling of the desired metal(s). Genetic modification is a potential solution for enhancing selectivity as well as the accumulating potential of cells (Pazirandeh *et. al.*, 1995). Genetic modification would be feasible, especially when the microbial biomass is produced from fermentation processes where genetically engineered microorganisms are used. Currently, many kinds of amino

acids and nucleic acids are being produced on an industrial scale by using genetically engineered microbial cells. Higher organisms respond to the presence of metals, with the production of cysteine-rich peptides, such as glutathione (GSH) (Singhal *et. al.*, 1997), phytochelatins (PCs) and metallothioneins (MTs) (Mehra & Winge, 1991), which can bind and sequester metal ions in biologically inactive forms (Hamer, 1986; Bae *et. al.*, 2000). The overexpression of MTs in bacterial cells will result in enhanced metal accumulation, thus offering a promising strategy for the development of microbial-based biosorbents for the remediation of metal contamination (Pazirandeh *et. al.*, 1995). In addition to the high selectivity and accumulation capacity, Pazirandeh *et. al.* (1995) demonstrated that uptake by recombinant *E. coli* (expressing the *Neurospora crassa* metallothionein gene within the periplasmic space) was rapid. Greater than 75% Cd uptake occurred within the first 20 min, with maximum uptake achieved in less than 1 h. However, the expression of such cysteine-rich proteins is not devoid of problems, due to the predicted interference with redox pathways in cytosol. More importantly, the intracellular expression of MTs may prevent the recycling of biosorbents, as the accumulated metals cannot easily be released (Gadd & White, 1993). Chen & Georgiou (2002) suggested a solution to bypass this transport problem by expressing MTs on the cell surface. Sousa *et. al.* (1996) demonstrated the possibility of inserting MTs into permissive site 153 of the *LamB* sequence. The expression of the hybrid proteins on the cell surface dramatically increased the whole-cell accumulation of cadmium. Also, the expression of proteins on the surface offers an inexpensive alternative for the preparation of affinity adsorbents (Georgiou *et. al.*, 1993). The use of PCs in a similar manner to MTs has also been suggested (Bae *et. al.*, 2000). PCs are short, cysteine-rich peptides, with the general structure (γGlu-Cys)nGly (n=2–11) (Zenk, 1996). PCs offer many advantages over MTs, due to their unique structural characteristics, particularly the continuously-repeating γGlu-Cys units. Also, PCs have been found to exhibit higher metal-binding capacity (on a per cysteine basis) than MTs (Mehra & Mulchandani, 1995). However, the development of overexpressing PC organisms requires a thorough knowledge of the mechanisms involved in the synthesis and chain elongation of these peptides. Several biosorbents, displaying metal-binding peptides on the cell surface, have been successfully engineered. A typical example includes creating a repetitive metal-binding motif, consisting of (Glu-Cys)nGly (Bae *et. al.*, 2000). These peptides emulate the structure of PCs; however, they differ in the fact that the peptide bond between the glutamic acid and cysteine is a standard α peptide bond. Phytochelatin analogs were found to be present on the bacterial surface, which enhanced the accumulation of Cd^{2+} and Hg^{2+} by 12- (Bae *et. al.*, 2000) and 20-fold (Bae *et. al.*, 2001), respectively. Attempts to create recombinant bacteria with improved metal binding capacity have so far been restricted mostly to *E. coli*. This is because *E. coli* greatly facilitates genetic engineering experiments and it is found to have more surface area per unit of cell mass, which potentially should result in higher rates of metal removal from solutions (Chen & Wilson, 1997). Nevertheless, a Gram-positive surface display system also possesses its own merits, compared to Gram-negative bacteria (Malik *et. al.*, 1998; Samuelson *et. al.*, 2000): (a) translocation through only one membrane is required; and (b) Gram-positive bacteria have been shown to be more rigid and, therefore, less sensitive to shear forces (Kelemen & Sharpe, 1979) due to the thick cell wall surrounding the cells, which potentially make them more suitable for field applications, such as bioadsorption. Samuelson *et. al.* (2000) generated recombinant *Staphylococcus xylosus* and *Staphylococcus carnosus* strains, with surface-exposed chimeric proteins containing polyhistidyl peptides.

Both strains of Staphylococcaceae gained improved nickel-binding capacities due to the introduction of the H1 or H2 peptide into their surface proteins. Owing to their high rate of selectivity, genetically engineered biosorbents may prove very competitive in the separation of toxins and other pollutants from diluted contaminated solutions.

3.3 Survivability and stability of GMOs

Although the utilization of GMOs in the field has been limited due to possible risks involved in the horizontal transfer of genetic material, the results that have been obtained are nevertheless important in assessing the benefits and obstacles associated with their applications in bioremediation. Such knowledge is necessary in view of the future possibility of releasing GEMs into contained environments for bioremediation. To be of practical use in the field, a bacterial GMO must be able to survive and grow in such environments. Important parameters in this regard are growth rate, inoculum size, environmental conditions, including spatial distribution, and the presence of competing microorganisms. The spatial distribution of a GMO introduced into the environment is important because it helps define its interactions with the members of the indigenous bacterial community and other components of the ecosystem (Dechesne *et. al.*, 2005). In general, a bacterium that has been recently isolated from a natural environment is more likely to survive when released back into that same environment. A crucial consideration regarding the introduction of engineered bacteria into field sites is their effect on the structure and function of natural ecosystems.

3.4 Natural horizontal transference of DNA in bacteria

It is perhaps most useful to consider horizontal transfer of recombinant DNA in the overall context of horizontal gene transfer among bacteria, which is a natural and presumably widespread phenomenon. The role of horizontal gene transfer in bacterial evolution has been demonstrated in many studies (Rensing *et. al.*, 2002; Dennis, 2005). It has been suggested that it is an important process contributing to the development of novel biodegradation capacities of microbial communities when they are exposed to organic pollutants (Rittmann *et. al.*, 1990; Dennis, 2005). The transfer of genes encoding biodegradation functions appears to occur through the action of conjugative plasmids, transposable elements, and "integrative and conjugative transposons" (also known as "genomic islands") (Springael & Top, 2004; Top *et. al.*, 2002; Van Der Meer & Sentchilo, 2003). There is evidence to suggest that the genes in at least some of these elements were assembled in stepwise processes (Springael & Top, 2004). That such horizontal transfer is apparently quite common is suggested by the variety of specific examples. Direct measurement of horizontal transfer has been carried out under both well-defined conditions and in microcosms, the latter serving as models for *in situ* situations. An interesting example of horizontal gene transfer in a completely natural environment is provided by the *pheBA* operon, which originated from the strain EST1001 of *Pseudomonas* sp. It encodes two enzymes involved in phenol catabolism and, like several examples described above, it is carried on a conjugative plasmid with transposable element characteristics. It was transferred by conjugation to *P. putida* PaW85 and this strain has already been released into the field for the large-scale bioremediation of river water contaminated with phenolics, originating from a fire in an oil shale mine (Peters *et. al.*, 1997). Six years later, despite the absence of the PaW85 strain, the operon was once again detected in the watershed,

apparently having been transferred to nine *Pseudomonas* strains belonging to four different species (*P. corrugata*, *P. fragi*, *P. stutzeri* and *P. fluorescens* biotypes B, C and F). In eight cases the operon was plasmid-borne, and in one case it had integrated into the host chromosome. The environment in which these bacteria were found was subject to continual pollution with phenolics. This presumably provided positive selection for their perseverance, which appears to have had continual beneficial effects regarding bioremediation in this location. The examples discussed above can be viewed as evidence that horizontal gene transfer of recombinant genes could occur rapidly by the same mechanisms. On the other hand, it may be that any predicted horizontal transfer of DNA from GMOs may occur naturally with the same genes in non-recombinant organisms at rates which make any contribution from GMOs insignificant. As discussed above, there is concern that GMOs introduced into polluted sites to enhance bioremediation may have adverse environmental effects because of horizontal transfer of recombinant DNA (Davison, 1999). In many cases, released GMOs do not survive long, and disappear before they have any effect on biodegradation (Davison, 1999). In other cases, however, transfer of plasmid from an introduced GMO to an indigenous microorganism may occur even though the introduced strain does not survive (Davison, 1999; Peters *et. al.*, 1997). Thus, in general it appears that the potential impact of introduction of GMOs on native microbial populations is not uniform and therefore must be evaluated on a case-by-case basis.

3.5 Containment strategies to diminish horizontal transfer of DNA from GMOs

As mentioned above, one important concern with the use of GMOs bearing recombinant genes on plasmids arises from the instability of the plasmids, which can lead to loss of the desired phenotype. On the other hand, the relative chemical and physical stability of plasmids can contribute to their spreading, along with both the recombinant genes and selective markers (e.g., antibiotic resistance), to other bacteria (Pieper & Reineke, 2000). One solution is to replace antibiotic resistance selective markers with selective markers that do not center on antibiotic resistance (Sanchez-Romero *et. al.*, 1998; Herrero *et. al.*, 1990). One way to obviate the problem of plasmid transfer is to use "mini transposons" for the stable integration of genes into the chromosome of recipient strains (De Lorenzo *et. al.*, 1998; Herrero *et. al.*, 1990). If the chosen GMO recipient proves not to be able to support plasmid maintenance, the transformant bearing gene(s) of interest must undergo transposition of the cloned gene into the host chromosome, where it will remain integrated in a stable manner. Some mini-transposon vectors have also been engineered for use on heavy metals or herbicides, rather than antibiotic resistance, as a selectable marker, to eliminate the problem of horizontal transfer of this characteristic altogether (Herrero *et. al.*, 1990). Other "biological containment strategies" that can minimize this problem are suicide systems, where the GMO dies after it has completed its required task (De Lorenzo *et. al.*, 1998; Molin *et. al.*, 1993; Molina *et. al.*, 1998; Pandey *et. al.*, 2005; Paul *et. al.*, 2005; Ronchel *et. al.*, 1998; Ronchel & Ramos, 2000). Such suicide mechanisms are based on the controlled expression in the gene host that encodes proteins that are lethal to it. A common system induces the "suicide gene(s)" when the pollutant that the host degrades is absent. Expression of these genes, which cause holes to form in the cell membrane, leads to cell death.

Composting is another containment strategy for using GMOs in field applications; this process has recently been reviewed by Singh *et. al.* (2006). The conditions during the composting process include elevated temperatures (as high as 80–90◦C), decreases in pH due

to organic acid production, and the production of toxic metabolites that can greatly decrease microbial populations. The subsequent lysis of dead microbes releases their DNA into the environment, where it is subject to degradation. These effects presumably minimize horizontal gene transfer from the GMOs, suggesting that composting could be a safe solution for their disposal after they have completed their required functions.

4. Conclusion

The use of genetic engineering to produce microorganisms capable of degrading specific contaminants or to enhance such processes in native organisms with such capabilities has become a popular way of increasing the efficiency of bioremediation in laboratory studies. Techniques used can include engineering with single genes, pathway construction, and alteration of the sequences of existing genes (both coding and controlling sequences). But, before releasing a GMO into the environment, the researchers should emphasize the ethical responsibilities to be considered before using such novel strategies for bioremediation. In this context, several points should be taken into account. The stability of GMOs and their horizontal transfer of engineered DNA are crucial issues regarding the potential impact of their release into the field for bioremediation. In order to determine how released GMOs are affecting the environment, it is necessary to be able to detect and enumerate them in complex samples. Important parameters in this context are survival, number, activity, and dispersion of released GMOs (Widada *et. al.*, 2002). Ideally such methods should be applicable in the field and in real time, and should be simple and inexpensive while also being accurate (Wu *et. al.*, 2001). In addition to the GMO itself, it is useful to track the recombinant DNA with which the GMO has been engineered so as to monitor potential loss of these genes and their possible horizontal transfer to other microorganisms.

In conclusion, a number of important molecular tools have been developed for genetic and metabolic engineering of microorganisms for the degradation of environmental contaminants. These new tools will make the construction of new or improved strains much easier and quicker than in the past. However, these genetic modifications should beunderstood in full and any research must always determine the actual risks and benefits involved.

5. Acknowledgments

6. References

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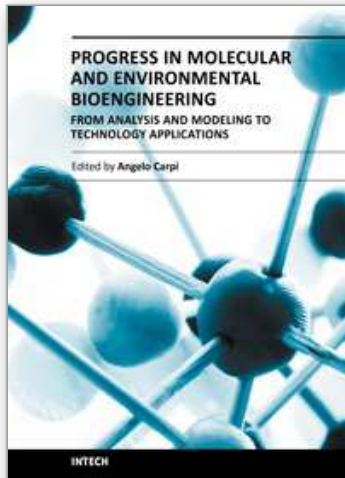
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