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Advances in Bioremediation of Wastewater and Polluted Soil

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ADVANCES IN BIOREMEDIATION OF WASTEWATER AND POLLUTED SOIL

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Notice

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Meet the editor

Dr. Naofumi Shiomi studied recombinant yeast and its utilization as a researcher at the Laboratory of Production Technology of Kanena Corporation for 15 years until 1998 and earned his PhD in Engineering from Kyoto University. He now works as a professor at the School of Human Sciences of Kobe College in Japan, where he teaches applied microbiology, biotechnology and life science in his “Applied Life Science” laboratory. He has studied bioremediation for 17 years at Kobe College and has published more than 40 papers and several book chapters on recombinant microorganisms and bioremediation. His recent research has also focused on the prevention of obesity and aging.

Contents

Preface

Consumable water comprises only 0.01% of the total water on earth and has been carefully used with great consideration of its limitedness. However, rapid population increases and the development of economies have led to excessive use of aquatic resources and pollution of vast amounts of river water and groundwater. For instance, acid rain, which is produced by sulfur and nitrogen oxides exhausted to the air from factories, has damaged the aquatic resources and ecosystems in ponds and forests. Domestic and industrial wastewaters have also polluted soils and groundwater in many countries because they are directly drained into the rivers without previous treatment to remove the harmful compounds. Moreover, the excessive use of fertilizers and herbicides has resulted in serious pollution of soil and groundwater. In China, for example, it has been estimated that 40% of the river water (95% in urban areas) is unsafe for consumption due to pollution.

Pollution by heavy metals as well as chemical compounds is also severe. Industrial and domestic wastes containing heavy metals are filled up or discarded without the removal of heavy metals. In the EU, for example, wastes of electrical and electric equipment (WEEE), such as television sets and cell phones, are expected to increase to more than 12 million tons by 2020, and most of them are filled without previous treatment. Strict Restriction of Hazardous Substance and 94/62/EC directives to decrease the emission in the EU have led to the export of WEEE to countries in Asia, thereby leading to pollution in those areas. Heavy metals contained in batteries, such as lithium, nickel-cadmium and lead batteries, also cause pollution. Industrial wastewater containing heavy metals, which is produced by metallurgical industries to produce batteries, leads to the pollution of rivers and soils.

Recently, the lack of aquatic resources has reached a critical condition. Many individuals living in polluted areas have developed symptoms of heavy metals poisoning. Furthermore, food shortages will most likely occur in the near future due to shortages in agricultural water, as well as drinking water. It is reported that the current drinking water shortage affects 700 million people worldwide, and 1.8 million children die annually due to water shortage or pollution. Therefore, for the future of human beings and other creatures, strict regulations on the emissions of harmful compounds and remediation of the soil and groundwater must be immediately implemented.

Among the various processes utilized for the remediation of wastewater and soil, bioremediation using microorganisms and plants has been remarkable due to its high safety and inexpensive running cost compared to physical and chemical methods. The USA invested 10 billion dollars in phytoremediation in the 1990s, and the market of phytoremediation is predicted to increase to over several trillion dollars in the EU. However, current bioremediation processes do not often achieve sufficient remediation, and more effective processes are de-

sired because of the insufficient performance or insufficient adaptability to various conditions.

This book discusses the recent advances in bioremediation of wastewater and polluted soil. In the first chapters of this book, respected researchers in this field describe how the optimization of microorganisms, enzymes, absorbents, additives and injection procedures can help to realize excellent bioremediation. The general concept of bioremediation of polluted water is reviewed in chapter 1. In chapters 2–4, the utilization of Haloarchaea, Synechocystis and other microorganisms identified using metagenomics are introduced as effective reaction agents for bioremediation. Because bioremediation often occurs in specific environments, the selection of microorganisms which can actively work in said environments is a key factor for optimal bioremediation. The contents of these chapters will assist the selection of microorganisms for bioremediation. Moreover, in chapters 5–7, the authors discuss optimal biosurfactants for adsorption, enzymes for the degradation of dyes and the effects of additives for bioremediation, respectively. The proper selection of absorbents, enzymes and additives is another key factor for optimal bioremediation. In the latter chapters, other respected researchers in this field introduce bioremediation processes that have been performed in the field. The eliminations of hexavalent chromium (VI) and organochlorine pesticides by fungi are introduced as concrete examples in chapters 8 and 9. Moreover, in chapters 10 and 11, phytoremediation of landfill soil and bioremediation of lead are discussed, including novel methods for bioremediation.

This book will be useful for those studying or developing new bioremediation processes and students studying environmental science and engineering. It will provide important information about recent advances in bioremediation and novel ideas for effective bioremediation. Finally, I would like to thank Ms. Ana Pantar, Ms. Ivona Lovric and the publishing process managers of the InTech Publishing Group for their great support and assistance throughout the writing and publication process of this book.

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Chapter 1

Bioremediation of Polluted Waters Using Microorganisms

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Additional information is available at the end of the chapter

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Abstract

Water pollution is an issue of great concern worldwide, and it can be broadly divided into three main categories, that is, contamination by organic compounds, inorganic compounds (e.g., heavy metals), and microorganisms. In recent years, the number of research studies concerning the use of efficient processes to clean up and minimize the pollution of water bodies has been increasing. In this context, the use of bioremediation processes for the removal of toxic metals from aqueous solutions is gaining considerable attention. Bioremediation can be defined as the ability of certain biomolecules or types of biomass to bind and concentrate selected ions or other molecules present in aqueous solutions. Bioremediation using microorganisms shows great potential for future development due to its environmental compatibility and possible cost-effectiveness. A wide range of microorganisms, including bacteria, fungi, yeasts, and algae, can act as biologically active methylators, which are able to at least modify toxic species. Many microbial detoxification processes involve the efflux or exclusion of metal ions from the cell, which in some cases can result in high local concentrations of metals at the cell surface, where they can react with biogenic ligands and precipitate. Although microorganisms cannot destroy metals, they can alter their chemical properties via a surprising array of mechanisms. The main purpose of this chapter is to provide an update on the recent literature concerning the strategies available for the remediation of metal-contaminated water bodies using microorganisms and to critically discuss their main advantages and weaknesses. The focus is on the heavy metals associated with environmental contamination, for

instance, lead (Pb), cadmium (Cd), and chromium (Cr), which are potentially hazardous to ecosystems. The types of microorganisms that are used in bioremediation processes due to their natural capacity to biosorb toxic heavy metal ions are discussed in detail. This chapter summarizes existing knowledge on various aspects of the fundamentals and applications of bioremediation and critically reviews the obstacles to its commercial success and future perspectives.

Keywords: Metals, microorganisms, bioremediation, polluted water

1. Introduction

Environmental contamination by heavy metals from anthropogenic and industrial activities has caused considerable irreparable damage to aquatic ecosystems. Sources include the mining and smelting of ores, effluent from storage batteries and automobile exhaust, and the manufacturing and inadequate use of fertilizers, pesticides, and many others. The metals and metalloids that contaminate waters and are most commonly found in the environment include lead, chromium, mercury, uranium, selenium, zinc, arsenic, cadmium, silver, gold, and nickel. These metals are the subject of concern due to their high toxicity. Apart from being hazardous to human health, they also have an adverse effect on the fauna and flora, and they are not biodegradable in nature. Thus, there is a need to seek new approaches in developing treatments to minimize or even eliminate metals present in the environment.

Several different physicochemical and biological processes are commonly employed to remove heavy metals from industrial wastewaters before their discharge into the environment [1]. Conventional physicochemical methods such as electrochemical treatment, ion exchange, precipitation, osmosis, evaporation, and sorption are not cost-effective, and some of them are not environmentally friendly [2, 3]. On the other hand, bioremediation processes show promising results for the removal of metals, even when present in very low concentrations where physicochemical removal methods fail to operate. Furthermore, this is an eco-compatible and economically feasible option. The bioremediation strategy is based on the high metal binding capacity of biological agents, which can remove heavy metals from contaminated sites with high efficiency. In this regard, microorganisms can be considered as a biological tool for metal removal because they can be used to concentrate, remove, and recover heavy metals from contaminated aquatic environments [4]. Several studies have been conducted using microorganisms for the uptake of heavy metals in polluted waters as an alternative strategy to conventional treatments [5–7]. Bioremediation by microorganisms is very useful due to the action of microorganisms on pollutants even when they are present in very dilute solutions, and they can also adapt to extreme conditions. Although the mechanisms associated with metal biosorption by microorganisms are still not well understood, studies show that they play an important role in the uptake of metals and that this action involves accumulation or resistance.

In this chapter, previously published research data on the potential of the microorganisms that have been used for the bioremediation of metals is discussed. In-depth descriptive information

on the bioremediation process uses various microorganisms, including algae and bacteria, and the mechanisms of action, bioremediation efficiency, and current applications are provided together with suggestions to overcome the limitations associated with their large-scale and more efficient application. Future prospects for the potential use of genetic engineering techniques to develop prominent recombinant novel microorganism variants that are more efficient and improvements in the operation conditions of bioremediation technologies will also be discussed and explored.

2. Status of heavy metal pollution

The term "heavy metal" generally refers to metallic elements with an atomic weight higher than that of Fe (55.8 g mol^{-1}) or density greater than 5.0 g cm^{-3}, and these metals are naturally present in the environment. However, some metals with an atomic weight lower than that of Fe, for example, Cr, and others which are considering metalloids, such as As and Se, are also commonly referred to as heavy metals [8]. Heavy metals can play a role as micronutrients, such as Cu, Fe, Mn, Mo, Zn, and Ni, but they can also be toxic to humans, e.g., Hg, Pb, Cd, Cu, Ni, and Co [9], depending on the exposure levels.

Contamination by heavy metals causes many deleterious effects, which affect not only fauna and flora but also human health [10, 11]. Heavy metal ions have a strong electrostatic attraction and high binding affinities with the same sites that essential metal ions normally bind to in 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 [12]. Heavy metals are notable contaminants because they are toxic, nonbiodegradable in the environment, and easily accumulated in living organisms [13]. The contamination of waters with heavy metals occurs through natural and anthropogenic activities, mainly related to industrialization. Table 1 shows the natural and anthropogenic sources of some of the most widespread study heavy metals as environmental pollutants, together with a brief list of their adverse health effects and their applications [14]. Although studies on bioremediation generally consider the total amount of metal present in the environment, the toxicity of these metals is dependent on their chemical form. The wide range of chemical forms in which heavy metals can be present in the environment includes cationic/anionic species and complexes (hydroxylated or complexed to Cl), and their oxidation state varies depending on the medium pH and composition.

Heavy metals contaminated in soil can accumulate and persist for long periods of time and may be harmful to vital processes involved in microbial nutrient cycling [15]. The toxicity and mobility of heavy metals are strongly dependent on their chemical form and specific binding properties. Changes in the environmental conditions in soils, such as acidification and variations in the redox potential, can cause the mobilization of heavy metals from the solid phase to the liquid phase, thereby allowing the potential contamination to the plants grown in these soils [16]. In water bodies, a heavy metal in relatively high concentrations affects the biota due to its toxicity, or it can be bioaccumulated, which increases its effect further along

Element	Contamination sources		Uses	Adverse health effects
	Natural	Anthropogenic		
Cd	Zn and Pb minerals, phosphate rocks	Mining waste, electroplating, battery plants	Automobile exhaust	Respiratory, cardiovascular, renal effects
Cr	Chromite mineral	Electroplating, metal alloys, industrial sewage, anticorrosive products	Pesticides, detergents	Mental disturbance, cancer, ulcer, hypokerotosis
Cu	Sulfides, oxides carbonates	Electroplating, metal alloys, domestic and industrial waste, mining waste, pesticides	Most uses are based on electrical conductor properties	Anemia and other toxicity effects induced indirectly through interaction with other nutrients
Pb	Galena mineral	Battery plants, pipelines, coal, gasoline, pigments	Batteries, alloys	Neurotoxic
Ni	Soils	Metal alloys, battery plants, industrial waste, production of vegetable oils	Batteries, electronics, catalysts	Skin allergies, lung fibrosis, diseases of the cardiovascular system
Zn	Minerals (sulfides, oxides, silicates)	Metal alloys, pigments, electroplating, industrial waste, pipelines	Fertilizers, plastics, pigments	Abdominal pain, nausea, vomiting and diarrhea, gastric irritation, headache, irritability, lethargy, anemia

Table 1. Contamination sources, uses, and adverse health effects of some heavy metals

the food chain. The progressive increase in the concentration of a contaminant such as a metal, as it advances in the food chain, is known as biomagnification. This occurs due to the need for a large number of organisms from lower trophic levels to feed a member of a higher trophic level and thus contaminants that cannot be metabolized but are fat soluble can accumulate in the fatty tissues of living organisms.

Various studies have been conducted to minimize or eliminate the heavy metals present in the environment. Conventional processes include precipitation, reverse osmosis, adsorption onto activated carbon or alumina, and redox processes [17]. However, these technologies are considered to be inefficient because of expensive cost [12]. In bioremediation by microorganisms typically employing one type of organism or a consortium of microorganisms, high toxic chemicals are converted into less toxic chemicals by biological means [18]. The technology makes use of the metabolic potential of microorganisms to clean up contaminated environments [19] and has been proposed as an attractive alternative owing to its lower cost and higher efficiency [20] compared with other physicochemical methodologies [12]. Microorganisms can decompose or transform hazardous substances into less toxic metabolites or degrade them to nontoxic end products. Microorganisms can also survive in contaminated habitats because they are metabolically able to exploit contaminants as potential energy sources [11].

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In bioremediation, microorganisms with biological activity, including algae, bacteria, fungi, and yeast, can be used in their naturally occurring forms.

The number of publications on the use of microorganisms for the removal of heavy metals in contaminated environments has been increasing steadily over the past 10 years. Figure 1 shows the main types of microorganisms used in these processes, based on a search for papers reporting microorganisms and bioremediation studies, indexed in the ISI Web of Science for the period of 2004 to 2014. It can be observed in Figure 1 that the microorganisms that have been most commonly used are bacteria and fungi, although yeast and algae are also frequently applied.

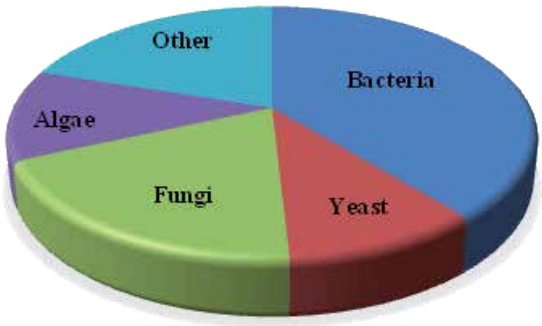

Figure 1. Types of microorganisms used in bioremediation processes.

Figure 2 gives some indication of which metals are used in bioremediation processes employing microorganisms, and chromium, copper, cadmium, and lead together account for 70% of applications, although nickel and zinc are also used. Other metals that are used to a lesser extent include arsenic and mercury.

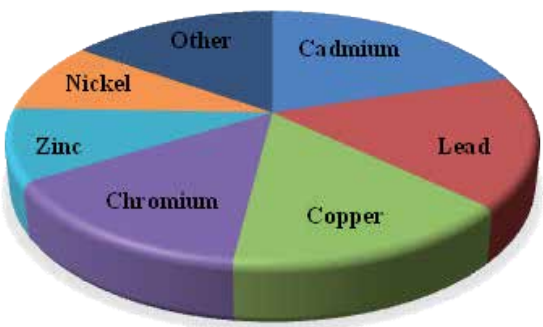

Figure 2. Metals used in bioremediation process employing microorganisms.

3. Types of organisms used in bioremediation

Typically, bioremediation is based on the cometabolism action of one organism or a consortium of microorganisms [18]. In this process, the transformation of contaminants presents a little efficiency or no benefit to the cell, and therefore this process is described as nonbeneficial biotransformation [21, 22]. Several studies have shown that many organisms (prokaryotes and eukaryotes) have a natural capacity to biosorb toxic heavy metal ions [23]. Examples of microorganisms studied and strategically used in bioremediation treatments for heavy metals include the following: (1) bacteria: *Arthrobacter* spp. [24], *Pseudomonas veronii* [25], *Burkholderia* spp. [26], *Kocuria flava* [27], *Bacillus cereus* [28], and *Sporosarcina ginsengisoli* [29]; (2) fungi: *Penicillium canescens* [30], *Aspergillus versicolor* [31], and *Aspergillus fumigatus* [32]; (3) algae: *Cladophora fascicularis* [33], *Spirogyra* spp. and *Cladophora* spp. [34], and *Spirogyra* spp. and *Spirullina* spp. [35]; and (4) yeast: *Saccharomyces cerevisiae* [36] and *Candida utilis* [37].

Prokaryotes (bacteria and archaeans) are distinguished from eukaryotes (protists, plants, fungi, and animals). The cellular structure of eukaryotes is characterized by the presence of a nucleus and other membrane-enclosed organelles. Also, the ribosomes in prokaryotes are smaller (70S) than in eukaryotes (80S) [38]. The way in which microorganisms interact with heavy metal ions is partially dependent on whether they are eukaryotes or prokaryotes, wherein eukaryotes are more sensitive to metal toxicity than prokaryotes [12]. The possible modes of interaction are (a) active extrusion of metal, (b) intracellular chelation (in eukaryotes) by various metal-binding peptides, and (c) transformation into other chemical species with reduced toxicity. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products [39]. Bacteria and higher organisms have developed mechanisms associated with resistance to toxic metals and rendering them innocuous [20]. Several microbes, including aerobes, anaerobes, and fungi, are involved in the enzymatic degradation process. Most of bioremediation systems are run under aerobic conditions, but anaerobic conditions make it possible microbial organisms to degrade otherwise recalcitrant molecules [39].

Because several different types of pollutants can be present at a contaminated site, various types of microorganisms are required for effective remediation. Some types of microorganism are able to degrade petroleum hydrocarbons and use them as a source of carbon and energy. However, the choice of the organisms employed is variable, depending on the chemical nature of the polluting agents, and needs to be selected carefully as they only survive in the presence of a limited range of chemical contaminants. The efficiency of the degradation process is related to the potential of the particular microorganism to introduce molecular oxygen into the hydrocarbon and to generate the intermediates that subsequently enter the general energy-yielding metabolic pathway of the cell. Some bacteria search the contaminant and move toward it because they flexibly exhibit the potential as a chemotactic response [40].

Numerous microorganisms can utilize oil as a source of food, and many of them produce potent surface-active compounds that can emulsify oil in water and facilitate its removal [21]. Bacteria that can degrade petroleum products include species of *Pseudomonas*, *Aeromonas*, *Moraxella*, *Beijerinckia*, *Flavobacteria*, *Chrobacteria*, *Nocardia*, *Corynebacteria*, *Modococci*, *Strepto-*

myces, *Bacilli*, *Arthrobacter*, *Aeromonas*, and cyanobacteria [40] and some yeasts [21]. For example, *Pseudomonas putida* MHF 7109 can be isolated from cow dung microbial consortia for the biodegradation of selected petroleum hydrocarbon compounds, such as benzene, toluene, and o-xylene (BTX) [23].

The application of biotechnology to the treatment of heavy metals is a relatively new subject. A better understanding of the processes through which microorganisms capture heavy metals, particularly the metabolism and detoxification pathways, has been accumulated. It can help the solution with maximum efficiency in dealing with environmental problems associated with heavy metal contamination [41]. The changes arising from the biotechnological approach include bioleaching, bioextraction, biosorption, bioencapsulation, and bioremediation [42]. In this regard, genetic engineering is a fundamental approach to modulate the metabolic pathways of these microorganisms and to inhibit the toxic the action of the metals by the modulated activity. The modified microorganisms can change the inorganic form into the organic form by some reactions, for instance, by transforming the metals through oxidation–reduction reductions, thus increasing the solubility.

Besides the increase of the solubility by microorganisms modifying microorganisms to increase their resistance through factors involving the solubility of heavy metals, their interaction with other factors (e.g., complexation reactions, changes in pH, sorption, precipitation, bioaccumulation, and encapsulation) can result in increased solubility or render the heavy metals inert in the environment [18]. Genetic engineering can be applied to modify the microorganisms and achieve interesting features such as accelerated growth, tolerance to extreme environmental conditions and pH variations, and low cost cultivation. Recent studies have demonstrated the ability of certain fungi (e.g., *Aspergillus* and *Penicillium*) and some yeasts (e.g., *S. cerevisiae*) to remove heavy metals from certain environments. The species *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces boulardii*, *Enterococcus faecium*, and *Staphylococcus aureus* have also been used for the removal of heavy metals from water bodies [43]. The process of metal accumulation on the cell surface is dependent on the metabolic activity of the microorganism as well as the characteristic of cell surface, and it is known as bioaccumulation [44]. It has been noted that metal ions interact with the proteins necessary for the proper functioning of the cell structure, affecting its metabolic functions. Genetic engineering, which allows the improvement of the metabolic structure of microorganisms, enables the high accumulation of metals or reduces the toxicity of metals, thereby promoting the decontamination of water bodies.

Many papers on bioremediation with wild or genetic modified microorganisms have been published over the years. Figure 3 shows the data obtained from a search of the web covering a period of 20 years (1995–2014), which deal with the development of methodologies for the decontamination of environments containing various heavy metals.

With the recent advances in genetic engineering, it is now relatively easy to construct genetically engineered microorganisms (GEMs) through reshuffling the genes, promoters, etc., and this can enhance their performance *in situ*. Several GEMs have been successfully constructed and experimentally tested for efficient bioremediation under laboratory conditions [45]. Recombinant DNA techniques can be used to enhance the ability of an organism to metabolize a xenobiotic through the detection of genes associated with degradation, transforming them

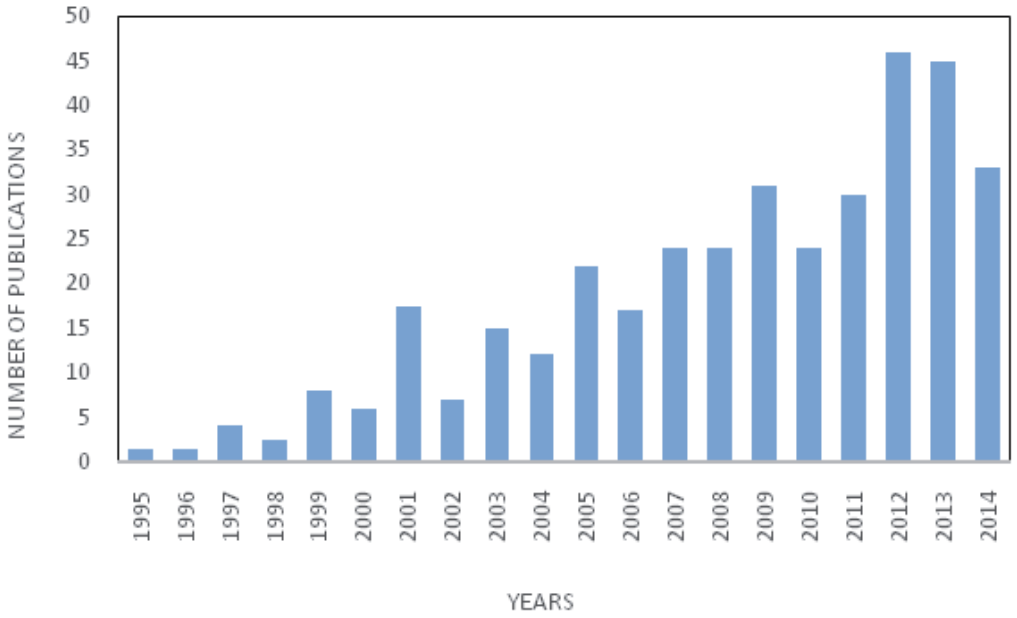

Figure 3. Scientific publications on bioremediation using microorganisms.

into appropriate bioremediation agents. Recombinant DNA technology explores the use of different approaches including PCR, antisense RNA technique, and site-directed mutagenesis.

Engineered strains of *Deinococcus geothermalis* have been developed for the bioremediation of environments containing mixed radioactive waste at high temperatures. Recombinant strain of *Acenitobacter baumanii* was found to enhance degradation rates at sites contaminated with crude oil [45]. In the presence of metals, some higher organisms produce cysteine-rich peptides, such as glutathione (GSH), phytochelatins (PCs), and metallothioneins (MTs), which can bind and sequester metal ions in biologically inactive forms. The overexpression of MTs in recombinant bacterial cells resulted in enhanced metal accumulation, thus offering a promising strategy for the development of microbial-based biosorbents [12].

Recent studies show that certain GEMs have increased ability to metabolize specific chemicals such as hydrocarbons and pesticides [12, 23].

Genetic engineering techniques and studies on the metabolic potential of microorganisms have allowed the design of genetically modified microorganisms capable of degrading specific contaminants. This approach offers an opportunity to create an artificial combination of genes that do not exist together in nature. The most commonly used techniques include engineering with single genes or operons, pathway construction, and alternation of the sequences of existing genes [22]. Genetic and biochemical techniques, such as PCR, *in situ* hybridization, and use of antibodies, can also contribute greatly to our knowledge regarding the potential activity of the microorganisms present at polluted sites. DNA tests can indicate the presence

of particular microbes potentially involved in biodegradation, and the use of enzyme-specific antibodies can reveal the induction of catabolic enzymes. Changes in the composition of bacterial populations may be observed during treatment, and differences can be noted in comparison with nonpolluted sites. DNA probes targeting specific genetic sequences, i.e., the genes responsible for the degradation ability of the microorganism, can be used to characterize a contaminated site throughout the bioremediation program, to determine the overall community structure and catabolic activity [46].

The first two genetically modified bacterial strains were *Pseudomonas aeruginosa* (NRRL B-5472) and *P. putida* (NRRL B-5473), and these contained genes for naphthalene, salicylate, and camphor degradation. *Pseudomonas fluorescens* HK44, which can degrade naphthalene, represents the first example of a microorganism genetically engineered for bioremediation purposes [22]. The associated research demonstrated that the genes responsible for the naphthalene degradation pathway were arranged under a common promoter, which resulted in the simultaneous degradation of naphthalene [22]. Other authors have shown that some bacteria, such as *Geobacter metallireducens*, can remove uranium from drainage waters in mining operations and from contaminated groundwater [21].

4. Mechanisms associated with bioremediation by microorganisms

Figure 4 shows the major groups of microorganisms commonly used for the bioremediation of metals, which include bacteria, microalgae, fungi, and yeast.

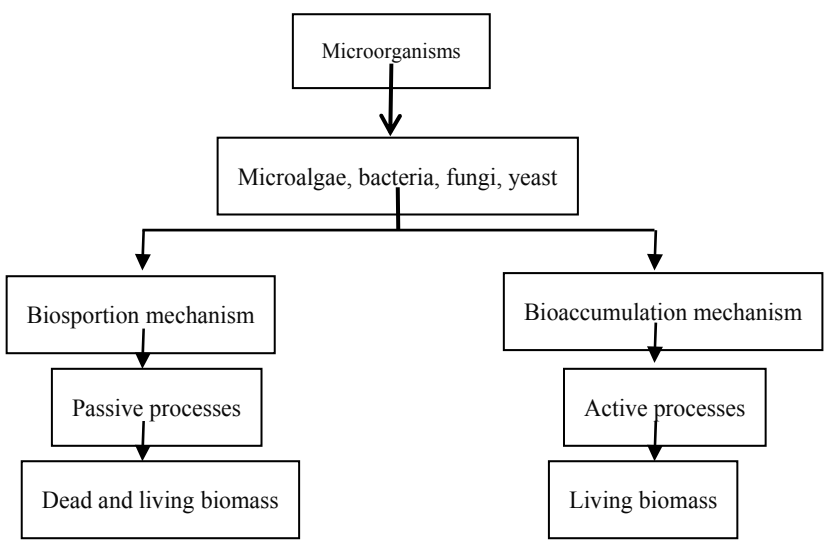

Figure 4. Microorganisms employed in the bioremediation and processes/mechanisms involved in the case of dead and living biomass.

Bioremediation can be separated into two categories, biosorption and bioaccumulation. Biosorption is a passive adsorption mechanism that is fast and reversible [6, 49]. The metals are retained by means of physicochemical interaction (e.g., ion exchange, adsorption, complexation, precipitation, and crystallization) between the metal and the functional groups present on the cell surface [6, 47–50]. Several factors can affect the biosorption of metals, such as pH, ionic strength, biomass concentration, temperature, particle size, and presence of other ions in the solution [48]. Both living and dead biomass can occur for biosorption because it is independent of cell metabolism. On the other hand, bioaccumulation includes both intra- and extracellular processes where passive uptake plays only a limited and not very well-defined role [6]. Therefore, living biomass can only occur for bioaccumulation.

Table 2 shows a comparison of the main parameters associated with biosorption and bioaccumulation processes. In general, the biosorption process needs inexpensive cost because the biomass can be obtained from industrial waste, and it can be regenerated and reused in many cycles. Bioaccumulation, on the other hand, needs expensive cost because the process occurs in the presence of living cells in which reuse is limited. Also, important factors to be considered include selectivity of metals and the potential for regeneration. The selectivity in biosorption is generally low because the bind only occurs by physicochemical interaction. It can be increased through modification of the biomass. Nevertheless, processes involving bioaccumulation generally perform better than those involving biosorption.

The structure of the cell wall of a microorganism contains various macromolecules, such as polysaccharides and proteins, with a high number of charged functional groups, including carboxyl, imidazole, sulfydryl, thioether, phenol, carbonyl, amide, ester sulfate, amino, and hydroxyl groups [51–53]. The positively charged metal present in the solution gravitates toward these functional groups and adsorption occurs. The form in which microorganisms are cultivated can influence the cell wall composition, and this can be exploited to improve the adsorption capacity of the microorganisms [6]. Bacteria can remove heavy metals from wastewater via functional groups, such as ketones, aldehydes, and carboxyl groups present in their cell walls and thereby produce less chemical sludge [54]. Both gram-positive and gram-negative bacteria are used for the uptake of metals. Green, red, and brown algae are also used as biosorbents. Some functional presents in bacteria such as uronic acid of carboxyl groups and sulfate groups, xylans, galactans, and alginic acid are capable of performing ion exchange. The advantage of using algae as biosorbents is that they generally do not produce toxic substances, unlike other microorganisms such as bacteria or fungi [55].

Fungi and yeasts also used for the adsorption. The most advantage of fungi is highly variable, ranging in size from mushrooms to microscopic molds. They are easy to grow and produce a substantial biomass. The cell walls of fungi are rich in polysaccharides and glycoproteins, which contain, for instance, amine, imidazole, phosphate, sulfate, sulfhydryl, and hydroxyl groups [56, 57]. However, the cell walls of yeasts contain a microfibrillar structure composed of more than 90% polysaccharides. The main groups present in these walls are amine, hydroxide, carboxyl, sulfate, and phosphate groups [58].

Characteristics	Biosorption	Bioaccumulation
Cost	Usually low. Biomass can be obtained from industrial waste. Cost associated mostly with transportation and production of biosorbent.	Usually high. The process occurs in the presence of living cells that have to be supported.
pH	The solution pH strongly affects the sorption capacity of heavy metals. However, the process can occur within a wide pH range.	Significant change in pH can strongly affect living cells.
Selectivity	Poor. However, this can be increased by modification/biomass transformation.	Better than in the case of biosorption.
Rate of removal	Most mechanisms occur at a fast rate.	Slower rate than in the case of biosorption because intercelluar accumulation takes a long time.
Regeneration and reuse	Biosorbents can be regenerated and reused in many cycles.	Reuse is limited due to intercellular accumulation.
Recovery of metals	With an adequate eluent the recovery of heavy metals is possible.	Even if possible, biomass cannot be used for other purposes.
Energy demand	Usually low.	Energy is required for cell growth.

Table 2. Comparison of biosorption and bioaccumulation processes [51].

Most heavy metals cannot be biodegraded and they tend to accumulate in the microorganism [59]. Several factors influence metal accumulation, such as the degree of exposure, metal concentration, temperature, and salinity, and therefore it is difficult to obtain detailed information on how the accumulation occurs in the bioremediation [60]. The process of accumulation is complex and varied according the pathway of metabolism is regulated by the metal concentration [61]. Mechanisms of metal ion uptake based on surface binding and metals ions entering the cell membrane have been proposed [62–65].

5. Considerations regarding metal uptake capacity of microorganisms

The pathway via which the metal binds to a specific site of the biomass is of great importance in relation to the efficiency of a bioremediation process. For example, the ingestion of sediments by microorganisms is considered a principal route of exposure to metals, although free metal ions in sediment pore waters are generally considered to be the most bioavailable form of metals. Thus, metal accumulation is affected by the feeding behavior of microorganisms [61]. After the ingestion of heavy metals, a process of metal excretion and/or detoxify begins to avoid potential toxic effects. However, microorganisms will not suffer the toxic effects of the presence of metals when they are stored in detoxified forms [61]. Moreover, the metal–biomass interaction is dependent on the type of metal that can bind to oxygen-containing or S- and N-containing ligands. Although this may be a simple overview of the mechanisms involved, it

can act as a starting point for proposing new approaches related to the efficiency of metal uptake by microorganisms [50].

Otherwise, microorganisms can synthesize metal binding proteins, such as MTs or PCs, and the proteins are strongly related to the capacity of metal adsorption, accumulation, and resistance [50]. In particular, metalloproteins are a large group of these proteins, which play an important role mainly in regulating the amount of metals within the cells.

Metal binding proteins present outside of cell membrane attract metal ions exist in solution and assist the transport to cytosol, where metallochaperones (specialized protein chelators) transfer metals to the appropriate receptor protein. The binding sites of the metal binding proteins have been improved to other protein, such as heterologous metalloproteins by using genetic technique. Some researchers developed heterologous metalloproteins with higher affinity and metal-binding capacity and/or specificity and selectivity, which was expressed in bacteria to improve their capacity to adsorb metals [50]. The technique changing the proteins on the cell surface, into heterogeneous one by using recombinant DNA has emerged as a novel approach to enhance the capacity of adsorption. Both bacteria and yeasts have been investigated for this purpose. A wide diversity of metal-binding proteins, such as glutathione (GSH), GSH-related phytochelatins (PCs), cysteine-rich metallothioneins (MTs), and synthetic phytochelatins (ECn), have been used to enhance the bioaccumulation of heavy metals [66]. For example, the recombinant bacterial strain cloned mercury operon, which coded the regulatory gene (MerR) and other genes involved in the transport, was constructed. The strain showed high resistant to mercury by the detoxification of mercury ions within the cell [66].

The expression of metal-binding proteins or peptides in microorganisms to enhance heavy metal accumulation and/or tolerance has great potential. Several different peptides and proteins have been explored [20, 50]. Different resistance mechanisms can be activated, for example, the production of peptides of the family of metal binding proteins, such as MTs or phytochelatins (PCs); the regulation of the intracellular concentration of metals, with the expression of transporters of proteins of ligand–metal complexes from the cytoplasm to the inside of vacuoles; and the efflux of metal ions by ion channels present in the cell wall. The genes to show the tolerance toward toxicity of metals are often encoded on the transposons or plasmids, which facilitate their dispersion from cell to cell [12]. The tolerance is caused by either the activity bacterial metal resistance result from either the active efflux pumping of the toxic metal out of the cell or enzymatic detoxification (generally via redox chemistry) where a toxic ion is converted into a less toxic or less available metal ion.

Several metal-binding peptides have been studied with the aim of increasing Cd resistance or accumulation by *E. coli* cells. Naturally occurring Cd-binding proteins and peptides, such as MTs and PCs, are very rich in cysteine residues. In addition, histidines are known to have high affinity for transition metal ions such as Zn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+}. Therefore, various peptides comprising different sequences of cysteines or histidines was used to bind Cd [20], and consequently Cd tolerance and accumulation could be enhanced in *E. coli* cells. It would be of interest to evaluate Cd-binding peptides and proteins engineered into more environmentally robust bacteria, such as *Pseudomonas*, for their potential use in bioremediation [20].

Hexavalent chromium is mobile, highly toxic, and considered as a priority environmental pollutant. Chromate reductases found in chromium-resistant bacteria have the potential for use in bioremediation process because they are known to catalyze the reduction of Cr(VI) to Cr(III) [67]. The enzymatic reduction of Cr(VI) to Cr(III) involves the transfer of electrons from electron donors, like NAD(P)H, to Cr(VI) with the simultaneous generation of reactive oxygen species (ROS) [67]. Microorganisms that have the ability to reduce Cr(VI) are referred as chromium-reducing bacteria (CRB). Gram-positive CRB shows to have significant tolerance to the toxicity of Cr(VI) even at high concentrations, whereas gram-negative bacteria are much more sensitive to Cr(VI) [67]. Some genes responsible for resistance to Cr(VI) have been determined in bacteria. For example, the chrR gene located on the chromosome of *P. aeruginosa* confers resistance to chromate. *Ochrobactrum tritici* contains several genes associated with chromate resistance, namely, chrB, chrA, chrC, chrF, and ruvB. The presence of enzymes that play a role in reducing Cr(VI) have been reported for different microorganisms. The enzymes such as quinone reductases, nitroreductases, and NADPH-dependent enzymes vary in their ability to transform chromate and involve different pathways. Several bacteria reduce Cr(VI) through membrane-bound reductases, such as flavin reductase, cytochromes, and hydrogenases. These enzymes can form part of the electron transport system and use chromate as the terminal electron acceptor [67]. Table 3 shows ability of typical microorganisms (algae, bacteria, fungi, and yeasts) to remove heavy metals from certain environments [68–80]. As can be seen, a wide range of microorganisms have been considered for the development of efficient technology for the removal of heavy metal ions from polluted effluents.

Microorganism	Type	Metal	Reference
Algae	*Ascophyllum nodosum*	Pb, Ni	68
		Pb, Cu, Cd, Zn	69
	Chlorella pyrendoidosa	U	70
	Cladophora fascicularis	Pb	33
	Fucus vesiculosus	Cr	71
		Pb	68
		Cd	72
	Hydrodictylon, Oedogonium, and *Rhizoclonium* spp	V, As	73
	Spirogyra spp. and *Cladophora* spp.	Pb, Cu	34
	Spirogyra spp. and *Spirullina* spp.	Cr, Cu, Fe, Mn, Zn	35
Bacteria	*Bacillus cereus*	Cr	28
	Burkholderia species	Cd, Pb	26
	Kocuria flava	Cu	27
	Pseudomonas veronii	Cd, Zn, Cu	25
	Sporosarcina ginsengisoli	As	29
	Stenotrophomonas spp.	Au	74

Microorganism	Type	Metal	Reference
Fungi	*Agaricus bisporus*	Cd, Zn	75
	Aspergillus fumigatus	Pb	32
	Aspergillus versicolor	Cr, Ni, Cu	31
	Aspergillus, *Mucor*, *Penicillium* and *Rhizopus*	Cd, Cu, Fe	77
	Aspergillus niger, *Aspergillus foetidus*, and *Penicillium simplicissimum*	Ni, Co, Mo, V, Mn, Fe, W, Zn	78
	Ganoderma lucidum, *Penicillium* spp.	Ar	76
	Penicillium canescens	Cr	30
Yeast	*Candida tropicalis*	Cd, Cr, Cu, Ni, Zn	79
	Candida utilis	Cd	37
	Pichia guilliermondii	Cu	79
	Saccharomyces cerevisiae	Cr, Ni, Cu, Zn	36
	Streptomyces longwoodensis	Pb	80

Table 3. Sorption potential of certain microorganisms to remove heavy metals.

6. Conclusions

Natural and anthropogenic activities generate large quantities of aqueous effluents containing toxic metals. Many studies have been conducted in recent decades aimed at lowering metal concentrations derived from natural resources. In addition, considerable effort has been made to develop efficient and cost-effective technologies and apply them to industrial wastewater treatment. The potential for microorganisms to remove metals from solutions through passive and active mechanisms has been shown to be an interesting approach to metal uptake in polluted waters, and the efficiency of such processes is dependent on the experimental conditions, the target pollutant and various other factors.

The application of this type of bioremediation process in large scale remains, however, a challenge, and a preventive approach to metal pollution problems is therefore encouraged. Further investigations aimed at the identification of the mechanisms involved the characterization of biosorbents, and advances in genetic engineering are required.

Many microorganisms can break down metals naturally, but this is not a sufficient solution on a global scale. Therefore, as a means to resolve this problem, engineered microorganisms can be developed with the help of genetic engineering. A better understanding of the way in which both eukaryotes and prokaryotes metabolize heavy metals and the detoxification pathways will help future researchers to deal with this type of environmental problem with maximum efficiency. The choice of the most promising type of biomass must be made, taking into account its cost and availability, and this is necessary on an industrial scale. The micro-

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organisms should be easy to obtain and to cultivate. For example, industrial-scale application would not be of interest if the microorganism is difficult to cultivate, a rare species or a species in danger of extinction.

Although some progress has been made in the recognition of the importance of microorganisms for the decontamination of polluted waters, some important points still need to be addressed. However, a new challenge has emerged for science. Thus, further studies need to focus on the development of new clean environmentally acceptable technologies with commercial feasibility.

Nomenclature

BTX	Benzene, toluene, and *o*-xylene
CAPES	Coordination of Improvement of Higher Education Personnel
CNPq	National Council for Scientific and Technological Development
DNA	Deoxyribonucleic acid
ECn	Synthetic phytochelatins
FAPEG	Research Foundation of the State of Goiás
FAPEMIG	Research Foundation of the State of Minas Gerais
GEM	Genetically engineered microorganisms
GMMs	Genetically modified microorganisms
GSH	Glutathione
MTs	Metallothioneins
NRRL B-5472	*Pseudomonas aeruginosa*
NRRL B-5473	*Pseudomonas putida*
PCR	Polymerase chain reaction
PCs	Phytochelatins
RNA	Ribonucleic acid

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Chapter 2

New Uses of Haloarchaeal Species in Bioremediation Processes

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Additional information is available at the end of the chapter

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Abstract

The extreme conditions under which haloarchaea survive make them good bioremediation agents in water treatment processes and in saline and hypersaline environments contaminated with toxic compounds such as nitrate, nitrite and ammonia, chlorine compounds such as perchlorate and chlorate, heavy metals, and aromatic compounds. New advances in the understanding of haloarchaea metabolism, biochemistry, and molecular biology suggest that general biochemical pathways related to nitrogen (Nitrogen cycle), metals (iron, mercury), hydrocarbons, or phenols can be used for bioremediation proposals.

The main goal of the chapter is to present a review about the main characteristics of the archaeal species and their possible uses for bioremediation processes paying special attention to the *Halobacteriaceae* family. Several examples about the role of these microorganisms in salty brines or soils with high concentrations of nitrogenous compounds, heavy metals, aliphatic or aromatic hydrocarbons, or oxyanions are also discussed.

Keywords: Haloarchaea, wastewater treatments, bioremediation, denitrification, nitrogen, carbon metabolism

1. Introduction

The main benefit of using bioremediation is that microorganisms can destroy hazardous contaminants or turn them into less harmful forms. These microorganisms act against the contaminants if there are a variety of compounds aiding them to generate both energy and nutrients in order to grow more cells. In a few cases, the natural condition of the contaminated site provides all the essential material in sufficient quantities so that bioremediation can occur without the need for human intervention, which is known as "intrinsic bioremediation" [1]. Often, bioremediation requires engineered systems to supply microbe-stimulating materials, which is called "engineered bioremediation" and relies on accelerating the desired biodegradation by encouraging growth of further organisms and optimizing the environment where detoxification takes place. Engineered bioremediation may be chosen over intrinsic bioremediation due to the time factor and liability. Where an impending property transfer or potential impact of contamination calls for rapid pollutant removal engineered bioremediation maybe more appropriate as it accelerates biodegradation. However, intrinsic bioremediation is an option where the natural occurrence of contaminant biodegradation is faster than contaminant migration. These rates depend on both, the type and concentration of contaminant, the microbial community, and the subsurface hydrogeochemical conditions [1]. Moreover, the lack of a sufficient microbial population can also hinder the cleanup rate.

Terrestrial subsurface ecosystems constitute one of the largest habitats and represent an important resource of microbial diversity. The organisms within provide critical services including mitigation of contaminants. Research in this area has intensified over the last two decades leading to significant discoveries in ecology, physiology, and phylogeny of subsurface microorganisms. Despite considerable progress, the structure–function relationships remain largely uncharacterized. Attempts to correlate microbial abundance and composition with variables likely to control metabolism have for the most part been unsuccessful. New technologies now give us the opportunity to gain further insights [2]

A critical factor as to whether bioremediation is an appropriate remedy depends on if the contaminants are susceptible to biodegradation by the site organisms, or alternatively, if the relevant organisms can be added. While those already present can detoxify a vast array of contaminants, some are more easily degraded than others. On the whole, those most easily degraded are petroleum hydrocarbons; however, technologies which stimulate organisms' growth to degrade further contaminants are emerging and are being field tested with success [1].

Bioremediation is a branch of environmental biotechnology often used to hasten this process and it guarantees the restoration of damaged ecosystems, using the metabolic capabilities of bacteria, fungi, yeast, algae, and microbial mats to degrade all contaminants harmful to living organisms. Bioremediation follows two main strategies: i) biostimulation, stimulation of indigenous microbial populations; ii) bioaugmentation, the introduction of viable microbial populations. Microorganisms are ideally suited to the task of contaminant destruction because they have enzymes that allow them to use environmental contaminants as food and because they are so small that they are able to contact contaminants easily [1]. Without the activity of

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microorganisms, the earth would literally be buried in wastes, and the nutrients necessary for life would be locked up in detritus.

Coastal marine sediments subjected to high anthropogenic inputs can accumulate large amounts of contaminants, which represents a major concern for the potential detrimental consequences on the health of the ecosystem and the subsequent provision of goods and services. In particular, the contamination by metals, due to their persistence and toxicity even at low concentrations, represents a serious and widespread environmental problem. Threats for ecosystem health do not rely only upon the concentration of metals in the sediment, but also upon their oxidation/reduction state and their partitioning in the different geochemical phases [3].

The presence of heavy metals in the environment has been a major concern because of their toxicity. Their elimination from wastewater before being released into the environment is important for the maintenance of the ecosystem and from an economic point of view. Techniques such as ion exchange, precipitation, filtration, electrochemical treatment, or reverse osmosis are used to do away with metals such as Cu, Co, Zn, Hg, etc.; however, these methods are rather costly when the metal concentrations are less than 0.01% [4].

The type and the concentration of carbon source and also the C/N ratio, have had a dramatic effect on the rate of heterotrophic denitrification. Microorganisms, water streams, and environmental conditions vary. In contrast, autotrophic denitrifiers utilize inorganic carbon (carbon dioxide or bicarbonate) as a sole source of carbon. Some advantages of autotrophic over heterotrophic denitrification are: avoiding of the poisoning effect of some organic carbon, low biomass buildup and less sludge production which results in reduction of reactor clogging and easier posttreatment. Since some wastewaters have a very low concentration of biodegradable organic materials, autotrophic denitrification requires the addition of an electron donor substrate. Extensive studies have been carried out on elemental sulfur and H_2 as electron donors for autotrophic denitrification systems [5].

Anaerobic ammonium oxidation (anammox) has received special attention because it is an efficient biological alternative to conventional nitrogen removal from wastewaters [6]. Under anaerobic conditions, ammonium is oxidized to nitrogen gas with nitrite as the electron acceptor: $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$, and carbon dioxide is used for growth of the anammox microorganisms involved. Compared with traditional nitrification–denitrification process, anammox consumes 100% less biodegradable organic carbon and at least 50% less oxygen and has, therefore, lower operating cost [5]. If the anammox process is combined with a preceding nitrification step, only part of the ammonium needs to be nitrified to nitrite, while the anammox process combines the remaining ammonium with the nitrite to yield dinitrogen gas.

Many studies have revealed that, while conventional cleanup technologies have prevented the contamination problem from spreading, in most cases they are incapable of restoring the water to meet health-based standards in a reasonable time frame. Soil cleanup procedures have been more successful in meeting regulatory standards. However, conventional soil cleanup methods may transfer contaminants to the air, posing risks that are not always acceptable to residents near the contaminated site. The limitations of conventional groundwater cleanup

technologies and the hazards of conventional soil cleanup methods have spurred investigations into in situ bioremediation, which uses microorganisms to destroy or immobilize contaminants in place.

In developing countries, 90% of untreated wastewater goes into the rivers so the access to safe drinking water is limited. The increasing water demand not only affects surface freshwater like rivers and lakes, but it also degrades groundwater resources [5]. The eutrophication and the presence of excess nitrogen in the environment have caused serious alterations of the natural nutrient cycle between the living world and the soil, water, and atmosphere [5]. The intensification of agricultural production and continuous industrial development have contributed to an increase in the nitrate content in drinking water. This is particularly evident in rural areas, where in private wells the concentration of nitrate nitrogen is often over twenty times above the permissible level. Therefore, it is now necessary to develop a technology which effectively reduces nitrate concentration in drinking water [7].

The engineering of bioremediation processes relies on information about the site and about candidate microorganisms. Process analysis usually begins with fixed waste characteristics but with options for microbial cultures, reactor types, waste pretreatment, and process operating conditions. Laboratory measurements are necessary to explore these options and to design an efficient process. These tests examine degradation rates as functions of critical operating parameters such as pH, oxygen and nutrient concentrations, microbial composition, soil particle size, temperature, and redox potential, shaping the design of a bench-scale process. Mass transfer effects such as agitation and aeration are also explored, although at a small scale. These tests constitute the basis for scale-up to the field scale and for the implementation of process control [8]. A main objective of biological remediation design is to remove the limiting factors in the growth of bacteria [9]. The main objective of site characterization is to identify the contaminants, their concentration, and the extent of contamination.

2. General characteristics of archaeal species and their potential availabilities to support bioremediation strategies

The word "archaea" means "ancient things" (from Greek) and it refers to a group of prokaryotic single-celled microorganisms characterized for the extreme conditions they need to be alive. Archaea, which are single-celled prokaryotic microorganisms, were first classified as a separate group of prokaryotes in 1977 by Woese and Fox [10]. Extreme conditions are necessary for archaea to live [10] and extremophiles such as methanogens, thermoacidophiles, halophiles, or alkalophilic microorganisms are included in the group.

Haloarchaea (salt-loving organisms) can grow in media with high salt concentration in a range of 12% to 30% salt (2-5 M NaCl). The cellular machinery of haloarchaea can work even in such high concentration of salt, because it accumulates potassium ion to counteract high concentration of sodium ion. Whereas biodegradation and bioremediation by non-extremophilic microorganisms have been extensively study, the use of extremophilic microorganisms is less studied and particularly haloarchaea.

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Enzymes and metabolic pathways specific in archaea were suggested by the comparison between genome sequences of archaea and others [11]. The metabolic pathways of carbohydrates, carbon and nitrogen assimilation or fixation, and sulfur metabolism were involved in the specific pathways, which are different from classical pathways existing in bacteria and eukarya. The enzymes of archaea may be used for the bioremediation.

2.1. Carbohydrate metabolism

In archaea, the modified version of the Embden-Meyerhoff-Parnas and Entner-Doudoroff pathways, and pentose degradation pathway have been described [12]. The Embden-Meyerhoff-Parnas is an optimized pathway of glycolysis for the conversion/oxidation of glucose to two molecules of pyruvate yielding ATP and intermediates for other metabolic pathways. The main modifications in this pathway include the presence of the enzymes as ADP-dependent glucokinase and phosphofructokinase, phosphoenolpyruvate synthase, pyruvate:phosphate dikinase or nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase. This modified pathway is present, for example, in members of *Thermococcales* [13], *Archaeoglobales* [14], and *Desulfurococcales* [15]. The main difference of the classical Entner-Doudoroff pathway is that this pathway is divided into two branches: semi-phosphorylated or nonphosphorylated. The highest enzymes of both branches are phosphoenolpyruvate synthase, pyruvate:phosphate dikinase and nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase, as in the Embden-Meyerhoof-Parnas pathway. The semi-phosphorylated pathway is found in haloarchaea [16], and the nonphosphorylated in the genera *Thermoplasma* [17], *Sulfolobus* [18], and *Thermoproteus* [19].

Pentoses are ubiquitous in nature as part of nucleic acids. The degradation of pentoses has been described in some haloarchaea [16, 20] and species for the genera *Sulfolobus* [21]. This pathway in archaea is similar to that identified in bacteria.

2.2. Carbon fixation/assimilation

Regarding the assimilation of carbon, different metabolic pathways have been described in archaea. Acetyl-CoA assimilation was performed by ethylmalonyl-CoA pathway and the glyoxylate cycle. In addition to this glyoxylate cycle, several haloarchaea can assimilate acetate [12]. Recently, Khomyakova et al. [22] have proposed a new acetyl-CoA assimilation pathway in *Haloarcula marismortui*, the methyl aspartate cycle.

Metabolic studies of autotrophic archaea have led to the discovery of several different CO_2-fixation pathways such as the reductive tricarboxylic acid cycle, the Calvin-Benson-Bassham cycle, the 3-hydroxypropionate cycle, and the reductive acetyl-CoA pathway. In anaerobic or microaerobic *Thermoproteales* and *Desulfurococcales,* the dicarboxilate-4-hydroxybutirate cycle is present [23,24]. The oxygen sensibility of some of the enzymes of this cycle restricts its use to this kind of microorganism. The hydroxypropionate–hydroxybutirate cycle was identified in members of the order *Sulfolobales* [25].

2.3. Nitrogen metabolism

Some archaea species have reductive pathways of nitrogen such as assimilatory pathway (nitrate assimilation and N_2 fixation) and dissimilatory pathway (nitrate respiration and denitrification). Nitrogen metabolism is much less known in archaea than in bacteria. However, in *Haloferax mediterranei* some of these pathways are well known, making it a good candidate microorganism for bioremediation approaches [26-28]..

Many archaea are able to reduce nitrate by assimilatory or respiratory pathways through enzymes such as nitrate and nitrite reductases. These enzymes are found in a variety of halophilic and hyperthermophilic archaea [29,30]. Moreover, denitrification has been described for several halophilic archaea, such as *Haloferax* and *Haloarcula* strains [31, 32], and extreme thermophilic archaea, such as *Ferroglobus placidus* [33] and *Pyrobaculum aerophilum* [34]. However, fixation of N_2 in archaea is exclusive of methanogenic euryarchaeota [35]. Glutamine synthetase, glutamate synthase and glutamate dehydrogenase are the major pathways for ammonium assimilation in archaea. For example, both pathways have been identified in *Hfx. mediterranei* [36-38]. Recently, anammox systems have also been described from haloarchaea [39] and some thermophiles [40].

2.4. Sulfur compounds metabolism

Many archaea can utilize sulfur compounds as electron donors or acceptors for energy production [41]. Aerobic sulfur oxidation is common in Crenarchaeota (mainly in the order *Sulfolobales*); while anaerobic reduction of S^0 is a widespread ability in the Crenarchaeota and Euryarchaeota phyla [42]. Dissimilatory sulfate, sulfite, and thiosulfate reduction are present in some thermophile genera of Euryarchaeota and Crenarchaeota [43,44]. Anaerobic DMSO respiration has been found in some haloarchaea that can grow anaerobically using DMSO as electron acceptor, such as *Halobacterium* sp. strain NRC-1 [45].

3. Bioremediation processes involving haloarchaea species

The severe environment in which haloarchaea can survive makes this archaea a good agent for bioremediation in water treatment processes and in saline and hypersaline environments contaminated with toxic compounds such as nitrate, nitrite and ammonia, chlorine compounds such as perchlorate and chlorate, hydrocarbons, or heavy metals. New advances in the understanding of the haloarchaea metabolism, biochemistry, and molecular biology suggest that general biochemical pathways related to nitrogen (Nitrogen cycle), metals (iron, mercury), hydrocarbons, or phenols can be used for bioremediation processes. With regard to nitrogen species, it is interesting to note that denitrification and nitrification have been described so far as powerful pathways to remove nitrogenous compounds from wastewater. In this context, several bacterial species are shown to be excellent models for removing nitrogenous compounds contained in wastewater. However, the use of archaea to remove nitrogen from wastewater has been poorly studied. Denitrification and anaerobic ammonium oxidation (anammox) carried out by archaea can be practically used as efficient pathways to remove

nitrate, nitrite, or ammonium from soils or wastewater. The existence of anammox (anaerobic ammonium oxidation, a part of the nitrogen cycle) was at first hypothesized based on thermodynamic calculations, and the hypothesis was subsequently confirmed in a pilot denitrifying wastewater treatment plant. Moreover, recent studies have suggested that anammox species are present not only in wastewater but also in marine- and freshwater with limited oxygenation, including oceans, seas, estuaries, lakes, and rivers. For example, the *amoA* gene coding oxidizing enzyme of ammonia was found from Crenarchaeota isolated from suboxic zones of marine environment by metagenomics. These results indicate that the nitrifying archaea can provide sufficient amount of nitrite by anammox under oxygen-limiting conditions. Thus, application of anammox may offer an attractive bioremediation process to current wastewater treatment systems for the removal of ammonia-nitrogen. In order to design a cheap and efficient process to nitrogen loss, anammox reaction could be coupled to nitrification carried out by archaea.

3.1. Halophilic archaea in bioremediation of hydrocarbons

Many hydrocarbon contaminated environments are characterized by low or high temperatures, acidic or alkaline pH, high salt concentrations, or high pressure. The studies on the characteristics and degradation of hydrocarbon under these conditions suggest that the presence of extremophilic microorganisms living in extreme environments play an important role in the biological reaction and they could be useful for bioremediation. Halophilic extremophiles seem to be very important to the bioremediation of oil-polluted salt marshes and treatment of industrial saline wastewaters. However, their full potential has not been sufficiently exploited [46]. Information on hydrocarbon degradation under high salt concentration is limited. Moreover, some authors reported the negative impact of increasing salinity on hydrocarbons biodegradation [47]. Most of these studies were performed using halophilic or halotolerant bacteria or bacterial consortia [48], but the potential alternative microorganisms are probably the extreme halophilic archaea.

Extreme halophilic microorganisms belonging to the Archaea Domain present diverse nutritional demand and metabolic pathways; for example, members of *Haloarcula* and *Haloferax* species use a variety of carbohydrates, organic acids as sole carbon and energy sources [49]. However, little is known about the ability of haloarchaea to grow in the presence of hydrocarbons as sole carbon and energy sources, although there is a lot of information related to physiology of microorganisms belonging to the *Halobacteriaceae* family.

3.1.1. Halophilic archaea in bioremediation of crude oil and aliphatic compounds

Zvyagintseva et al. [50] have reported the ability of halophilic archaea to degrade crude oil in hypersaline environments. Significant amount of isoprenoid and *n*-alkane fractions in crude oil were degraded by halophilic-like isolates from the brines of the Kalamkass oil fields in Kazakhstan. Al-Mailem et al. [51] isolated four extreme *Haloferax* archaea strains from the hypersaline coastal area of the Arabian Gulf. They were identified as two *Haloferax* strains, *Halobacterium* sp. and *Halococcus sp.* These halophilic archaea can use crude oil as sole source of carbon and energy, and the growth was enhanced by increasing the NaCl concentration in

the medium. The optimum salt concentration value for growth was between 3.5 and 4.5 M. They also examined the effect of illumination and casamino-acids enrichment on the bioremediation of crude oil using hypersaline soil and red pond water samples from the supertidal ''sabkha'' coastal area south of Kuwait [52]. The results indicated that addition of casamino-acids and exposure to light enhanced the oil consumption. Additionally, the antibiotics contained in the medium inhibit the growth of most bacteria except archaeal. These results suggest that degradation of hydrocarbons are mainly performed by haloarchaea species.

Some other researchers have reported archaeal ability to metabolize aliphatic hydrocarbons. Table 1 shows the extreme halophilic archaea able to degrade hydrocarbons and their carbon source. Bertrand et al. [53] isolated a halophilic archaeon, strain EH4, from a salt marsh in France. This strain was originally assigned to *Halobacterium* based on its phenotypic and biochemical characteristics but later analysis of the 16S RNA of strain EH4 indicated that it was closely related (99%) to *Haloarcula vallismortis* [54]. This strain was able to metabolize saturated hydrocarbons (tetradecane, eicosane, hexadecane, heneicosane). The growth of EH4 in the medium containing eicosano was salt dependent, growth and degradation was maximum at 20% salinity and nondetectable below 10% salinity. Kulichevskaya et al. [55] have reported the isolation of an archaeon, *Halobacterium* sp. from hypersaline oil-contaminated wastewater in Russia. This strain grew optimally at 15-32% NaCl and showed a high capacity to degrade C_{10}-C_{30} *n*-alkanes in a medium containing 30% NaCl. The *Haloferax*, *Halobacterium*, and *Halococcus* strains isolated on the basis of crude oil bioremediation [51] also degraded n-alkanes and mono- and polyaromatic compounds.

MICROORGANISM	HYDROCARBON	REFERENCE
Strain EH4*	Tetradecane Hexadecane Eicosane Heneicosane	[53]
Halobacterium sp.	*n*-Alkane C_{10}-C_{30}	[55]
Haloferax sp. Halobacterium sp. Halococcus sp.	*n*-Alkane C_{10}-C_{34}	[51]
Haloarcula sp. Haloferax sp.	Heptadecane	[54]

*Bertrand et al. [53] labeled this strain as EH4 and assigned it to *Halobacterium sp.* based on phenotypic features. Tapilatu et al., [54] reassigned it to *Haloarcula vallismortis* based on 16 rRNA

Table 1. Extreme halophilic archaea able to biodegrade or grow on aliphatic hydrocarbons

Recently, the effect of vitamin and organic nitrogen on hydrocarbon removal was assessed by using halophilic bacteria and archaea from the Arabian Gulf. Al-Mailen et al. [56] proved the hydrocarbon remediation potential of five archaea in their natural hypersaline environments and how this potential can be enhanced by the addition of certain vitamins to the cultures. The most effective vitamins were thiamine, pyridoxine, and vitamin B_{12}. These results were

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obtained not only for individual microorganisms in pure cultures but also for microbial consortia. Therefore, the supplement of vitamins could be an effective practice to enhance bioremediation of oil-contaminated hypersaline environments [56]. Tapilatu et al. [54] have reported the isolation of four alkane-degrading halophilic archaeal strains: one (strain MSNC 2) was closely related to *Haloarcula* and the others (strains MSNC 4, MSNC 14, and MSNC 16) were identified as *Haloferax*. These strains could degrade 32-95% of heptadecane when they were cultured in the medium containing 0.5 g/l of heptadecane and 22.5% NaCl for 30 days at 40°C. The strain MSNC 14 was also able to degrade phenanthrene. Otherwise, surfactants and emulsifiers are used to solubilise and disperse hydrophobic compounds. Halophilic archaea could be employed to this purpose. Post and Al-Harjan reported that the ether-linked phytanyl membrane of *Halobacteriaceae*, which showed emulsification properties, was effective in enhancing the efficiency of oil recovery [57].

3.1.2. Halophilic archaea in bioremediation of aromatic hydrocarbons

Studies on aromatic hydrocarbons in earlier times were carried out with microorganisms isolated from samples of diverse hypersaline environments. Table 2 shows the typical halophilic archaea which can degrade aromatic compounds. In 1990, Bertrand et al. [53] isolated from salt-marsh an extremely halophilic archaea able to biodegrade aromatic carbons such as acenaphthene, phenanthrene, and anthracene. Later, Emerson et al. [58] described that *Haloferax* strain D1227 could grow in the medium containing monoaromatic compounds such as benzoate, cinnamate, and phenylpropionate by mineralizing as carbon sources, showing the physiological diversity of this group of archaea.

4-Hydroxybenzoic acid is a contaminant in certain highly saline industrial effluents. Fairley et al. [59] examined the metabolism of 4-hydroxybenzoic acid by *Haloarcula sp*. strain D1. The 4-hydroxybenzoic acid was changed to gentisate in the initial ring-cleavage reaction by the strain, although protocatechuic acid, hydroquinone or catechol is produced in case of the common pathways in aerobic bacteria, fungi, and yeast. In order to isolate new halophilic archaea able to grow in aromatic compounds, Cuadros-Orellana et al. [60] chose five different and unrelated hypersaline sites, the Uyini Salar (Bolivia), solar salterns in Cahuil (Chile), solar salterns in Cabo Rojo (Puerto Rico), *sabkhas* (Saudi Arabia), and the Dead Sea (Jordan). In this study, forty-four new halophilic archaea able to grow in 4-hydroxybenzoic acid as sole carbon and energy sources were isolated (Table 2). Taxonomic characterization of these microorganisms revealed that the isolates represent at least four different groups of haloarchaea. They concluded that the ability to metabolize 4-hydroxybenzoic acid is widespread in the *Halobacteriaceae* family, and thus, these haloarchaea microorganisms are excellent candidates to bioremediate aromatic compounds of hypersaline environments and treatment of saline effluents. These authors also determined biodegradation kinetics of strain L1 isolated from the Dead Sea [61], and suggested that the strain L1 could degrade benzoic acid more efficiently than *Haloferax sp* D1227 [58]. Moreover, features about the benzoic acid catabolism were found in the *Haloarcula* sp. L1. When the strain L1 was grown in the medium containing benzoic acid, gentisic acid was produced, which was not usual in other microorganisms. Therefore, gentisic acid is an intermediate in the degradation of benzoic acid, hydroxybenzoic acid, cinnamate, and

phenylpropionate by the archaea *Haloferax sp.* D1227, and in the degradation of 4-hydroxibenzoic acid by the *Haloarcula sp.* strain D1 [59].

In relation to metabolism of the aromatic compounds in haloarchaea, the gentisate-1,2-dioxygenase genes (*gdoA*), which correspond to the ring-cleavage enzyme of gentisic acid, of *Haloferax sp.* D1227 and *Haloarcula sp.* strain D1 were cloned. Surprisingly, the expression pattern of the genes is different: in *Haloarcula* sp. D1, *gdoA* is expressed in the presence of 4-hydroxybenzoate but not benzoate; however, *gdoA* is expressed in *Haloferax sp.* D1227 during growth on benzoate, 3-hydroxybenzoate, cinnamate, and phenyl-propionate. Moreover, genes of Co-A synthetize (*acdB*) subunit and CoA-thioesterase (*tieA*) also existed at the upstream of the *gdoA* gene. The pattern of these genes expression is also different between the two species, obtaining only expression of *acdB* and *tieA* in *Haloferax sp.* D1227 during growth on benzoate, cinnamate, and phenypropionate, but not on 3-hydroxybenzoate. This suggests that *acdB* and *tieA* are part of benzoate degradation pathway in *Haloferax sp.* D1227, while the *gdoA* genes encode part of a 4-hydroxybenzoate and 3- hydroxybenzoate pathways in *Haloarcula* sp. D1 and *Haloferax sp.* D1227, respectively [62].

During the last four years, the number of studies which describe the degradation of aromatic compounds by halophilic archaea have increased. As already mentioned, Tapilatu et al. [54] isolated four haloarchaea from hypersaline environment. The MSNC 14, one of the strains (Table 2), was able to degrade 43% of phenanthrene after 30 days of incubation, although the degradation of anthracene and dibenzothiophene was not detected. Al-Mailen et al. [51] isolated four strains (Table 2) from a hypersaline coastal area for the Arabian Gulf on a mineral salt medium containing crude oil vapor as unique source of carbon and energy. The four strains were able to biodegraded not only aliphatic hydrocarbons but also aromatic hydrocarbons after three weeks of incubation. In particular, *Halobacterium* and *Haloccocus* could grow in the presence of benzene, toluene, and p-hydroxybenzoic acid, and the two *Haloferax* strains could grow with toluene and phenanthrene, and one of them also with benzene, but both failed to grow on p-hydroxybenzoic acid. This study also revealed that the biodegradation rates increased in proportion to NaCl concentration in the medium, and thus supported the idea that extreme halophilic archaea are suitable biological material to bioremediate oil-polluted hypersaline environments. Bonfá et al. [63] revealed the usefulness of halophilic archaea in the bioremediation of wastewater of petroleum production, which contained high saline concentration and various aromatic acids and hydrocarbons. Aromatic compounds (p-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene) could be also degraded by nine halophilic archaea (Table 2) isolated from Çamalti Saltern, Turkey [64]. This study broadens the understanding of metabolism of aromatic compounds, and the activities of catechol 1,2 dioxygenase and protocatechuate 3,4 dioxygenase were identified as the enzymes involved in *ortho* cleavage pathway. *Ortho* cleavage pathway is widely distributed in soil bacteria and fungi, constituting the major pathway for aromatic compounds catabolism in these organisms. Therefore, these enzymes may be important to remove aromatic hydrocarbons.

These results suggest the following: *Halobacteriaceae* family, which can degrade aromatic compounds, exists widely, and can be used to degrade aromatic compounds in oil-polluted hypersaline environments [60]. Thus, the bioremediation process using those strains is

promising, as the remediation processes using physical and chemical methods are complicated and expensive [65]. However, more precise understanding of the mechanism of carbon-cycling cleavage and their enzymes and genes may be necessary to achieve the bioremediation [66].

MICROORGANISM	AROMATIC COMPOUNDS	REFERENCE
Strain EH4*	Acenaphthene Phenanthrene Anthracene	[53]
Haloferax strain D1227	Benzoate Cinnamate Phenylpropionate	[58]
Haloarcula sp.	4-hydroxybenzoic acid	[59]
Halobacterium species	4-hydroxybenzoic acid	[60]
Haloferax species	4-hydroxybenzoic acid	[60]
Strain L1	Benzoate 4- hydroxybenzoic acid	[61]
Haloferax strain MSNC14	Phenanthrene	[54]
Haloferax sp. strain HA-1	Benzene Toluene Phenanthrene Naphthalene	[51]
Haloferax sp. strain HA-2	Toluene Phenanthrene Naphthalene	[51]
Halobacterium sp. HA-3	Benzene Toluene p-Hydroxybenzoic acid	[51]
Halococcus sp. HA-4	Benzene Toluene Naphthalene p-Hydroxybenzoic acid	[51]
Halorubrum ezzemoulense	p-Hydroxybenzoic acid Naphthalene Pyrene Phenanthrene	[64]
Halorubrum sp.	p-Hydroxybenzoic acid Naphthalene Pyrene Phenanthrene	[64]

MICROORGANISM	AROMATIC COMPOUNDS	REFERENCE
Haloarcula hispanica	p-Hydroxybenzoic acid	[64]
	Naphthalene	
	Pyrene	
	Phenanthrene	
Halobacterium salinarum	p-Hydroxybenzoic acid	[64]
	Naphthalene	[56]
	Pyrene	
	Phenanthrene	
Halobacterium piscisalsi	p-Hydroxybenzoic acid	[64]
	Naphthalene	[56]
	Pyrene	
	Phenanthrene	
Haloferax mucosum	Phenanthrene	[56]
Haloferax lucentense	Phenanthrene	[56]
Haloferax sulfurifontis	Phenanthrene	[56]

*Bertrand et al. [53] labeled this strain as EH4 and assigned it to *Halobacterium sp.* based on phenotypic features. Tapilatu et al. [54] reassigned it to *Haloarcula vallismortis* based on 16 rRNA.

Table 2. Extremely halophilic archaea able to biodegrade or grow on aromatic compounds

3.2. Halophilic archaea in bioremediation of heavy metal ion

Some heavy metals such as iron, cobalt, copper, manganese, molybdenum, and zinc are trace elements required at a certain level, and thus are necessary for life. However, they are excessively damaging to organisms. Other heavy metals such as mercury, aluminium, cadmium, gold, lead, and arsenic are toxic and are not beneficial to organisms. Both essential and nonessential metals at high concentration, directly or indirectly compromise DNA, protein, and membrane integrity and function [67, 68].

Frequently, heavy metals are found in saline and hypersaline environments due to the evaporation in such environments and also as a result of industrial activities. Therefore, some halophilic archaea have developed tolerance to heavy metals. Wang et al. [69] reported that the *Halbacterium sp.* NRC-1, which had a plasmid carrying genes of arsenite and antimonite extrusion system, showed high resistance to arsenic. Kaur et al. [70] studied the haloarchaeal strategies of adaptation to high metal concentration, using *Halobacterium sp.* NRC-1 as model organism. Sublethal levels of Mn(II), Fe(II), Co(II), Ni(II), Cu(II), and Zn(II) were used to investigate the change on transcriptional level using microarray technology. All growths were inhibited at high concentrations of metals, but the susceptibility was different. Consequently, the effective inhibitory concentrations of Zn (II), Fe (II), Co (II), Cu (II), and Mn (II) were 0.05, 7.5-8.5, 0.6, 1.2, and 2 mM, respectively. Some of the adaptation mechanisms detected include previously known mechanisms such as efflux of metal ions by P1 ATPases, downregulation

of Mn(II) uptake, ion scavenging, protein turnover increase, and minimizing ROS production depletion. Novel discoveries include: control of transcriptional regulation by a TRASH domain, ability of ZntA to confer resistance to several metals, a global control mechanism mediated by GTFs and key metalloregulatory proteins, and simulation of Fe deficiency by Mn(II) [70]. Authors developed a systems-level model to provide an integrated perspective of responses to these metals.

Srivastava et al. [71] have reported the intracellular synthesis of silver nanoparticles by the haloarchaeal isolated *Halococcus salifodinae* BK_3 when the cells were grown in the medium containing silver nitrate. They also described the intracellular synthesis of selenium nanoparticles (SeNPs) by the haloarchaeon *Halococcus salifodinae* BK18, when the cells were grown in the presence of sodium selenite. Also, cadmium tolerance has been reported in haloarcheal strains from solar salterns of Ribandar and Siridao in India [73].

Biosorption of metals by the organisms at the surface or by the exopolysaccharides (EPS) secreted to form the biofilms enables organisms to tolerate metals [74]. The adsorption of heavy metal by EPS has been attributed to interaction between metal cations and negative charges of acidic functional groups of EPS [75]. Kawakami et al. [76] found that *Halobacterium salinarum* CCM 2090 has a Ca(II)-dependent aggregation system. Calcium ion is adsorbed on the surface of the cells and induces ionic cross-bridging between the EPS, resulting in aggregation of the haloarchaeal cells. Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} could replace Ca^{2+}. However, Mg^{2+}, Sr^{2+}, Mo^{2+}, Cd^{2+}, Sn^{2+}, Hg^{2+}, and Pb^{2+} induced no flocculation of cells of this halophilic archaeon. In addition, Popescu and Dumitru [75] reported the two *Haloferax* strains had the capacity to reduce the concentration of Pb, Cr, Zn, and Ni ions from media with high salinity, by biosorption process. Knowledge regarding molecular mechanisms underlying resistance to metal is cursory. Therefore, more precise understanding of the mechanism is necessary to facilitate the use of haloarchaea for bioremediation of metal-polluted hypersaline environments [74].

3.3. Halophilic archaea in bioremediation of wastewater

Wastewater treatments (WWT), such as breakdown of sewage influent, are generally performed by microorganisms. These microorganisms are able to live in the sludge of treatment plants and holding tanks. They obtain nutrients by degrading the solids in WWT to various compounds. Some wastewater treatment systems are efficient and desirable from an economical point of view. However, other systems are not efficient because of the undesirable effects the system itself promote on the microorganisms. Therefore, the establishment of optimal conditions (such as nutrients, pH, temperature, and oxygen availability) for comfortable growth is most important in order to treat WWT effectively.

Modern biological treatment of wastewater involves not only C removal, but also elimination of other nutrients such as P and N. Combined and sequential actions are required for such treatment successively by several groups of microorganisms, such as heterotrophic bacteria, phosphate-accumulating organisms (PAO), or microorganisms able to perform nitrification, denitrification, or anammox [77]. However, it is difficult to design this kind of treatment process, because the system becomes extremely complex to exhibit a satisfactory performance

and it requires expensive costs from economical point of view. Figure 1 shows the nitrogen transformation pathways by archaea. Archaea, which can degrade ammonia, are now one of the main candidates for wastewater treatment.

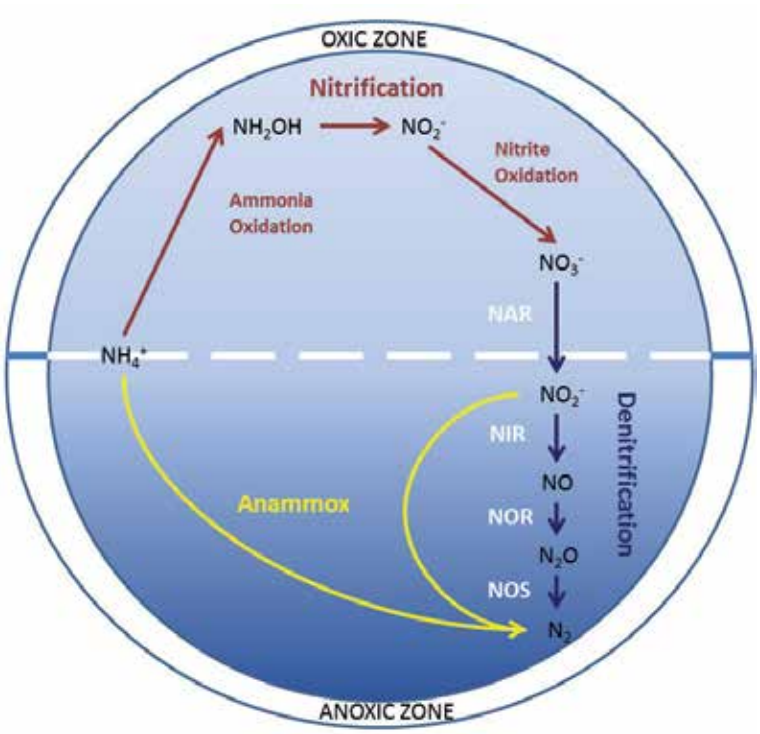

Figure 1. Archaeal nitrogen transformations in wastewater treatment environment. The enzymes involved in denitrification process are shown in the figure: NAR, nitrate reductase; NIR, nitrite reductase; NOR, nitric oxide reductase; NOS, nitrous oxide reductase.

Although there are natural microorganisms used in wastewater treatment, the bioremediation process requires further addition of various types of microorganisms known as bioremediators. Since there is an organism available to treat any organic molecule (the microorganism does this by extracting the energy from the molecule bonds), it is a very effective treatment. However, it is essential to distinguish the types of microorganisms present in wastewater as well as the pollutants to be removed and where they are to be located in the wastewater process. When these details are known, it is quite easy to select the best microorganisms to be used along with their best locations in the wastewater treatment processing plant.

Figure 2 shows the nitrogen sources produced by artificial activities and their metabolic cycles. The wastewater containing excess nitrogen compounds is constantly discharged from houses and factories, and overall nitrogen species in wastewater and soil are increased. Due to the accumulation of this nitrogen species (NH_4^+, NO_2^-, and NO_3^-, etc.), in groundwater or tap water, the wastewater should be removed or reused [81, 82]. Conventionally,

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biological nitrogen removal is achieved by nitrification followed by a denitrification process: firstly, aerobic nitrification of NH_4^+ to NO_2^- or NO_3^- with O_2 as the electron acceptor; secondly, anoxic denitrification of NO_2^- or NO_3^- to gaseous N_2 using organic matter as carbon and energy source [82].

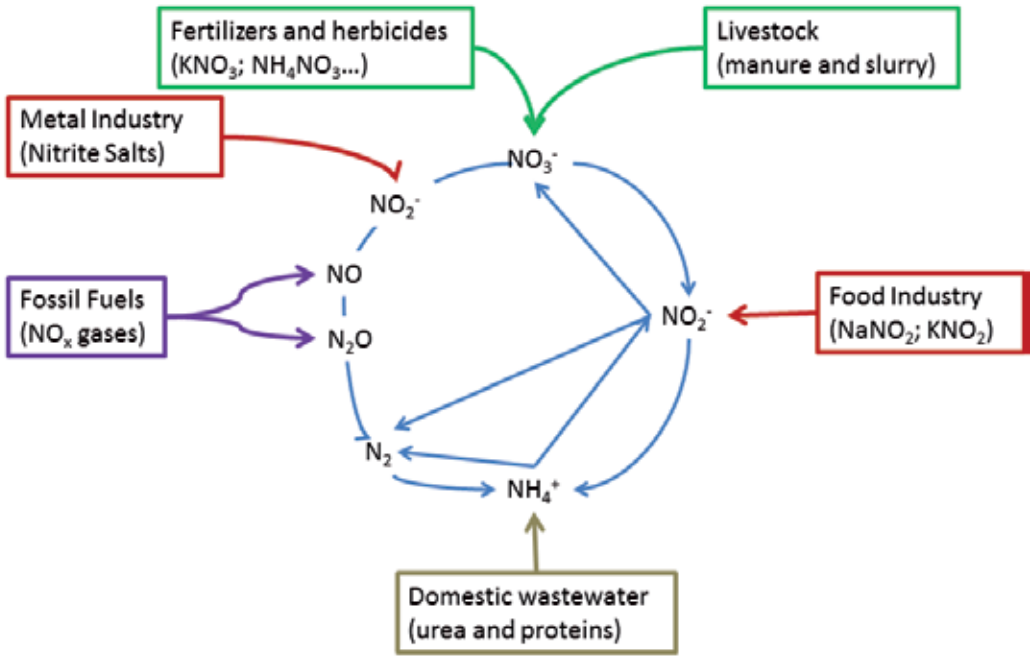

Figure 2. N-cycle scheme. The various sources of pollution of nitrogen cycle species are represented.

Figure 3 shows the typical processes in wastewater plant. Treatments were performed by following three processes:

a. Primary Treatment: Flocculation, setting, and anaerobic degradation are carried out in the primary treatment. The solid matter in wastewater containing metal salt is clumped together (flocculated) by storing in the settling tank, and they finally precipitated and composed sludge at the bottom of the tank. The sludge is then subjected to anaerobic degradation. Methane gas, which is used as a fuel, is produced during the degradation, and the efficiency can be enhanced by the addition of suitable anaerobic microorganisms. Flocculation, settling, and anaerobic degradation are essential to remove the nonorganic matter before the secondary treatment.

b. Secondary Treatment: degradation by microorganisms is aerobically carried out in the secondary treatment. The residual effluent is moved to the aeration tank using pumps, and the remaining suspended solids (including fecal matter) are degraded by the addition of microorganisms and the supplement of air. The values of biological organic matter (BOD) can be decreased by this process.

c. Tertiary Treatment: this treatment removes the nitrogen (mainly through denitrification processing) and phosphates (usually by chemical precipitation from the effluent).

Other compounds such as minerals or metals (such as iron, sulfur, manganese), and runoff pollutants such as fertilizers, hydrocarbons, and tar can also be removed. Therefore, extremophilic microorganisms able to deal with these compounds have become of great interest in designing new strategies to treat wastewater.

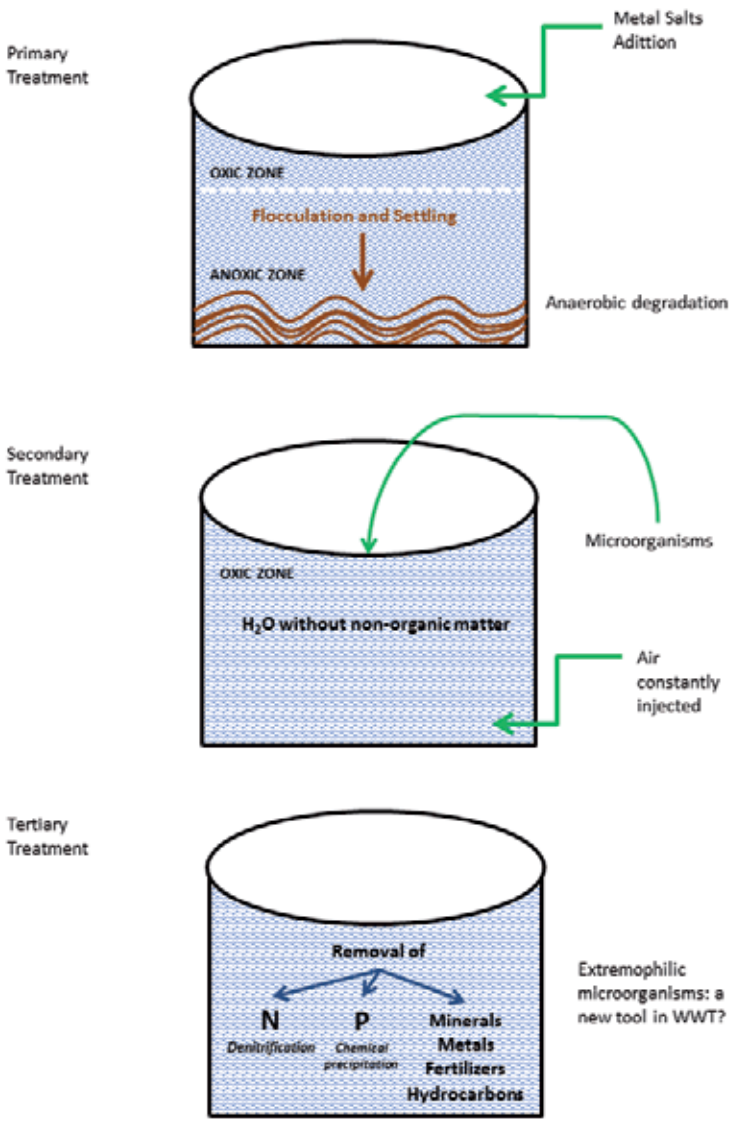

Figure 3. Primary, secondary (the tank contains the supernatant followed by primary treatment), and tertiary treatments used in wastewater treatment plants.

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Activated sludge process is currently the most used for the treatment of both domestic and industrial wastewater. The bioreactor has two chambers: one in which the wastewater is agitated and maintained at constant oxygen, where microorganisms degrade the contaminants; second, where the biologically treated water settles to form a sludge that will be removed. Due to the characteristics of the process, the decantation system limits the efficiency of the treatment as a result of problems such as the production of foam, floatability of the sludge, or the large size required for the installation of the wastewater treatment plants [78].

Alternative treatment technologies are thus of great interest when searching for more efficient strategies regarding nutrient removal and the generation of effluents with enough quality for direct reclamation. One new treatment is based on membrane bioreactors (MBRs) (Figure 4): the MBR system consists of a cylindrical bioreactor equipped with two ultrafiltration membrane units submerged inside the aerated bioreactor, and the extraction of the effluent water takes place by mechanical suction. The membranes are continuously aerated, maintain solids in suspension, and supply oxygen to the process [78].

Submerged biofilters (SBs) (Figure 4) are another reduced-size and low-cost alternative of proven efficiencies for the design of WWTPs, which are of simple control and maintenance, and minimize undesirable odors and noise in the vicinity of the installations. SBs consist of two methacrylate cylindrical columns each, packed with clayey schists biofilm support [79, 80]. The columns are connected with a valve that allows a separated cleaning of the biofilters. They operate downflow (denitrifying column, anoxic) and upflow (nitrifying column, aerated) [78].

Another recent process for nitrogen removal has gained importance in the last years: the anaerobic ammonium oxidation (anammox). This reaction is based on energy conversion from anaerobic ammonium oxidation using nitrite as the electron acceptor. It was discovered in 1995 in a pilot plant treating wastewater at Gist-Brocades, Delft, in The Netherlands [7, 83]. This process offers a novel, energy-saving and cost-effective biological nitrogen removal technique.

In the past years, partial nitrification, anammox and denitrification simultaneously in a single reactor (SNAD technology), was developed for the complete removal of nitrogen sources. Under oxygen limitation, ammonium is oxidized to nitrite by aerobic ammonium oxidation; the nitrite in the reactor can be used by anammox microorganisms with ammonium, and finally to dinitrogen gas with small amounts of nitrate produced. Afterward, COD as electron donor can deoxidize nitrate to dinitrogen gas through denitrifying process for the complete nitrogen removal performance. The interaction of aerobic nitrifying, anaerobic ammonium oxidizing, and anaerobic denitrifying microorganisms under oxygen limitation has the potential to make an almost complete conversion of ammonium and organic carbon to dinitrogen gas and carbon dioxide [84].

Although the importance of eukaryotic and bacteria organisms in aerated activated sludge has long been recognized, the role played by archaea in aerobic and anaerobic WWPTs has not been mentioned. However, recent researches suggest that growth and activity of archaea were significant in the treatment of activated sludge and wastewater [85-87]. The roles of methanogenic archaea within a broad range of activated sludge, submerged biofilters, and membrane

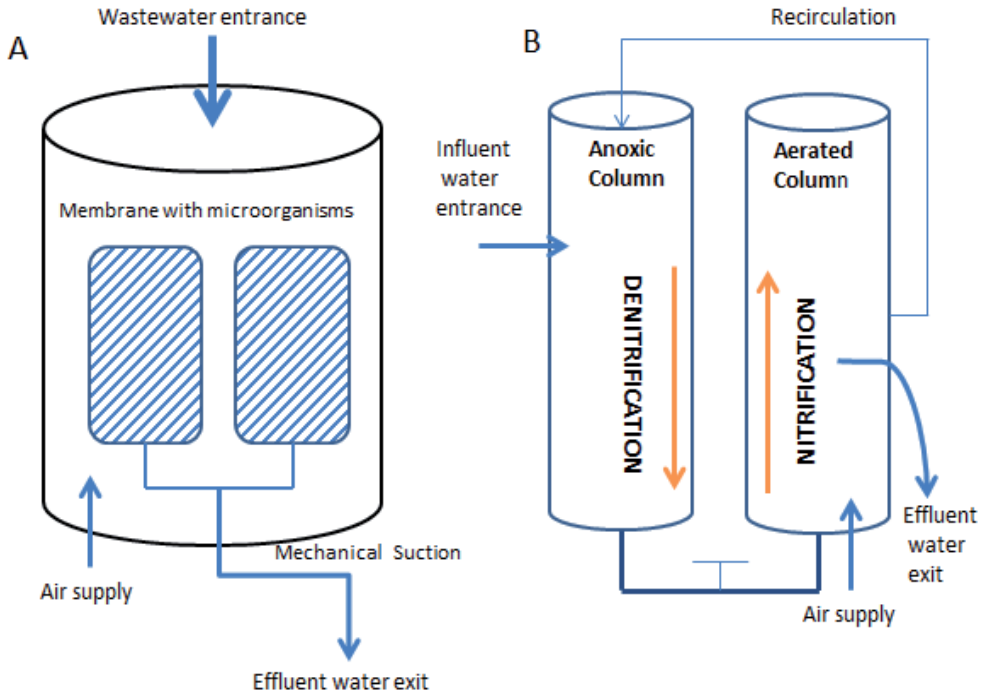

Figure 4. Schematic diagram of the two new types of WWTPs. (A) Submerged membrane bioreactor (MBR). (B) Submerged biofilter (SB).

bioreactors have been studied in recent research [78, 88-89]. Under oxic conditions, no methanogenesis was detected; however, once oxygen is depleted, methane production ensued. The results suggest that methanogenic archaea can be activated under anoxic conditions [88].

The composition of the wastewater is a key determinant of archaeal community composition in WWPTs [88, 90-91]: the microbial population in industrial wastewater treatments, rich in ammonia, phenol, and with high salinity, are closely related to *Methanobrevibacter smithii*, the predominant methanogen in human intestines [78]. The manufacturing of chemical compounds (pesticides, herbicides, explosives, etc.) usually generates effluents containing complex mixtures of salts and nitrate or nitrite. Also, the increase of salinity in soils and waters in the last few decades has given advantage to some species like *Hfx. mediterranei.* For example, *Hfx. mediterranei* is resistant to very high nitrate (up to 2 M) and nitrite (up to 50 mM) concentrations, which are the highest described from a prokaryotic microorganism [92]. Therefore, it could be useful for bioremediation applications in sewage plants where high salts, nitrate, and nitrite concentrations are detected in wastewaters and brines. In a recent study [93], this haloarchaea was able to eliminate 60% of the nitrate and 75% of nitrite initially present in the brines (initial concentration was 40 mM nitrite). Moreover, it has also been described that nitrate reductase involved in denitrification reduces efficiently other oxyanions such us bromate and (per) chlorate [94]. These results suggest that *Hfx. mediterranei*, and in general, halophilic archaea, are able to carry out denitrification, thus providing excellent models to

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explore large-scale bioremediation processes to remove nitrogen compounds from brines and salty water.

4. Conclusions

Bioremediation provides a technique for cleaning up pollution by enhancing the same biodegradation processes that occur in nature. Depending on the site and its contaminants, bioremediation may be safer and less expensive than alternative solutions such as incineration or land filling of the contaminated materials. It also has the advantage of treating the contamination in place so that large quantities of soil, sediment, or water do not have to be dug up or pumped out of the ground for treatment. Therefore, bioremediation is considered as of now one of the best options to treat contaminated environments. Taking into account the amazing metabolic features that define haloarchaea metabolism, these microorganisms may become good candidates to improve bioremediation procedures, or even new bioremediation strategies could be defined using them. Thus, aerobic and anaerobic haloarchaea could be considered to design co-metabolic in situ bioremediation to remediate different pollutants in water and soils. Although the potential use of haloarchaea in bioremediation has been extensively demonstrated, different aspects of their metabolism remain poorly known. Therefore, more studies from biochemical and molecular biology points of view are required to properly comprehend haloarchaeal metabolism regulation. Moreover, new niches and extreme microecosystems in terms of temperature, salt concentration, and pH should be explored to identify and locate new microorganisms able to deal with heavy metals, hydrocarbons, chlorinated compounds, and, in general, all pollutants affecting soil and water.

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Chapter 3

The Potential of the Photoautotroph Synechocystis for Metal Bioremediation

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Additional information is available at the end of the chapter

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Abstract

The photoautotrophic cyanobacterium *Synechocystis* PCC6803 has received much attention as a model photosynthetic cell factory for the production of a range of important biotech products. The biomass remaining from this activity may then have further utility in processes such as metal bioremediation. In addition *Synechocystis* being an inhabitant of many natural aquatic environments is seen as an environmentally friendly alternative to using chemical precipitation methodologies for metal remediation. *Synechocystis* produces a range of extracellular polysaccharide substances (EPS) that can undergo modification as a function of culture age and growth nutrients which have been implicated in metal biosorption. Many studies have demonstrated that high levels of charged groups present in EPS are important in forming polymeric matrices with metallic ions allowing their biosorption. Genetic studies has revealed genes involved in such metal binding indicating that EPS can be modified for potential enhancement of binding or modification of the types of metals bound. The utility of metal binding to live and dead biomass of *Synechocystis* has been demonstrated for a range of metals including Cr(VI), Cd(II), Cu(II), Pb(II), Sb, Ni(II), Mn(II), Mn(IV), As(III), As(V), Cs and Hg. The potential of using *Synechocystis* as a biosorption platform is discussed.

Keywords: *Synechocystis*, EPS, metal biosorption, metal binding, metal remediation

1. Introduction

Heavy metals because of their chemical nature cannot be biodegraded by microorganisms to non-toxic species and therefore build up in the environment. Many metals undergo a change in chemical state from one form to another but ultimately they accumulate in the environment and potentially enter the human food chain through uptake by plants or animals. Removal of metals by chemical technologies has been widely used but has proven expensive or inappropriate in the case of low level metal contamination. Thus attention has focussed on newer technologies such as metal biosorption as an alternative to chemical removal.

Biosorption can simply be defined as 'the removal of substances from solution by biological material' [1]. The process is energy independent and differs from bioaccumulation which is an energy dependent transport process associated with accumulation or transport of a metal into the cell. Biomaterials and particularly biomass have a bioaffinity for metals via a number of different physico-chemical interactions with the metal. These include sorption (ad- and ab-), ion exchange and surface complexation and precipitation. There has been a large increase in published work on biosorption but so far little by way of exploitation of the process on a large scale other than by traditional sewage treatment methodologies [1]. Most biological material either living or dead can biosorb a variety of materials including metals with the vast majority of the sorption being adsorption to surface groups associated with the particular biological material. Thus far there appears to be no clear winner in terms of the best candidate as a biomass material although many bacteria and algae including cyanobacteria have been examined.

Within the domain bacteria, cyanobacteria are the only organisms to carry out oxygenic photosynthesis and are phylogenetically most closely related to gram positive microorganisms [2]. Amongst the many thousands of genera of cyanobacteria, a number of model organisms have emerged. Amongst these is *Synechocystis* which is classified within the Phyllum Cyanobacteria and is a member of the Order Chroococcales. The genus *Synechocystis* was originally described as a botanical taxon [3], with the type species being *S. aquatilis*. Members of the genera *Synechocystis* are non-nitrogen fixing, unicellular cyanobacteria which primarily inhabit fresh or marine water environments. As shown in (Table 1), more than 20 species have been characterised within the genera *Synechocystis*. These have been sub grouped on the basis of sequence and GC content into 3 groups, a high and low GC freshwater group, and a marine group [4]. *Synechocystis* PCC 6803 [5, 2] belongs to the marine group although originally isolated from a freshwater lake in the US.

Synechocystis PCC 6803 is naturally transformable [6] and can grow heterotrophically on glucose [7]. These characteristics make it of interest as a model cyanobacterium for genetic manipulation. The original PCC 6803 strain, designated the Kunisawa strain was isolated in Berkley in 1968 [8], and since then there have been a number of sub-strains derived from this original strain including Kazusa (non-transformable), China (increased settling), Amsterdam, Mu and a number of New Zealand derivatives based on individual lab publication and morphological variation. All strains (original and sub strains) demonstrate 16s rDNA sequence identity but differ phenotypically in certain traits. Phenotypic differences include increased

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settleability, motility differences, sensitivity to glucose, propagation rate and transformability [2]. Indeed, recent sequencing of a number of these 'sub'strains has revealed a small number of single nucleotide polymorphisms that may be responsible for the number of phenotypic differences observed. This phenomenon in *Synechocystis* has been termed microevolution [2].

Synechocystis aquatilis Sauvageau 1892	*Synechocystis bourrellyi* Komárek 1976
Synechocystis buzasii Palik 1948	*Synechocystis consotia* Norris 1967
Synechocystis crassa Woronichin 1929	*Synechocystic diplococca* Pringsheim 1970
Synechocystis endobiotica Elenkin and Hollerbach 1938	*Synechocystis endophytica* Smith
Synechocystis fuscopigmentosa Kováčik 1988	*Synechocystis limnetica* Popovskaya 1968
Synechocystis major Geitler 1995	*Synechocystis minima* Woronichin 1927
Synechosystis miniscula Woronichin 1926	*Synechocystis parvula* Perfiliev 1923
Synechocystis pevalekii Ercegović 1925	*Synechocystis planctonica* Proškina-Lavrenko 1951
Synechocystis primigenia Gardner 1927	*Synechocystis sallensis* Skuja 1929
Synechocystis salina Wislouch 1924	*Synechocystis septentrionalis* Skuja 1956
Synechocystis skujae Joosten 2006	*Synechocystis thermalis* Copeland 1936
Synechocystis trididemni Lafargue 1979	*Synechocystis negrescens* Stearn 1973
Synechocystis willei Gardner	

Table 1. *Synechocystis* strains as designated in the AlgaeBase [9] with the discoverer and the year of discovery when known. In some instances these strains may be called after the discoverer such that it is not uncommon to have the 'Sauvageau strain' for *S. aquatilis*.

Cyanobacteria and in particular *Synechocystis* species, have recently received much attention as potential cell factories for the production of a wide variety of compounds of biotechnological interest. Such compounds include isobutanol, [10], 1,2-propanediol [11], isopropanol [12], 2,3-butanediol [13], ethanol [14], 3-hydroxybutyrate [15], free fatty acids [16], fatty alcohols [17], endogenously produced alka(e)nes [15], carotinoids [18], sesquiterpene β-caryophyllene [19], isoprene [20], squalene [21], poly-β-hydroxybutyrate (PHB) [22], polyhydroxyalkanoate [23], ethylene [24], cellulose [25], sucrose and glucose/fructose carbon substrates [26], mannitol [27], lactic acid [28], acetone [29] and H_2 [30]. Given the large interest in experimental production systems using *Synechocystis*, there has been an equivalent interest in utilising the biomass produced to add to the economics and increase the industrial potential of such systems. Biosorption of metals to various biomass types has emerged as one potential candidate for biomass utilisation following its use in other production processes. However, although there have been a wide range of biological materials studied [1], there is no clear best candidate as yet. Biomass with Gram negative peptidoglycan or Gram positive surface phosphate groups have clear advantages, while bacterial surface S-layers, proteins and sheaths all contribute to binding, making microbial candidates important. Attention has focussed on the potential of many cyanobacterial species for bioremediation, in particular, the model organism *Synechocystis*, given its potential availability from engineered production systems as discussed above.

The *Synechocystis* genus is of interest in this respect because of its natural freshwater or marine habitat. In addition *Synechocystis* produces EPS (Extrapolysaccharide Substances) which can be either cell attached or released exopolysaccharides (RPS) [31]. This EPS can biosorb a range of material including metals which can be produced as a by-product of other biotechnological processes at scale. Thus while there may be many candidates for biomass biosorption, the sorption efficiency, scale and economics may well be key factors in adoption of a particular technology or species for an *in situ* industrial process.

2. Characteristics of EPS produced from *Synechocystis*

The abbreviation EPS has variously been used to indicate extracellular polysaccharide, exopolysaccharides, exopolymers and extracellular polymeric substances. The material has been shown to contain nucleic acids, proteins, humic substances and lipid depending on its origin and environmental source. In addition, EPS may contain material derived from cell lysis and adsorbed materials that adhere to the natural polymers secreted from the surrounding environment. This adds to the complexity of EPS from a structural perspective. Most of this EPS is associated with the formation of aggregates or biofilms. The extracellular polysaccharide material varies in consistency, thickness and response to dye staining [32]. In laboratory culture, EPS is not an essential component, but in nature offers adaptive functionality. The characteristics of this material have been widely examined and there appears to be quite a large diversity in chemical composition and functionality. For example, in many cyanobacteria, this extra polysaccharide material plays a role in protecting cells from environmental extremes and stress. In certain strains, the release of exopolysaccharides together with sucrose and trehalose has been associated with desiccation resistance [33] and stabilization of cells when dried by air. In natural strains, dense layers may make strains less popular as food for predators relative to strains devoid of such material [34]. Attachment of benthic cyanobacteria to sediments, plant cells and other surfaces has been associated with extracellular polysaccharides and their hydrophobic nature [35]. It has also been proposed that secreted exopolysaccharides may play a role in precipitation of particles such as clay in aquatic environments clarifying the surrounding water. With precipitation more light is available for photoautotrophic metabolism [36]. The exopolysaccharides have also been proposed to disperse the cells themselves, facilitating optimum nutrient uptake [37]. In strains of *Microcystis*, the extracellular polysaccharide plays a role in metal ion accumulation, providing essential minerals for cyanobacterial metabolism. Such metals are often the few essential nutrients needed by photoautotrophic strains [38]. Protection of oxygen sensitive nitrogenase (used to fix nitrogen) has also been proposed as a function for attached extra polysaccharide material which can function by limiting oxygen transport to cells [39].

In addition to these important roles, extracellular polysaccharide plays a key role in cell aggregation and in biofilm formation [32]. As a major structural component of biofilm, EPS plays a role in allowing microorganisms to exist in large cell densities of mixed populations. This allows extensive communication to occur and exchange of genetic material via horizontal gene transfer. Participating cyanobacteria can thus adapt and evolve through the acquisition

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of genetic material from cells present in the biofilm community. In cell suspensions, EPS is distributed between the cell surface [in the case of capsular or cell-bound EPS] and the aqueous phase containing slime or free EPS, or as a hydrated matrix in biofilm with a composition that depends on growth phase and solution chemistry [40]. This mixture mediates adhesion and binding through interfacial processes including covalent or ionic bonding, dipole interactions, steric interactions, and hydrophobic association.

Many factors associated with EPS and surface layers of the cyanobacterial biomass can affect metal biosorption. pH has a major effect, where binding is decreased as a function of low pH, while other factors include whether the biomass is free or immobilised, the growth, age and metabolic state of the biomass, surface area of the cells or biomass for binding, the presence of competing ions in the effluent, the equilibrium binding concentrations, the flow rates, the nature of the metal complex and the temperature of the binding reaction. Thus there are numerous parameters that need careful attention to ensure optimal biosorption.

2.1. Composition of the EPS and extrapolysaccharide material

Bacterial EPS can exist in many forms; as cell-bound capsular polysaccharides, unbound "slime", and as an O-antigen component of lipopolysaccharide [41]. EPS is generally observed as a sheath or capsule. This is a thin layer surrounding the cell membrane with concentric or radial fibres which vary in volume and layer composition. The material may also be observed as a slime layer, which is more loosely associated or as a soluble form which is released [42]. Much of the EPS or slime layers have limited association with the surface of the bacterial cell whereas capsular polysaccharides can be strongly connected to cell surfaces by means of a covalent attachment to phospholipid or lipid A molecules at the surface [43]. This division in nomenclature may become masked as capsular material is released and becomes free as a result of growth or leakage of the material into the growth medium.

In general, bacterial polysaccharides are composed of repeating monosaccharide units, forming homo- or hetero- polysaccharides linked via glycosidic linkages. Capsular polysaccharides are usually linear with molecular weights up to 1000 kDa. These are linked to a lipid anchor which in the classical *E. coli* system can be lipid A in the case of lipopolysaccharides. The chemical structure of lipid A is highly conserved, with the general backbone consisting of a β-1-6-linked disaccharide of 2-amino-2-deoxy-D-glucose to which fatty acids, often 3-hydroxyalkanoic acids, are linked by ester or amide linkages [41]. The inner region of the core oligosaccharide consists generally of 3-deoxy-D-manno-oct-2-ulosonic acid and L-glycero-D-manno-heptose. These together with phosphate, and less frequently hexuronic acid and other sugar acid residues, give rise to the anionic nature of both the inner core region and of lipid A. The phosphate groups present often act to link amino alcohols to 4-amino-4-deoxy-L-arabinose or other amino sugar residues present [41]. The complexity can be increased by the presence of stereoisomers, enantiomers, structural modifications of monosaccharide units and branching [44]. Table 2 illustrates the composition of EPS produced by bacteria. The exact nature of cyanobacterial EPS is largely uncharacterised but given the conservation of EPS composition in bacterial species, it can be assumed to be generally similar amongst cyanobacteria. EPS in cyanobacteria is characterized by having relatively few sugar types consisting of

the pentoses- xylose, arabinose and ribose; the hexoses -galactose, glucose (found in 90% of polymers) and mannose (found in 80% of polymers); and derived hexoses rhamnose (found in 80% of polymers), fucose, glucuronic and galacturonic acids, with occasionally methyl and amino sugars [32]. The presence of glucuronic and galacturonic acids associated with EPS further accounts for the anionic nature of many such polymers.

Amongst the cyanobacteria, there is quite a wide variation in the quantity of such polymeric material produced, varying from 144 mg. L^{-1} day^{-1} in the case of *C. capsulate* ATCC 43193 to 2 mg. L^{-1} day^{-1} in the case of *Synechocystis* PCC 6803 [45], which is amongst the lowest producer of this exopolysaccharide material. There have been few studies on whether compositional change occurs in such material. In *Synechocystis*, it has been shown that strains can modify the composition of this material, particularly as a function of culture age and in response to nutrients. In many cyanobacteria, the exopolysaccharide material may be a subcomponent of the extrapolysaccharide material that remains attached to the cyanobacterial cell itself and leaks or is physically broken away due to the habitat location, flow conditions or activity of the organism. This may occur as the extrapolysaccharide layers enlarge and may become solubilized or fractured as a natural part of the growth cycle. In the case of *Synechocystis*, the small amounts released suggest that it occurs at a low level and is not a common behaviour. In *Synechocystis*, neither addition of up to 0.5M of sodium chloride, nor glyoxylate, nor altering the light intensity during growth affected release of exopolysaccharides [45] suggesting that leakage occurred rather than an active release process. The components in *Synechocystis* PCC 6803 and PCC 6714 appeared different from other cyanobacteria. Substituent group analysis showed an absence of acetate or pyruvate with some sulfate substituent detected and a rather high protein content [46]. It had been thought that sulfated exopolysaccharide material was only found in eukaryotic algae so its presence in *Synechocystis* is somewhat unusual. Such sulfated material is thought to have antiviral activity and may be a defence strategy in natural environments [32]. Acetate groups have been proposed to hinder cation binding so their absence in *Synechocystis* is of significance in its potential role in metal binding. Extrapolysaccharide material with high levels of charged groups (such as sulfated derivatives) are important in forming polymeric matrices [47] with metallic ions and indeed natural clay particles being a prerequisite for good metal binding.

Type of Microbial Compound	**Chemical Group and biosorption**
Peptidoglycan	Carboxy groups cation binding
Gram positive surface groups	Phosphate groups cation binding
EPS and related polysaccharide components	Polysaccharide groups uronic acid and sulfate
Microbial surface proteins	Charged Amino acid groups
Archael glucoproteins	Carbohydrate groups + charged amino acids
Algal cellulose	Hydroxyl groups
Fungal chitins, glucans, mannans	Amino groups of chitin

Table 2. Types of microbial surface compounds associated with biosorption.

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2.2. EPS associated genes in *Synechocystis* PCC 6803

In a study to determine key genes associated with the production of EPS in *Synechocystis*, four *Synechocystis* PCC 6803 genes, slr1875 (exoD), sll1581 (gumB), sll0923 (gumC) and sll5052 (gumD), sharing sequence homology with non-photosynthetic bacteria (in brackets), were determined [31]. The expression of these genes was shown to be dispensable for cell growth under standard laboratory conditions. In the wild type PCC 6803 strain, analysis of the EPS showed it formed a thick layer that enclosed the cell, while in the slr1875 and sll1581 deletion mutants, this layer decreased as did the tolerance of *Synechocystis* to salt and heavy metal stresses. The surface charge of *Synechocystis* PCC 6803, which plays a major role in cell interactions with other cells or surfaces, was determined by measuring the zeta potential with electrophoretic mobility. The zeta potential of the wild type strain and mutant strains were −33 mV and between −20 mV and −25 mV respectively indicating that the zeta potential can be correlated with the total amount of EPS and the resulting density of ionic surface charges produced by the cells [31].

Genome comparison of *in silico* translated genes from *Synechocystis* and *E. coli* was used to locate genes in *Synechocystis* that may modulate the cell surface moieties. The *Synechocystis* genes slr0977 and slr0982 (located in a cluster of transport genes) were shown [48] to encode homologs of the *E.coli* proteins Wzm and Wzt, the permease and oligosaccharide binding proteins functioning as an ABC-transporter. Mutation of these genes in *Synechocystis* also resulted in flocculating strains with modulated adherence properties and altered EPS.

3. Biosorption and general characteristics of absorption of metals with *Synechocystis* strains

Heavy metals are discharged from various industries, such as smelters, electroplating facilities, metal refineries, textile, mining, ceramic and glass industries. Some of the chief metals studied in terms of biosorption are those that have the potential to cause most pollution and include lead, antimony, copper, mercury, cadmium, chromium and arsenic as well as radionuclides of elements such as Cobalt, Strontium, Uranium and Thorium [1]. These all have different properties, may exist as complexes, have different oxidation states and their nature may depend on the pH of the medium. The remediation of trace amounts of metals can be carried out via electrolytic extraction, separation processes such as reverse osmosis or dialysis, chemical precipitation or solvent extraction, evaporative methods, or absorption methods such as carbon ion-exchange resin adsorption. However, because of the global problem of metal remediation and the cost of clean-up, new methodologies have been investigated and biosorption falls into this category.

Biosorption offers the following advantages: the volume of chemical and biological sludge can be minimised, there are potentially low operating costs, the possibility of metal recovery and regeneration of the biosorbent afterwards. In recent years, there has also been a significant effort to search for new methods of metallic trace element removal that can be used *in situ* at

contaminated sites. The mechanisms by which metal ions can attach to microbial surfaces can include van der Waals forces, electrostatic interactions, precipitation extracellularly, covalent bonding, redox interactions leading to oxidation or volatilisation (as with mercury) and precipitation, or a combination of such mechanisms. The negatively charged groups (carboxyl, hydroxyl and phosphoryl) of the bacterial cell surface can adsorb metal cations. Cation exchange capacity or ability to bind metals, which can be useful in predicting microorganism-metal interactions, has been determined from pH titration curves for the cyanobacteria *Anacystis nidulans* and *Synechocystis aquatilis* and the green alga *Stichococcus bacillaris*. The results suggest that the exchange capacity is dependent on the external pH of the environment [49]. Thus the physico-chemical environment plays a major role in addition to the binding materials themselves. Many organisms capable of secreting EPS are potential candidates [41] but the ability of cyanobacterial species to grow photoautotrophically in contaminated oligotrophic marine or fresh water environments together with the potential biomass availability, their high sorption characteristics and their non-pathogenic nature makes them ideal candidates for such studies. The cyanobacterial cell surface with EPS consisting of polysaccharide, protein and lipid, together with adsorbed material make them ideal candidates. In addition, model strains such as *Synechocystis* PCC 6803 offer a tool kit of genomic techniques to explore biosorption and examine potential genetic improvements that may be possible.

3.1. Absorption of Cr(VI) and CD(II) by *Synechocystis*

Ozturk *et al* recently reported the removal of Chromium, Cr (VI) and Cadmium, Cd (II) by *Synechocystis sp*. BASO671 [50]. In their experiments, strains with a biomass density of 2.5 at OD_{665nm} were exposed to 10 ppm Cr(VI), Cd(II) and a Cr(VI) + Cd(II) mixture for 7 days in BG11 medium (the standard laboratory growth medium for *Synechocystis*), at 25°C with a light (3000 lux) and a dark cycle of 12/12 h, with shaking. Metal removal was determined as metal in the medium, metal adsorbed onto the surfaces of the cells, and metal accumulated within the cells determined by atomic absorption. Consequently, around 90% of the 10 ppm Cd(II) was absorbed onto the cell surfaces and none accumulated intercellularly. With Cr(VI), some 14% of the 10 ppm Cr(VI) was found to be intercellular with none adsorbed onto the cell surface [50]. In the case of Cd(II), there was an extremely fast adsorption to the surface layers. When mixed metal solutions were added, the preference for Cd(II) binding was confirmed with less binding of Cr(VI). The results suggest that competition for functional groups on the surface of cells may favour one type of metal species over another and suggests that biosorption may be highly dependent on the initial binding kinetics. This study also highlighted a number of interesting issues relating to the production of EPS. The productivity of EPS in strains exposed to Cd(II) compared to strains exposed to Cr(VI) was superior with both strains producing less than controls without metal exposure [50]. However, higher metal exposures (beyond 10 ppm) appeared to enhance the production of EPS, further suggesting the possibility of a stress response to the metal species.

The nature of the monomer composition of *Synechocystis* EPS was monitored as a function of the addition of the single metals [Cd(II) and Cr(VI)] and the mixture of both [50]. Relative to the control, Cr(VI) decreased the uronic content (~25%) of the EPS, while Cd(II) and the mixture

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increased the uronic acid content (~25%). There was no change in glucose content, a general reduction in rhamnose content, an increase in xylose content (~100%) with Cr(VI), which reduced to zero with Cd(II). Glucuronic and galacturonic levels were increased by the presence of both metals [50]. These results suggest that not only is EPS induced in response to metals but that the nature of the EPS alters and that this alteration may be metal specific, at least in the case of chromium and cadmium in *Synechocystis*.

SEM (Scanning Electron Microscopy) and EDS (Energy Dispersive X-ray Spectroscopy) analysis of *Synechocystis* exposed to Cd(II) and Cr(VI) was also carried out and demonstrated that surface roughness was increased, with direct metal binding observed via EDS [50]. Fourier Transform Infra-Red Spectroscopy (FTIR) was also utilised to determine the nature of the functional groups involved in metal binding. Metal binding changed peaks in various parts of the FTIR spectrum, at 3400 cm^{-1} (hydroxyl and amino groups), at 2933 cm^{-1} (aromatic groups), at 1600-1725 cm^{-1} (carboxylic acid groups) and at 1034-1025 cm^{-1} (possibly carboxyl groups of polysaccharides), indicating a role for these groups in metal binding [50].

To determine the optimal biosorption process, a comparative study was carried out using dried, immobilised and live cultures of *Synechocystis sp*. with calcium alginate beads used as the immobilization substrate [51]. The removal efficiency by biosorbent was studied as a function of pH (2-8), temperature (20–40°C), initial cadmium ion concentration (50–300 mg/L), and contact time (0–120 min). The maximum biosorption capacities of the dried, immobilized dried, and immobilized live *Synechocystis sp*. and plain Ca-alginate beads were 75.7, 4.9, 4.3, and 3.9 $mg.g^{-1}$ respectively, under optimum conditions, with the biosorption equilibrium taking 15 min. These results indicated that dried *Synechocystis* biomass was superior for Cd(II) ion removal from aqueous solution by a factor of 15 fold. Interestingly, the dried material could be reused up to 5 times via adsorption and desorption cycles without significant loss in the biosorption capacity [51].

Given the large number of variables that might affect metal biosorption, an approach using response surface methodology (RSM) was employed to study the removal of Cd(II) by *Synechocystis*. RSM allows the study of the effect of several factors influencing the response to metals by varying these factors simultaneously [52]. Utilisation of this approach (RSM) has led to optimization of the critical parameters responsible for higher Cd(II) removal by *Synechocystis pevalekii*. The optimum value of pH, biomass concentration, and metal concentration were pH 6.48, 0.25 mg protein.ml^{-1} and 5 ug.ml^{-1} respectively. Modelling data predicted that 4.29 μg.ml^{-1} Cd(II) would be removed and when experimentally determined, it was found that 4.27 μg.ml^{-1} Cd(II) removal occurred [52]. This data correlated well with model data and indicated the potential utility of such models for predicting biosorption rates.

3.2. Binding of other important metals by *Synechocystis*

Binding of EPS from *Synechocystis* to Cu(II) was investigated using fluorescence spectroscopy [53]. Under different test conditions, *Synechocystis sp*. PCC 6803, grown in BG-11 media, with 72 μmol photon. m^{-2} s^{-1} of light intensity, a photoperiod of 14 hours light to 10 hours dark at 25^0C, was subjected to 0.5-4 μg.ml^{-1} of Cu (II). Three fluorescence peaks were found in the excitation-emission fluorescence spectra of EPS. Fluorescence of peak A (Ex/Em= 275/452 nm)

and peak C (Ex/Em= 350/452 nm) originated from humic-like substances and fluorescence of peak B (Ex/Em= 275/338 nm) was attributed to protein-like substances. Fluorescence of peaks A, B, and C could be quenched by Cu(II). The binding constants indicated that binding to peak A>peak B>peak C, implying that the humic-like substances in EPS have greater Cu(II) binding capacity than the protein-like substances. The binding site number in EPS-Cu(II) complexes for peaks A, B, and C was less than 1 suggesting negative co-operativity between multiple binding sites and the presence of more than one Cu binding site.

Adsorption of metals to cells can be determined through isotherms, which are defined as the amount of adsorbate (in this case metals) bound to adsorbent either as a function of concentration in liquid phase or pressure in the gas phase at constant temperature. The most common isotherms for the evaluation of adsorption kinetics are listed in Table 3. The reader is referred to [1] for a detailed examination on biosorption isotherms and equilibrium sorption studies in relation to metal biosorption. Absorption isotherms (Table 3) for Cu(II) were determined and indicated that physical adsorption followed Langmuir behaviour with the equilibrium being obtained rather slowly and possibly showing monolayer binding [54]. Absorption was shown to be a function of pH with copper hydroxides limiting absorption at alkaline pH [54]. The results suggested that not only is biomass important in metal absorption but also illustrates the importance of pH dependence with alkaline or acidic conditions promoting complexing of metallic ions rather than biomass absorption. For example, it was observed in the case of Cd(II) that complex forms were less likely to be adsorbed onto EPS of *Synechocystis aquatilis* particularly in the presence of chloride [55]. In mixed metal streams there may be competition between various metal cations for binding onto EPS. Whereas little work has been carried out on this area in *Synechocystis*, various selectivity series have been published which reflect such competition, e.g. binding of $Al^{3+} > Ag^{+} > Cu^{2+} > Cd^{2+} > Ni^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+} > Cr^{3+}$ for *Chlorella vulgaris*, and binding of $Cu^{2+} > Sr^{2+} > Zn^{2+} > Mg^{2+} > Na^{+}$ for *Vaucheria* sp. [57, 1]. The presence of ions that can complex with the metal may have dramatic effects on the overall biosorption process, again indicating that variability is dependent not only on biomass factors but also on compositional aspects of the effluents being treated.

Many industries, such as coatings, automotive, storage batteries, aeronautical and steel industries generate large quantities of wastewater containing various concentrations of lead. Data from storage battery producers demonstrated that the pH value of wastewater discarded by these industries ranged between pH 1.6 and 2.9, while the concentration of soluble lead was in the range of 5–15 $mg.L^{-1}$ [57]. The relationship between binding of Pb(II) and Cd(II) on the cell ultrastructure, growth and pigment content of *Synechocystis* PCC 6803 [58] was examined and a dependence on metal concentration was demonstrated. At low level absorption, few growth effects were observed, however as levels of Pb(II) increased to greater than 4 $mg.L^{-1}$, cell ultrastructure changes were observed including thylakoid deterioration suggestive of high levels of accumulation of intercellular Pb(II). Such accumulation could be useful in Pb contaminated environments. In similar studies, the optimum initial pH of biosorption was found to be pH 4.5 with the equilibrium Pb(II) uptake of 2.265 $mg.g^{-1}$ at this pH [57].

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Name of the isotherm	Equation	Principle	Reference
Henry's adsorption isotherm	$q_e = KC_e$	The amount of the adsorbate is proportional to the concentration of the adsorbent	[59]
Freundlich isotherm	$q_e = K_f C_e^{1/n}$	Describes the non-ideal and reversible adsorption not restricted a monolayer	[60]
Langmuir isotherm	$q_e = \frac{Q_{max} b C_e}{1 + bC_e}$	Assumes monolayer adsorption and can only occur at a finite number of definite localized sites, which are identical and equivalent	[60]
Brunauer-Emmett-Teller (BET) isotherm	$\frac{C_e}{q_e(C_s - C_e)} = \frac{1}{q_s C_{BET}} + \frac{(C_{BET} - 1)}{q_s C_{BET}} \frac{C_e}{C_s}$	Describes multilayer adsorption systems with relative pressure	[61]
Temkin isotherm	$q_e = B\ln A_T + B\ln C_e$ $B = \frac{RT}{b_T}$	Adsorbent–adsorbate interactions with temperature effects	[62]

Table 3. Isotherms utilized for adsorption kinetics.

Glossary of Terms: q_e, amount of adsorbate bound to the adsorbent at equilibrium ($mg.g^{-1}$); Kf, Freundlich isotherm constant ($mg.g^{-1}$) ($dm^3.g^{-1}$) related to adsorption capacity; C_e, equilibrium concentration ($mg.L^{-1}$); n, adsorption intensity; $Q_{max,}$ maximum monolayer coverage capacities ($mg.g^{-1}$); b, Langmuir isotherm constant ($dm^3.mg^{-1}$); C_{BET}, BET adsorption isotherm relating to the energy of the surface interaction ($L.mg^{-1}$); C_s, adsorbate monolayer saturation concentration ($mg.L^{-1}$); qs, theoretical isotherm saturation capacity ($mg.g^{-1}$); AT, Tempkin isotherm equilibrium binding constant ($L.g^{-1}$); B, constant related to heat of sorption ($J.\ mol^{-1}$); b_T, Temkin isotherm constant; R, Universal gas constant (8.314 $J.mol^{-1}.^0K^{-2}$); T, temperature at 298^0K.

In an experimental system treating mixed metal wastes in an algal pond using *Synechocystis salina*, it was shown that 60% Cr(VI), 66% Fe(II), 70% Ni and 77% Hg was removed after 13 days of treatment. This reduction correlated with surface absorption [63], however details of the initial metal concentrations were not given.

Antimony (Sb), a non-essential element in biological systems, poses a major problem in mining areas, particularly in China. Around 80% of the world's reserves are deposited here, leaving aquatic environments in the mining areas polluted by long term leaching [64]. Conventional methodologies to remove Sb are limited to precipitation methods such as alum, lime or ferric salts precipitation. Biosorption using *Synechocystis* has been investigated as a potential economic alternative. Here, the added attraction of using *Synechocystis* lies in the fact that it is

a common inhabitant of aquatic environments in the South China region. Absorption of Sb by EPS in *Synechocystis* FASHB898 was examined. It was observed that some 50% of the Sb was absorbed in the first 30 minutes, with equilibrium being reached after 1 hour. Sorption concentrations of 2.61 $mg.L^{-1}$ of Sb per gram dry weight of biomass were determined [64]. It was shown that using initial Sb concentrations of 100 $mg.L^{-1}$ that up to 1.92 $mg.g^{-1}$ was absorbed by EPS, with some 2.64 $mg.g^{-1}$ being located intercellularly. The results of FTIR analysis confirmed that Sb binds to EPS via protein and carbohydrate group interactions as indicated for many other metals. Again it has been suggested that EPS absorption may act as a stress barrier to protect the cells from such metals [64] in natural environments.

In a study examining resistance to Nickel (Ni), 10 different *Synechocystis* strains were initially examined for nickel resistance. The EC_{50} values of the 10 isolates ranged from 2.56 to 17.41 $mg.L^{-1}$, while the EPS concentrations of the 10 isolates ranged from 44 to 143 $mg.L^{-1}$. *Synechocystis sp*. BASO403 and *Synechocystis sp*. BASO404 were chosen on the basis of greatest resistance and highest EPS to examine Ni(II) biosorption [65], thus illustrating the potential utility of certain *Synechocystis* strains for (Ni) removal.

Engineered nanoparticles, particularly particles containing titanium dioxide (TiO_2) are finding application in industry particularly in paints, cosmetics and as part of solar cells. Although relatively inert, TiO_2 can be activated by UV light producing reactive oxygen species which can be antibacterial [66]. Thus with the increased potential use of such nanomaterial's, biological treatment regimens could be compromised by the killing effects on bacterial communities in treatment facilities. It has been demonstrated that *Synechocystis* PCC 6803 has significant ability to biosorb TiO_2 [67]. The response of wild-type *Synechocystis*, which possesses abundant EPS surrounding the cells, to that of an EPS-depleted mutant was also examined and indicated that the EPS play a crucial role in S*ynechocystis* protection against cell killing caused by TiO_2 nanoparticles [67] indicating that it may have potential in remediation of this emerging class of compound.

Manganese (Mn) uptake to cells of *Synechocystis* was measured in cells incubated with Mn solutions. *Synechocystis* cells were shown to be able to take up 150 μM of Mn(II) or Mn(IV) in 48 hours [68]. The predominant accumulation of Mn was associated with the outer membrane for both Mn substrates. Large manganese deposits were found associated with the EPS of *Synechocystis* cells. TEM analysis demonstrated that Mn accumulation occurred on the cell surface and analysis demonstrated that the attached material was manganese phosphate. This bound material withstood multiple washes and appeared to be quite stably bound, indicative of tight binding and its potential as a biosorption material [68].

Arsenic (As) is a widely used component of batteries, a dopant in semiconductors and in optoelectronics. Additionally, it is used in some pesticides and herbicides. Toxicity to humans occurs mainly via drinking water and it is thus important to remove even trace amounts from water. Arsenic is present in two biologically active forms, As(V) and As(III), depending on the redox potential of the environment. Oxidation of As(III) to As(V) is a detoxification process, since As(V) is less toxic than As(III) [69] while arsenate methylation is also a common detoxifying mechanism in many microbial systems. Examination of the response of *Synechocystis* PCC 6803 to arsenic revealed that the organism can grow and accumulate arsenic to high levels. Biomass of *Synechocystis* could accumulate up to 0.38 $g.kg^{-1}$ dry weight when treated with 100 μM sodium arsenite over a 14 day period [70]. When treated with arsenate for six weeks,

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Synechocystis produced volatile arsenicals. An *Ars*M homolog of a known arsenic methylases from *Synechocystis* sp. PCC 6803 was purified and shown to play a role in methylating arsenite *in vitro* with trimethylarsine as the end product. This illustrated the potential utility of this organism in detoxification of arsenic compounds. Amongst a number of cyanobacteria examined, *Synechocystis* was shown to have one of the highest levels of tolerance to arsenic and to be able to accumulate arsenic at a high rate [71]. Genomic studies on tolerance to arsenic have shown that arsenic resistance in *Synechocystis* PCC 6803, in addition to *ars*M (the methylase), was mediated by the *ars*BHC operon which was regulated by *ars*R and two additional arsenate reductases encoded by the *ars*I1 and *ars*I2 genes [72]. *Ars*B encoded an arsenite transporter, *ars*H an FMN-quinone reductase and *ars*C a FMN-quinone reductase. Using a gene array study, a highly orchestrated response to arsenic was observed in *Synechocystis* with 421 genes involved, of which, 179 were induced while 242 were repressed on arsenic addition based on transcriptomic studies [72]. These arrays of genes, whose expression was modified by arsenic were shown to be associated with the repression of growth, the lowering of energy metabolism and the induction of general stress responses which form part of the core transcriptional response to stress in many organisms. The most highly induced genes were those for the *ars*BHC operon [72].

In *Synechocystis* PCC 6803 similar systems for detoxification of mercury are observed as found in many other microbial systems. The protein Grx1, annotated as Slr1562 in the *Synechocystis* genome, selectively interacts with the putative mercuric reductase protein, Slr1849, in PCC 6803. Grx1 which is designated *Mer*A- like, appears to play a major role in catalysing NADPH-driven reduction of mercuric and uranyl ions [73]. In addition to a defence role against the toxicity of such metals, the presence of this system may also have a bioremediation role in mixed effluents. However, its potential has not been realised nor have comparative studies been carried out comparing its detoxification abilities with other organisms.

Sorption of caesium (Cs) by *Synechocystis* PCC 6803 has been examined at concentrations between 1 to 100 μM Cs in the presence of three clay types [74]. Binding was found to occur in two distinct phases, the first step was shown to be a rapid uptake not dependent on light to the clay-cell material and a second slower step which was inhibited by metabolic inhibitors. This data indicated a role for cell and energy dependent uptake, which was pH and salt dependent. The data indicated that the clay adsorption played a significant role supplemented by a slower binding step and accumulation by the cyanobacteria. The practical ability to remove caesium using *Rhodobacter* was analysed from contaminated mud in Japan after the Fukushima accident. Approximately 90% of the Cs found in the mud in a swimming pool could be removed by immobilized cells in a 3 day period [75]. The treatment was repeated 3 times and efficiencies remained high with 84% of the remaining material being sorbed on the second treatment and a further 78% sorbed on the third batch treatment. Here Cs attachment was not altered by nitric acid treatment below pH 2 indicating a strong sediment attachment whereas cell sorption showed major utility. This study indicated the potential for cell sorption in dealing with certain tightly attached radionuclides.

3.3. Reactor configurations for biosorption

Use of diverse biomass material as a biosorption candidate has been infrequently examined. Free biomass, such as microbial cells suffers from a number of disadvantages, including low

mechanical strength, the small size of individual microbial cells and the difficulty of separating cells once they have been utilised to adsorb metals in liquid effluents. Several processes using biomass immobilisation have been investigated to overcome these disadvantages. Immobilisation of biomass in bio-towers, trickle filters, airlift reactors or rotating systems where microbial biofilms play a key role have been examined [76]. As the immobilised biomass grows and its size increases, there is natural expansion and leakage of the biomass, which can then be collected as a microbial sludge. Provided the metals in the wastewater do not have a deleterious effect on the biofilm or other co- habiting organisms, this system can work well. The advantage of rotating immobilised systems, in the case of cyanobacteria, would be that they can still be exposed to light, as opposed to bio-tower systems. Moving sand bed reactors have also been used [77] to develop consortia to treat mixed metal pollutant effluents, which could also provide enough light for cyanobacterial consortia. Technologies and processes for metal recovery are reviewed in [78].

Dried or dead cells may absorb more metals than live cells and for this reason encapsulation of biomass may be advantageous [79], which would mean the utilisation of different process configurations. Although dead cells or biomass can be used, there is little data on the relative merits when compared to live cells. Generally, in addition to metallic pollution, natural waste materials may contain other substances that need remediation, and thus having live biomass may, on occasion, be more advantageous. It is envisaged however that should biosoption be employed at scale then some form of continuous flow through system would need to be employed. Many variables need to be considered; including biomass concentration, pollutant metal concentration, pH of the system, and flow rate. As such studies have been carried out at small laboratory scale there is little data available on large scale systems particularly with cyanobacteria.

Metals absorbed by EPS or biomass are often required to undergo elution in subsequent processes. The nature of such elution processes is dependent on whether the biomass needs to be reused or recycled. Acid or alkali desorption can generally be used for elution [1]. For particular cases, such as precious metal recovery, selective desorption may be used. In the case of radionucleotide recovery, this can occur via combustion and ash removal. In other cases simple liquid extraction may be used on occasion with a variety of solvents. The desorption procedures utilised are thus dependent on the metal, its value and whether the biomass will be reused.

4. Other biodegradative reactions associated with *Synechocystis*

The genome sequences of a number of *Synechocystis* strains have now been determined but there appears to be few genes associated with biodegradation in this organism. As the organism utilises a phototrophic metabolism, it appears to lacks transport systems for organic materials in general as it synthesises most of what it needs. Interrogation of the KEGG database [80] for metabolic activities associated with *Synechocystis* has revealed that the organism does not possess metabolic pathways to degrade organic pollutants. This may not be a general feature of all *Synechocystis* genera as there have been reports that some species may possess

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degradative abilities. *Synechocystis aquatilis* has been reported to degrade 85% of n-octadecane and 90% of pristine added to growth medium [81]. However other than this strain, which indeed may be unique, there appears to be limited metabolic degradative capability within the genus, particularly with respect to organic compounds.

5. Conclusion

Although there have been many studies on the biosorption potential of cyanobacteria, there remains some way to go before their potential may be realised. Many laboratory based studies do not translate to the field. This may be a factor of the altered physicochemical environment, the competition for binding sites for metals when there are mixed metal species present, or the presence of other competing substances in the polluting water. The debate between use of live and dead cells is also open, with some metals showing both EPS binding and bioaccumulation. Bioaccumulation of the metal species may thus be favoured by the use of live biomass. When live cells are used the uptake tends to be bi-phasic, with initial rapid uptake occurring followed by a slower metabolism driven accumulation [74, 79]. Live cells additionally may display the potential to mutate, become more resistant to the metals and adapt to increase metal loadings in the longer term. Indeed there is a trend to seek out specific metal resistant species as biosorbants, in many cases to verify their potential as a start point for further study [82]. Some studies support the use of dead or dried cells, which often show greater metal binding capacity and may be particularly important if the biomass is to be reused a number of times. There have also been attempts to utilise mixed consortia, using organisms with varying and mixed metal sorption capacities [83]. Such consortia, which may develop naturally in response to the metal loading, are difficult to characterise and members are often transient, making the assignment of roles to particular genera or species difficult.

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Chapter 4

Metagenomics — A Technological Drift in Bioremediation

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Additional information is available at the end of the chapter

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Abstract

Nature has its ways of resolving imbalances in its environment and microorganisms are one of the best tools of nature to eliminate toxic pollutants. The process of eliminating pollutants using microbes is termed Bioremediation. Metagenomics is a strategic approach for analysing microbial communities at a genomic level. It is one of the best technological upgradation to bioremediation. Identification and screening of metagenomes from the polluted environments are crucial in a metagenomic study. This chapter emphasizes recent multiple case studies explaining the approaches of metagenomics in bioremediation in different contaminated environments such as soil, water etc. The second section explains different sequences and function-based metagenomic strategies and tools starting from providing a detailed view of metagenomic screening, FACS, and multiple advanced metagenomic sequencing strategies dealing with the prevalent metagenomes in bioremediation and giving a list of different widespread metagenomic organisms and their respective projects. Eventually, we have provided a detailed view of different major bioinformatic tools and datasets most prevalently used in metagenomic data analysis and processing during metagenomic bioremediation.

Keywords: Metagenomics, Bioremediation, Microbial Metagenomes, Bioinformatics, Pollution

1. Introduction

From the day humans started invading this planet, Earth has been crammed with numerous toxic pollutants from multiple sources. Advance scientific technology has given rise to multiple

tools to reduce pollutants in different ways, and bioremediation is considered to be the best way to neutralise polluted environments on Earth [1, 2]. In this genomic era, metagenomic approaches have been developed and are known as effective methods of removing various kinds of pollutants [3, 4]. Metagenomics is a strategic approach of analyzing microbial communities at a genomic level. This provides a glimpse of the microbial community view of "Uncultured Microbiota". Recent studies suggest that microbial communities are the potential alternatives to eliminate toxic contaminants from our environment [5-8]. The term metagenomics was coined by Jo Handelsman et al. in 1998. They have accessed the collective genomes and the biosynthetic machinery of soil microflora during a study of cloning the metagenome [9]. Bioremediation has always been adapting new advances in science and technology for establishing better environments. Compared with the previous years, there has been a gradual increase of interest in metagenomics-based bioremediation studies [10-12]. These studies can prove that metagenomics is one of the best adaptations of bioremediation leading to the establishment of a pure nontoxic environment.

In this chapter, we discussed recent approaches of metagenomics in bioremediation with the help of recent multiple case studies. Preliminarily, we explained the methodology behind metagenomic analysis, starting from the sample screening and ending up with metagenomic analysis with respect to bioremediation. Metagenomic bioremediation reviews and extracts microbial communities applying their extensive biochemical pathways in degrading toxic pollutants. A part of our study aims to emphasize multiple case studies of metagenomic applications on air, water, and soil contaminations. Our analysis provided a topic-specific landscape with respect to metagenomic bioremediation of water contaminations, soil contaminations, and followed by air contaminations. The following part of our study focuses on recently developed sequence and function-based metagenomic strategies to analyze metagenomes from contaminated environments. In addition to this, our study explains the highly prevalent metagenomes derived from metagenomic communities which are also highly capable of degrading contaminations and toxins in the environment. Finally, we provided a landscape view of multiple bioinformatic tools used in the processing and analysis of metagenomic bioremediation data.

2. Applications of metagenomics in bioremediation

Environmental scientists consider metagenomic bioremediation as one of the potential tools to remove contaminants from the environment [13-15]. As cited earlier, recent multiple studies have reported metagenomic approaches in bioremediation. When this was compared with the other approaches of bioremediation, metagenomic bioremediation provided best outcomes with better degrading ratios. The results of a recent study emphasized the potential of metagenomic bacteria derived from petroleum reservoirs [16]. In this study, microbial strains and metagenomic clones have been isolated from petroleum reservoirs, and petroleum degradation abilities were evaluated either individually or in pools using seawater artificial ecosystems. The results showed that metagenomic clones were able to biodegrade up to 94% of phenanthrene and methyl phenanthrenes with rates ranging from 55% to 70% after 21 days

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[16]. The authors concluded that bacterial strains and metagenomic clones showed high petroleum-degrading potential.

Metagenomic approaches in bioremediation aid in comprehending the characteristics of bacterial communities in different kinds of contaminated environments. A metaproteogenomic study was carried out on long-term adaptation of bacterial communities in metal-contaminated sediments [17]. The aim of this study was to understand the effect of a long-term metal exposure (110 years) on sediment microbial communities. In this study, the authors selected two freshwater sites differing by one order of magnitude in metal levels. The samples extracted from the two sites were compared by shotgun metaproteogenomics which resulted in a total of 69–118 Mpb of DNA and 943–1241 proteins. The two communities were found to be functionally very similar. However, significant genetic differences were observed for three categories: synthesis of exopolymeric substances, virulence and defense mechanisms, and elements involved in horizontal gene transfer. This study can be considered as a best example of advanced metagenomic approaches applied in bioremediation of different contaminated environments.

3. Metagenomic bioremediation of different contaminations

The environment where human activity abounds is being more polluted and contaminated by different kinds of toxic contaminants [18-20]. The contaminations are diverse and cover almost all sources of life including water, soil, and air which are considered the most important sources of life [21-23]. Metagenomic analysis is applied to multiple kinds of polluted environments primarily soil- and water-contaminated environments [24, 25].

3.1. Metagenomic bioremediation of soil contaminations

Soil contamination is a serious contamination [26, 27] as soil is considered as one of the major sources of life [28]. Compared with other approaches of bioremediation, microbial and environmental researches are more inclined in applying metagenomic approaches to bioremediation [10, 29, 30]. A recent case study discusses the metagenomic analysis of arctic soils contaminated by high concentration of diesel in Canada [31]. As this study was on arctic soils, the objective framed was to trace out microorganisms and their functional genes which are abundant and active during hydrocarbon degradation at cold temperature. In this study, scientists have sequenced the soil metagenome and performed reverse-transcriptase real-time PCR (RT-qPCR) to quantify the expression of several hydrocarbon-degrading genes. Pseudomonas species were detected as the most abundant organisms in diesel-contaminated soils at cold environments. RT-qPCR assays confirmed that Pseudomonas and Rhodococcus species actively expressed hydrocarbon degradation genes in arctic biopile soils. The results of this study indicated that biopile treatment leads to major shifts in soil microbial communities which favors aerobic bacteria to degrade hydrocarbons [31].

3.2. Metagenomic bioremediation of water contaminations

Water pollution has dramatically increased in comparison with the conditions of the 20th century [32, 33]. Metagenomic application in the bioremediation of water contamination is one of the best ways to reduce water contaminations [34-37]. Recent multiple case studies suggest that metagenomic applications have been widely used for the identification and treatment of pollutants and contaminations in the sea, ground water, and drinking water [34-37]. A recent research performed at the Gulf of Mexico beaches precisely talks about the longitudinal metagenomic analysis of water and soil affected by deepwater horizon oil spill [34]. Approximately 7×105 cubic meters of crude oil were released into the Gulf of Mexico as a consequence of deepwater horizon drilling rig explosion, where thousands of square miles of the earth's surface were covered in crude oil. During this study, researchers performed high throughput DNA sequencing of close-to-shore water and beach soil samples before and during the appearance of oil in Louisiana and Mississippi. The sequencing results have identified an unusual increase in the human pathogen *Vibrio cholera*, a sharp increase in *Rickettsiales sp.*, and decrease of *Synechococcus sp.* in water samples [34]. In addition, a metagenomic analysis was also performed for the bioremediation of hexavalent chromium-contaminated water that existed in fixed-film bioreactor [38]. This study talks about hexavalent chromium (Cr^{6+}) contamination from a dolomite stone mine in Limpopo Province, South Africa, causing extensive groundwater contamination. To restrict any further negative environmental impact at the site, an effective and sustainable treatment strategy for the removal of up to 6.49 mg/l Cr^{6+} from the groundwater was developed. The microbial community shifted in relative dominance during operation to establish an optimal metal-reducing community, including *Enterobactercloacae*, *Flavobacterium sp.* and *Ralstonia sp.*, which achieved 100% reduction. This study provides a glimpse of effective demonstration of a biological chromium (VI) bioremediation system [38].

4. Metagenomic strategies and tools for bioremediation

Advanced scientific technology has given rise to the advancements in research tools applied in different fields of scientific research [39]. These technologically advanced inventions have driven scientific researchers towards finding out some unrevealed things of nature [40]. Multiple technologies have started getting embedded to metagenomics for a better understanding of biological and life sciences [41]. Thus, in this section, we have discussed recent major metagenomic strategies and tools applied in the process of metagenomic bioremediation.

4.1. Screening of metagenomes from polluted environments

Identification and screening of metagenomes from polluted environments are crucial in a metagenomic study. The microbial community interaction can be detected precisely when metagenomes are finely screened from a contaminated environment. A methodology proposed from a recent study [42] suggested an updated technology of high throughput genetic

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screening of a soil metagenomic library. The study was initiated by adding a typical composition of oligonucleotide probes to soil metagenomic DNA for hybridization. The pooled radiolabeled probes were designed to target genes encoding specific enzymes. The soil metagenomic DNA of fosmid clone library were spotted on high-density membranes before the addition of oligonucleotide probes. This next step was followed by affiliation of positive hybridizing spots to the corresponding clones in the library and sequencing of metagenomic inserts.

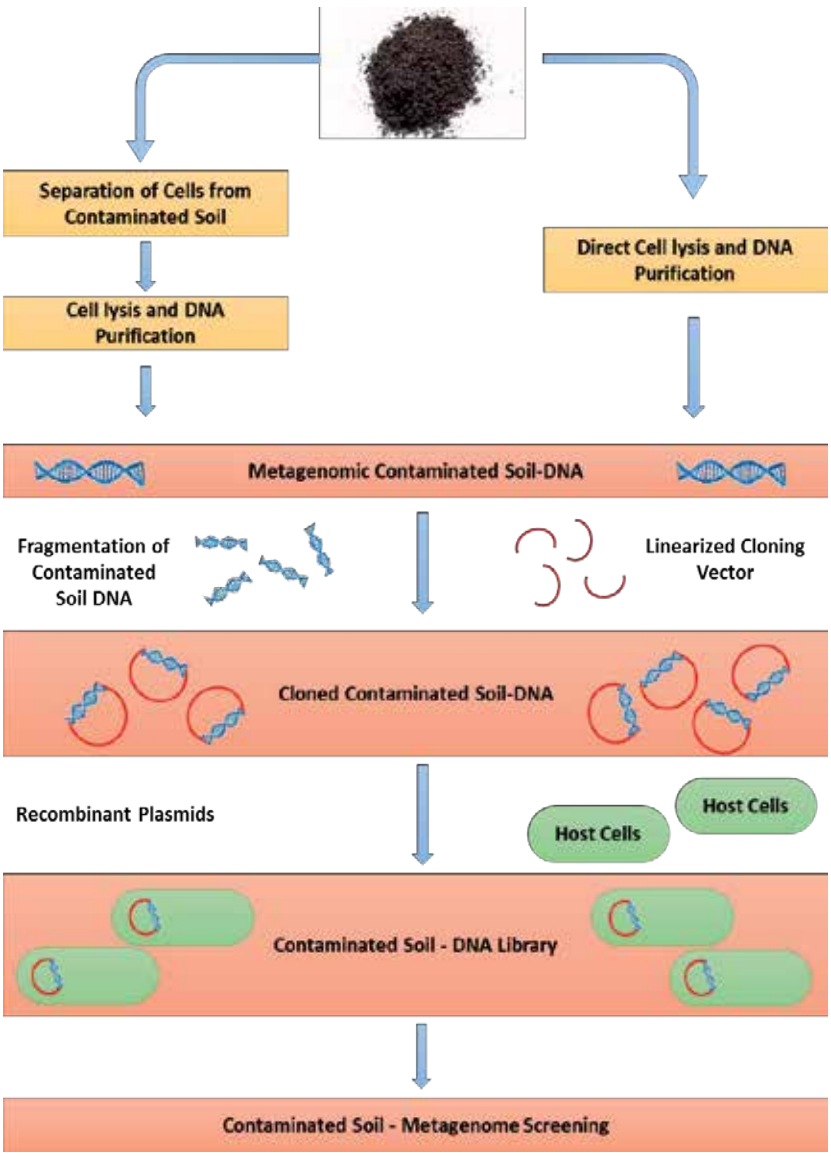

Figure 1. An ideal systematic workflow of steps involved in contaminated-soil metagenomics.

When assembly and annotation were completed, new coding DNA sequences related to genes of interest were identified with low protein similarity against the closest hits in the databases. This work basically highlights the sensitivity of DNA/RNA hybridization techniques as an effective and complementary way to recover novel genes from large metagenomic clone libraries with respect to soil microbiota. Nevertheless, multiple molecular biological-based techniques [43] may also be applied during the process of metagenome extraction and screening. The basic workflow of extracting metagenomes out of contaminated soil has been explained in Fig. 1. The steps were initiated by collecting contaminated soil from the environment. The collected contaminated soil sample can be processed in two ways; one is by direct cell lysis and DNA purification and second, by separation of cells from contaminated soil and then followed by cell lysis and DNA purification. The isolated DNA is then cloned using specific cloning vectors. The cloned contaminated soil DNA is then delivered into host cells using different gene delivery systems. The multiplied host cells containing contaminated soil DNA forms a Metagenome library and these contaminated soil metagenomes were then screened. A recent study conducted screening of biosurfactant producers from petroleum hydrocarbon contaminated sources in cold marine environments. In this study, the researchers have isolated and characterized 55 biosulphant microbiota of 8 different genera including 1 *Alcanivorax*, 1 *exiguobacterium*, and 2 *halomonas* strains [44].

4.2. Florescence-Activated Cell sorting (FACS)

Florescence-activated cell sorting is one of the most widely used cell sorting techniques which is applied to sort microbial cells based on florescence during the process of metagenomic screening [45], with an accuracy rate of 5,000 cells per second [46]. Figure 2 shows the schematic flow of SIGEX and intercellular biosensors methods. High-throughput screening does not require a selectable phenotype. This phenomenon has led to the focus on phenotypes such as pigments that are readily visible providing the use of fluorescence-activated cell sorting. Moreover, FACS can be used to detect expression of certain types of genes by regulation of a fluorescent biosensor present in the same cell as the metagenomic DNA [47, 48]. Hence, these screen methods will be a critical tool for rapid selection of cells from metagenomic libraries.

4.3. Metagenomic sequencing strategies

Genome sequencing technologies have been frequently upgraded [49] since the completion of the human genome at the beginning of the 21st century [50]. Multiple next-generation genomic sequencing strategies are applied to sequence the metagenomes of different microbial communities [51, 52]. Sequencing technologies were initiated by the Sangers sequencing method which was widely used during the process of human genome sequencing [53, 54]. Technological drift has gifted next-generation sequencing techniques like pyrosequencing [55, 56], ligation sequencing [57, 58], reverse terminator [60, 61], and single-molecule sequence by synthesis [62, 63], providing a high throughput that reads comparatively in less time [64-66]. A comparative overview of recent sequencing technologies applied in metagenome sequencing is provided in Table 1 for a more detailed understanding. However, most metagenomics researchers prefer the pyrosequencing method for sequencing the metagenomes of microbial communities [67-70].

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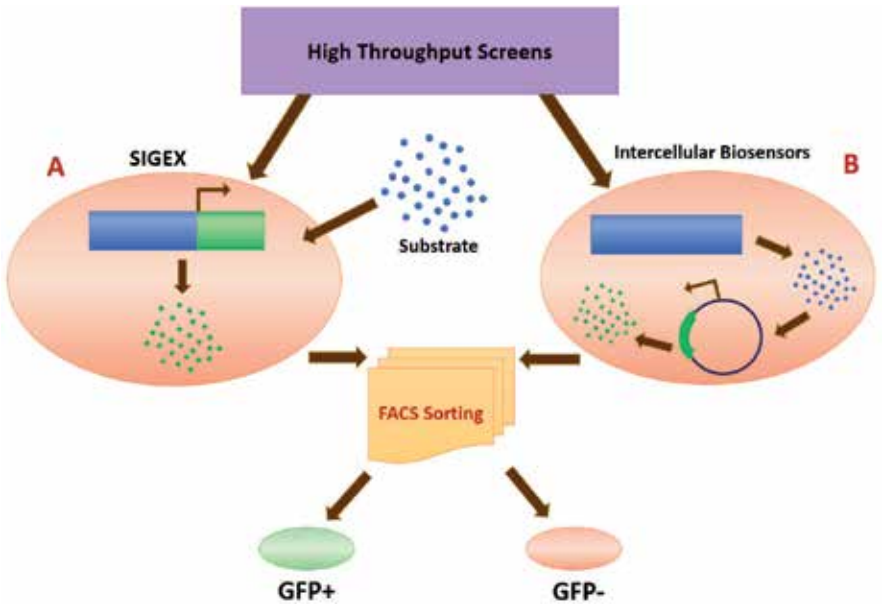

Figure 2. Systematic workflow representing the examples of high-throughput screens of (A) SIGEX and an (B) intracellular biosensor. SIGEX exploits the principle that catabolic genes are often substrate-induced by fusing a promoterless GFP to the metagenomic DNA and identifying clones in which GFP production is induced by the substrate of interest. An intracellular biosensor detects biologically active small molecules. GFP expression is dependent on the presence of a small molecule that activates a regulator. Finally, FACS is used to sort the GFP+ and GFP- cells separately.

Sequence Reaction Method	Read Length	Amplification	Data Production	Templates per run	Commercially Available as
Sanger's Method	~900 to 1,100	PCR	1 Mb per day	96	ABI 3730xl
Pyrosequencing	~400	Emulsion PCR	400 Mb per run in 7.5 to 8 hours	1,000,000	454 FLX Roche
Reverse Terminator	36 to 175	Bridge PCR	>17 Gb per run in 3 to 6 days	40,000,000	Illumina SOLEXA Genome Analyser
Ligation Sequencing	~50	Emulsion PCR	10 to 15 Gb per run in 6 days	85,000,000	ABI SOLiD
Single Molecule Sequence by Synthesis	30 to 35	None	21 to 28 Gb per run in 8 days	800,000,000	Helicos Heliscope

Table 1. A Comparative overview of next-generation sequencing technologies applied in metagenome sequencing

5. Prevalent metagenomes for bioremediation

Metagenomes extracted from uncultured microbial communities from multiple contaminant sites are screened and further identified for degrading properties [71]. Microbial communities

vary according to the characteristics of source and site of contamination [72]. A metagenomic analysis conducted on the heavy metal-contaminated groundwater revealed metagenomes of γ- and β-Proteobacteria dominated by *Rhodanobacter*-like γ-proteobacterial and *Burkholderia*-like β-proteobacterial species from the habitat of extremely high levels of uranium, nitrate, technetium and various organic contaminants [73]. Moreover, multiple metagenome projects have been taking place around the world; we have sorted out a list of multiple environmental metagenome projects with top microbe having the highest percentage of presence in the metagenomic community (Table 2). Studies on microbial adaptation of toxic environments may give rise to trace new metagenomic communities useful for efficient bioremediation. Specific functions and interactions of microbial communities with respect to contamination-degrading capabilities can be a result of environmental-based gene switching in the metagenomes.

Top Phylum	Percentage of Presence in Community	Domain	Metagenome Projects	Source
Actinobacteria	38.04	Bacteria	BASE - Biomes of Australian Soil Environments	Soil
Chlorobi	56.04	Bacteria	Antarctica Aquatic Microbial Metagenome	Environmental
Actinobacteria	38.21	Bacteria	American Lake Mendota metagenome	Water
Proteobacteria	31.62	Bacteria	Swedish Lake Vattern metagenome	Water
Proteobacteria	29.68	Bacteria	Detoxification of arsenic mediated by microbial sulphate reduction in Mediterranean marine sediments	Environmental
Proteobacteria	48.12	Bacteria	Illumina and 454-based metatranscriptomic analyses of a diatom-induced bacterioplankton bloom in the North Sea	Environmental
Unassigned Bacteria	34.8	Bacteria	Functional metagenomic profiling of Tibetan Plateau soils affected by permafrost or seasonal freezing	Soil
Euryarchaeota	22.71	Archaea	Lonar Lake Sediment prokaryotic metagenome	Water
Unassigned Bacteria	53.84	Bacteria	Metagenome of a microbial consortium obtained from the tuna oil field in the Gippsland Basin, Australia	Environmental
Actinobacteria	27.1	Bacteria	Meta soil	Soil

Table 2. List of multiple environmental metagenome projects with top microbe having the highest percentage of presence in the metagenomic community

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6. Bioinformatic tools for metagenomic bioremediation

In the last two decades, bioinformatics has been advanced and simultaneously adapted to multiple fields of science such as basic sciences and advanced applied sciences [74]. Our previous study has given a glance of basic applications of bioinformatics in bioremediation [75]. Bioinformatics holds multiple tasks in the field of metagenomic bioremediation, majorly during metagenomic data analysis [76, 77]. A special issue on bioinformatics approaches and tools for metagenomic analysis has provided an advanced view towards comprehensive bioinformatic tools and methodologies used in metagenomics [78].

Multiple metagenomic projects are generating a large chunk of metagenomic sequence data challenging bioinformatics to develop more robust and better tools to analyze metagenomic sequence data. A recent study reveals the metagenomic characterization of soil microbial community using metagenomic approaches [79]. In this study, researchers have used 33 publicly available metagenomes obtained from diverse soil sites and integrated some state-of-the-art computational tools to explore the phylogenetic and functional characteristics of the microbial communities in soil. Recently, multiple advancements have taken place in the field of bioinformatics with respect to metagenomic bioremediation. In this section, most of our study focuses on recent bioinformatic tools and datasets majorly used in the analysis of metagenomic data in bioremediation. A comparative overview of functions and suitability of mostly used tools for metagenomic analysis is given in Table 3.

6.1. MEGAN

Meta Genome Analyzer (MEGAN) is one of the most widely used software tools for efficiently analyzing large chunks of metagenomic sequence data [80, 81]. This tool is most preferably used to interactively analyze and compare metagenomic and metatranscriptomic data, taxonomically and functionally. To perform taxonomic analysis, the program places reads onto the NCBI taxonomy and functional analysis is performed by mapping reads to the SEED, COG, and KEGG classifications. In addition, samples can be compared taxonomically and functionally, using a wide range of charting and visualization techniques like co-occurrence plots. This software also performs PCoA (Principle Coordinate Analysis) and clustering methods allowing high-level comparison of large numbers of samples [82]. Different attributes of the samples can be captured and used during analysis. Moreover, MEGAN supports different input formats of data and is capable of exporting the results of analysis in different text-based and graphical formats. Multiple methods of analysis, acceptance and comparison of high throughput data, robustness and being easy-to-handle are some of the features that made MEGAN as one of the most used metagenome analyzers.

6.2. SmashCommunity

Simple Metagenomics Analysis SHell for microbial communities (SmashCommunity) is a stand-alone metagenomic annotation and analysis pipeline that shares design principles and routines with SmashCell [83]. It is suitable for data delivered from Sanger and 454 sequencing technologies. It supports state-of-the-art software for essential metagenomic tasks such as

assembly and gene prediction. It also provides tools to estimate the quantitative phylogenetic and functional compositions of metagenomes, to compare compositions of multiple metagenomes, and to produce intuitive visual representations of such analyses [84]. It provides optimized parameter sets for Arachne and Celera for metagenome assembly, and GeneMark and MetaGene for predicting protein coding genes on metagenomes. SmashCommunity also includes scripts for downstream analysis of datasets. They can generate intuitive tree-based visualizations of results using the batch access API of the interactive Tree of Life (iTOL) web tool. SmashCommunity can also compare multiple metagenomes using these profiles, cluster them based on a relative entropy-based distance measure suitable for comparing such quantitative profiles, perform bootstrap analysis of the clustering, and generate visual representation of the clustering results.

6.3. CAMERA

Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis (CAMERA) is a database and associated computational infrastructure that provides a single system for depositing, locating, analyzing, visualizing, and sharing data about microbial biology through an advanced web-based analysis portal [85]. CAMERA holds a huge chunk of data including environmental metagenomic and genomic sequence data, associated environmental parameters, pre-computed search results, and software tools to support powerful cross-analysis of environmental samples. CAMERA works on a pattern of collecting and linking metadata relevant to environmental metagenome datasets with annotation in a semantically aware environment that allows users to write expressive semantic queries to the database. It also provides data submission tools to allow researchers to share and forward data to other metagenomic sites and community data archives. CAMERA can be best considered as a complete genome-analysis tool allowing users to query, analyze, annotate, and compare metagenome and genome data [86].

6.4. MG-RAST

Rapid Annotation using Subsystems Technology for Metagenomes (MG-RAST) is an automated analysis platform for metagenomes, providing quantitative insights into microbial populations based on sequence data [87]. This pipeline performs quality control, protein prediction, clustering, and similarity-based annotation on nucleic acid sequence datasets using a number of bioinformatic tools. Users can upload raw sequence data in FASTA format; the sequences will be normalized and processed, and summaries will be automatically generated. The MG-RAST server provides several methods of access to different data types, including phylogenetic and metabolic reconstructions, and has the ability to compare metabolism and annotations of one or more metagenomes and genomes. In addition, the server also offers a comprehensive search capability. The pipeline is implemented in Perl by using a number of open-source components, including the SEED framework, NCBI BLAST, SQLite, and Sun Grid Engine.

Suitability	SmashCommunity	MG-RAST	IMG/M	RAMMCAP	MEGAN	MOTHUR
Single Genomes	-	-	+	+	-	-
Single Cell Genomes	-	-	-	-	-	-
Shotgun Metagenomes	+	+	+	+	+	-
16S rDNA Metagenomes	+	+	-	+	+	+
Functions						
Assembly	+	-	+	-	-	-
Gene Prediction	+	-	+	+	-	-
Functional Analysis	+	+	+	+	+	-
Taxonomy Assignment	+	+	+	+	+	+
Comparative Analyses	+	+	+	+	+	+
Data Management	+	-	+	-	-	-

+Yes, -No

Table 3. A comparative overview of functions and suitability of mostly used tools for metagenomic analysis

6.5. IMG/M

Integrated Microbial Genomes and Metagenomes (IMG/M) system supports annotation, analysis, and distribution of microbial genome and metagenome datasets. IMG/M provides comparative data using analytical tools extended to handle metagenome data, together with metagenome-specific analysis [88, 89]. IMG/M consists of samples of microbial community aggregate genomes integrated with IMG's comprehensive set of genomes from all three domains of life: plasmids, viruses, and genome fragments. Function-based comparison of metagenome samples and genomes is provided by analytical tools that allow examination of the relative abundance of protein families, functional families or functional categories across metagenome samples and genomes. It seems like registered users can gain more advantage out of IMG/M as the tools focus on handling substantially larger metagenome datasets, are available only to registered users as part of the 'My IMG' toolkit, and support specifying, managing, and analyzing persistent sets of genes, functions, genomes or metagenome samples and scaffolds.

7. Summary

Metagenomics is a strategic approach for analyzing microbial communities at a genomic level. This gives a glimpse towards the microbial community view of "Uncultured Microbiota". Bioremediation has always been adapting new advances in science and technology for

establishing better environments, and metagenomics can be considered as one of the best adaptations ever. Identification and screening of metagenomes from the polluted environments are crucial in a metagenomic study. The second section emphasizes recent multiple case studies explaining the approaches of metagenomics in bioremediation. Accordingly, the third section speaks about metagenomic bioremediation in different contaminated environments such as soil and water. The fourth section explains different sequences and function-based metagenomic strategies and tools starting from providing a detailed view of metagenomic screening, FACS, and multiple advanced metagenomic sequencing strategies. The fifth section deals with the prevalent metagenomes in bioremediation giving a list of different prevalent metagenomic organisms and their respective projects. The last section gives a detailed view of different major bioinformatic tools and datasets most prevalently used in metagenomic data analysis and processing during metagenomic bioremediation.

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Chapter 5

Biosurfactants as Useful Tools in Bioremediation

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Additional information is available at the end of the chapter

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Abstract

Environmental pollution by organic contaminants is a major problem today because it has affected many environments. Hydrophobic contaminants are of special concern since their molecules can be bound to the soil particles, but because of its low solubility in water and high interfacial tension, those contaminants cannot be easily removed. To help with desorption of contaminants, surfactants can be used in soil and water remediation technologies. Amphiphiles that can form micelles are termed as surface active agents or *surfactants* and are among the most commonly used chemicals in everyday life. Chemically produced surfactants have increasingly been replaced by biotechnology-based products, obtained either by enzymatic or microbial synthesis, because they can be produced using natural resources. The group of surface active biomolecules produced by living organism is called *biosurfactants*. Originally, biosurfactants attracted attention as hydrocarbon-dissolving agents in the late 1960s and as potential replacements for synthetic surfactants (carboxylates, sulfonates and sulfate acid esters) in the food, pharmaceutical, and oil industries. Synthetic surfactants currently used are usually toxic and hardly degraded and as such are also a contaminant in the environment. To replace synthetic surfactants, biosurfactant production needs to be cost-effective; therefore, it is important to develop culture conditions with low-cost materials using efficient biosurfactant-producing microbial strains. Although bacteria have been extensively studied for biosurfactant production, yeasts are also potential biosurfactant-producing microorganisms. Because of their unique structures, biosurfactants may have a greater range of properties that can be

exploited commercially. This review article will describe microorganisms related to biosurfactant production, including yeasts, as well as their role in bioremediation.

Keywords: Biosurfactant, soil yeast, microbial communities in soil, bioremediation

1. Introduction

Recently, there are many reports of soil and surface water locations that are contaminated with organic pollutants, with a great impact on soil and groundwater. Because of its low solubility in water and high interfacial tension, those contaminants cannot be easily removed. Bioremediation has become one of the methods used in the remediation of contaminated sites; bioremediation strategies are based on the use of different microorganisms: bacteria, yeasts, or fungi isolated from soil or from a place where there is a presence of contaminants such as hydrocarbons, which facilitate the cleaning of the contaminated sites. Bioremediation studies begin with the isolation and identification of microorganisms from soil and water that are able to degrade these contaminants. Some hydrocarbon-degrading microorganisms are also able to produce biosurfactants. Biosurfactants produced by the microorganisms in the environment help them to take the hydrocarbons as carbon source, either by making available the hydrocarbon by releasing biosurfactant into the environment or by changing its cell surface so that the contaminant can be absorbed.

Originally, biosurfactants attracted attention as hydrocarbon dissolving agents in the late 1960s as potential replacements for synthetic surfactants (carboxylates, sulfonates, and sulfate acid esters), especially in the food, pharmaceutical, and oil industries. Synthetic surfactants currently used are usually toxic and hardly degraded by microorganism, causing damage to the environment. Most of the biosurfactants are high molecular weight lipid complexes, which are normally produced under aerobic conditions. The classification of biosurfactants is based on their chemical composition, their mode of action, and the microorganisms that produce it. Biosurfactants can be of high or low molecular weight, and based on their composition, they can be glycolipids, phospholipids, lipopeptides, or a mixture of amphiphilic polysaccharides, proteins, lipoproteins, or lipopolysaccharides. Microorganisms also produce surfactants that are in some cases a combination of many chemical types referred to as the *polymeric microbial surfactants*. Because of their unique structures, biosurfactants may have a large range of properties that can be exploited commercially.

Regarding their mechanism of action, some compounds are better at decreasing the surface tension (biosurfactants), and others are able to produce stable emulsions (bioemulsifiers). Best known biosurfactants are produced by bacteria, and there are many studies on them, especially on *Pseudomonas* spp., strains that produce rhamnolipids. However, it is necessary to find new types of biosurfactants and bioemulsifiers, and the studies of other organisms are increasing recently. Yeast and fungi have demonstrated to produce biosurfactant and bioemulsifiers with very good results. The aim of this chapter is to describe microbial biosurfactant, especially those produced by yeasts and to propose their use in bioremediation.

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2. General characteristics of biosurfactants

Recently, there are many reports of soil and surface water locations that are contaminated with organic pollutants, with a great impact on soil and groundwater. During this process, molecules of the pollutant have bound to the soil particles as it has moved toward the groundwater and are therefore difficult to remove it from soil because many of these pollutants have low solubility and high interfacial tensions with water [1].

To help on the solution of this problem, surfactants can be used to clean the contaminated soil and water. Surfactants are one of the most commonly used chemicals in everyday life. Since the beginning of the 20th century, the production of a wide spectrum of synthetic surfactants from petroleum resources has increased intensively. The amphiphiles that can improve surface–surface interactions by forming micelles are termed as surface active agents or *surfactants* [2]. Surfactants are amphiphilic molecules consisting of a hydrophobic and a hydrophilic portion [3]. Usually, the hydrophobic portion is a nonpolar long chain of fatty acids, whereas the hydrophilic domain can be nonionic, positively or negatively charged, or amphoteric, frequently a carbohydrate, an amino acid, or a phosphate [4–6].

Increasing concentrations of surfactant into an oil/water or water/air systems causes a reduction in surface tension up to a critical point where the surfactant can form structures like micelles, bilayers, or vesicles. This concentration defines the critical micelle concentration (CMC). To determine this value, the solution containing the surfactant is diluted severalfold; surface tension is measured for each dilution, and the CMC is calculated from this value. Surface tension can be easily measured with a tensiometer. There are surfactant molecules that are able to reduce the surface tension of water from 72 to around 27 mN m^{-1} [7]. When water, oil, and a surfactant are mixed, the surfactant rests at the water–oil interface; these systems are called *emulsions* or *microemulsions* depending on their stability [8, 9]. These characteristics confer excellent detergency and emulsifying, foaming, and dispersing capacities, which make surfactants one of the most versatile chemicals in industrial processes [10].

Current, worldwide surfactant market is around $9.4 billion annually, while their production has been reported to be approximately 10 million tons, and their use is divided almost equally between household detergents and several industrial applications [10, 11]. Synthetic surfactants have increasingly been replaced by biotechnology-based compounds, derived either from enzymatic or microbial synthesis, because they can be produced using natural sources [12]. The group of surface active biomolecules produced by living organism is called *biosurfactants*.

Synthetic surfactants currently used are toxic and hardly degraded, causing damage to the environment. Initially, biosurfactants were considered to have applications in the food, pharmaceutical, and oil industries [13–15]. Biosurfactants have several advantages over chemical surfactants, including lower toxicity, higher biodegradability, effectiveness at extreme temperatures or pH values, biocompatibility, and digestibility. Also, biosurfactants can be produced using agroindustrial waste material; they can be economically produced and show better environmental compatibility. The microorganisms that produce the biosurfactant

can be modified by genetic engineering or biological and biochemical techniques. Because the possibility of practical applications for biosurfactants depends on whether they can be produced economically, there have been many efforts to optimize its biological production [16–18]. To replace synthetic surfactants, biosurfactant production needs to be of low-cost, and up to now, there are few studies on the use of low-cost materials on the pilot plant or industrial scale [4].

Most of the biosurfactants are lipid-containing molecules, which are normally produced under aerobic conditions [16]. The classification of biosurfactants is based on their chemical composition, their mode of action, and the microorganisms that produce it. Biosurfactants can be of high or low molecular weight, and based on their composition, they can be glycolipids, phospholipids, lipopeptides, or a mixture of amphipathic polysaccharides, proteins, lipoproteins, or lipopolysaccharides. Biosurfactants with low molecular mass are efficient in lowering surface and interfacial tensions, whereas biosurfactants with high molecular mass are more effective at stabilizing oil-in-water emulsion (Figure 1) [19]. Microorganisms also produce surfactants that are in some cases a combination of many chemical types referred to as the *polymeric microbial surfactants* [8].

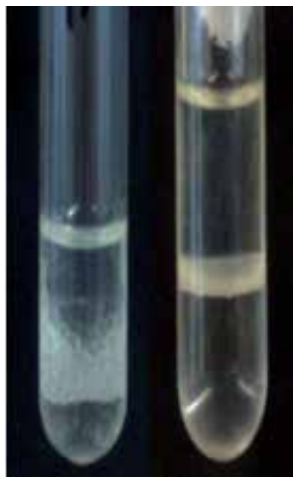

Figure 1. Stable emulsions (EI_{24}) produced by mixing a cell-free supernatant from a biosurfactant-producing yeast and hexadecane. The tube on the left shows a clear emulsion characteristic of polymeric biosurfactants. The tube on the right shows a compact stable emulsion that is characteristic of low molecular weight biosurfactants.

Low molecular weight biosurfactants are usually glycolipids or lipopeptides; the later are usually produced by bacteria from the *Bacillus* genus and is composed of a cyclic peptide and a fatty acid residue. Among the glycolipids, the most studied is rhamnolipid, which is produced by *Pseudomonas aeruginosa* strains; it is composed of a backbone of two rhamnose moieties and two fatty acid residues. Other glycolipid biosurfactants include trehalolipids, produced by *Rhodococcus erytropolis* and other bacterial genera, and sophorolipids, produced by several yeast strains (Figure 2a and 2b). The physicochemical properties of low molecular weight biosurfactants are influenced by the fatty acid residues that contain, and those in fact

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depend on the bacterial strain used and on the growth conditions and nutrients present. High molecular weight biosurfactants are usually a complex mixture of macromolecules containing proteins, polysaccharides, and lipid residues. The most studied polymeric biosurfactant is emulsan, produced by *Acinetobacter calcoaceticus* (Figure 2c) [1, 10]. Because of their unique structures, biosurfactants may have a greater range of properties that can be exploited commercially [20].

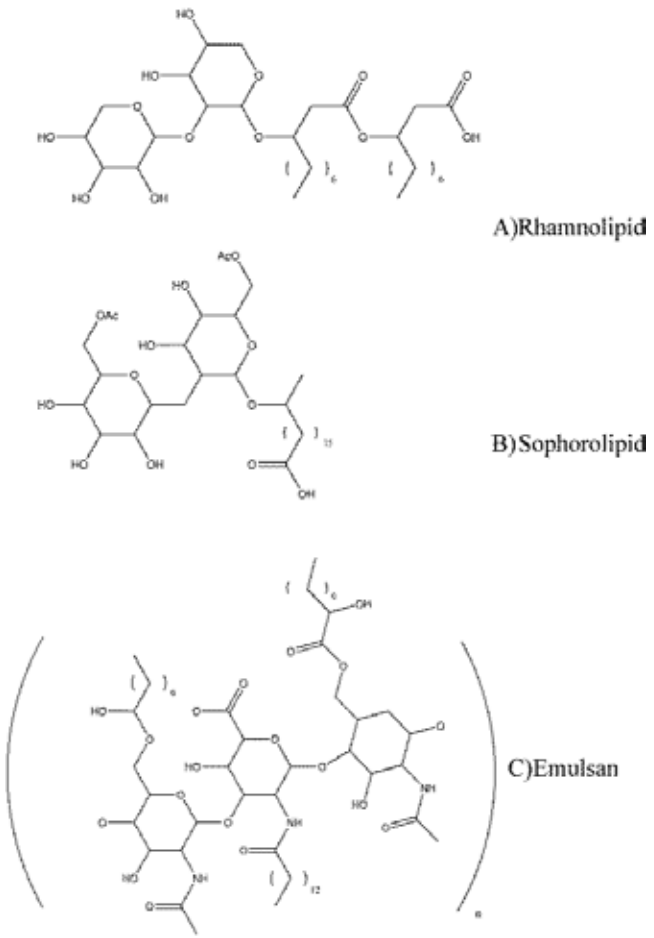

Figure 2. Chemical structures of some of the most common biosurfactants. Low molecular weight glycolipids: (a) rhamnolipid and (b) sophorolipid; high molecular weight glycolipids: (c) emulsan.

3. Biosurfactant-producing microorganisms

The ability of microorganisms to degrade hydrocarbons was first described in 1895 by Misyoshi, who reported the microbial degradation of paraffin. Many different microbial

species of bacteria, yeast, and mold are capable of degrading hydrocarbons, and bacteria are the best described biosurfactant producer [21]. The exact reason why some microorganism can also produce biosurfactants is still not clear [22].

Bushnell and Hass (1941) were the first to demonstrate the bacterial production of biosurfactants, using a strain of *Corynebacterium simplex* and a strain of *Pseudomonas* grown in a mineral media containing kerosene, mineral oil, or paraffin. Since then, numerous studies on the structure and mechanisms involved in the production and action of biosurfactants have been reported [22]. It can be stated that biosurfactants are produced by a variety of microorganisms, and there is also a wide variety on the chemical composition and nature of the biosurfactant produced, as well as on the location (membrane-bound, extracellular) of the produced molecule [23]. The most reported genera of biosurfactant-producing bacteria include *Pseudomonas* sp., *Acinetobacter* sp., *Bacillus* sp., and *Rhodococcus* sp., among others. Table 1 shows some of the most studied bacteria and the type of biosurfactant produced.

Microorganism	**Biosurfactant**	**Reference**
Pseudomonas aeruginosa	Rhamnolipids	[24]
Pseudomonas fluorescens	Ornithine lipids	[25]
Pseudomonas stutzeri		[25]
Pseudomonas cepacia		[25]
Acinetobacter calcoaceticus	Lipopolysaccharides (biodispersant)	[10]
Acinetobacter radioresistens	Heteropolysaccharide protein (alasan)	[10]
Bacillus subtilis	Lipopeptides and lipoproteins (surfactin)	[16]
Bacillus licheniformis	Lipopeptides (lichenysin)	[5]
Rhodococcus erythropolis	Trehalolipids	[26]
Mycobacterium sp.		[2]
Nocardia sp.		[5]
Tsukamurella sp.	Di- and oligosaccharide lipids	[27]

Table 1. Biosurfactant-producing bacteria

Microorganisms that produce biosurfactants are isolated mainly from sites that are or were contaminated with petroleum hydrocarbons: contaminated soils, effluents, and wastewater sites. Thus, these have an ability to grow on substrates considered potentially noxious for other nonbiosurfactant-producing microorganisms. Biosurfactants play a physiologic role in increasing bioavailability of hydrophobic molecules, which are involved in cellular signaling and differentiation processes, which facilitate the consumption of carbon sources present in soil [23, 28].

The physiological role of biosurfactants is not clear yet, but it might be related to an increase in the nutrient uptake of hydrophobic substrates, in enhancing the growth on hydrophobic

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surface, and in cellular motility and biofilm formation by reducing the surface tension at the phase boundary [10, 15]. The mechanism of uptake of liquid hydrocarbon substrates by microbial cells involves interfacial phenomena. The significant influence on the biodegradation process is observed after the addition of surface active compounds [21]. Another physiological role of biosurfactants can be their observed antimicrobial activity [3].

Biosurfactants are produced predominantly when hydrophobic substrates provided as carbon source is used for microbial growth; they can be either secreted extracellularly or attached to the microbial cell wall. On the contrary, some microorganisms may produce biosurfactants in the presence of different types of substrates, including carbohydrates and other water soluble compounds. It has been reported that the carbon source used for biosurfactant production influences the structure of the compound produced by the microorganism. It is also affected by nitrogen sources as well as by the presence of minerals such as iron, magnesium, manganese, phosphorous, and sulfur [3, 23]. This capacity of modification of the biosurfactant molecule by the composition of the culture media can be used to produce compounds with specific applications. Industrial production of microbial metabolites is a very complex process, and for industrial production, many variables are needed to be considered; in the case of biosurfactants, media composition is a key element to control yield and specific productivity [29]. The success of biosurfactant production depends on the development of cheaper processes and the use of low-cost raw materials, which account for 10% to 30% of the overall production cost. The literature shows that a wide range of carbon sources, including agricultural renewable resources, like sugars and oils, are suitable carbon sources for production of ecologically safe biosurfactants with good properties [30]. The use of agroindustrial waste products such as bagasses, molasses, and plant material residues can be good candidates for use in biosurfactant production.

Interest in microbially produced biosurfactants has led to a need for the further development of rapid and efficient qualitative and quantitative methods for screening and analyzing biosurfactant-producing microorganisms [20]. The development of rapid and reliable methods for screening and selection of microbes from thousands of potentially active organisms and the subsequent evaluation of surface activity holds the key for the discovery of new biosurfactants. Among the most important characteristics needed for rapid screening methods is the ability to identify microorganisms capable of biosurfactant production in large culture collections, as well as the use of reliable methods to quantify the compounds produced [31].

4. Biosurfactant-producing yeast

Yeasts are unicellular cells of dimorphic fungi that are usually classified in the subdivision Ascomycotina and Basidiomycotina. They are ubiquitous in most environments, although they are more related to sites with high organic matter content and/or high water availability. They have been isolated from leaves, flowers and fruits, trees exudates, insects, soils, and other natural environments. Nowadays, approximately 100 genera and 700 species of yeast have been classified based on their morphological, physiological, and biochemical characteristics.

The most frequently isolated yeast genera from soils are *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Lipomyces*, *Pichia*, *Rhodotorula*, *Schizoblastosporion*, *Sporobolomyces*, *Torula*, and *Torulopsis* [32, 33]. Yeasts are involved in the production of a wide variety of foods, including fermented foods, alcoholic beverages, and bread. Yeasts are also involved in industrial fermentations for the production of antibiotics and vitamins among other commodities [34].

There are only few studies on biosurfactants synthesized by yeasts because most reports are related to bacteria and marine microorganisms, but the number of reports has increased, especially for *Candida* sp., *Pseudozima* sp., and *Yarrowia* sp. [35]. Table 2 shows yeast strains and the type of biosurfactant produced.

Microorganism	Biosurfactant	Reference
Candida bombicola (most studied system)	Sophorolipid	[36]
Candida apicola		[37]
Candida rugosa		[15]
Candida mucilaginosa		[15]
Rhodotorula bogoriensis		[38]
Pichia anomala		[39]
Candida lipolytica	Carbohydrate–protein (Liposan)	[40]
Yarrowia lipolytica	Carbohydrate–protein–lipid complex (Yansan)	[41]
Saccharomyces cerevisiae 2031	Mannoprotein	[42]
Pseudozyma (*Candida antarctica*)	Mannosylerythritol lipids	[43]
Pseudozyma rugulosa NBRC 10877		[44]
Pseudozyma churashimaensis		[45]
Schizonella malanogramma	Erythritol and mannose lipid	[10]
Ustilago maydis		[10]

Table 2. Biosurfactant-producing yeast

Yeasts can be preferred to bacteria as sources for biosurfactants because of their GRAS (generally regarded as safe) status, that is, they do not present risk of inducing toxicity or pathogenic reactions. Yeasts are also known for producing biosurfactants in higher concentrations than bacteria, which is an advantage for the development of production schemes [28, 46]. On the other hand, when comparing bacteria and filamentous fungi to yeast, the latter has many advantages, including faster growth rate than filamentous fungi; still, they can resist unfavorable environments such as filamentous fungi, being useful in biological treatment of effluents [47].

Yarrowia lipolytica was the first yeast used experimentally for the degradation of aliphatic hydrocarbon; this yeast also produces a highly efficient emulsifier [48]. Most of the biosurfactants produced by yeasts are better emulsifiers than biosurfactants, mainly because of the chemical structure of the molecules [49]. The widespread occurrence of yeasts with hydrocarbon-degrading activities has been extensively investigated. *Candida* species, especially *Candida lipolytica*, has been isolated from diesel oil storage tanks and fuel systems. *Candida tropicalis* and *Candida maltosa* are also noted for their use of saturated hydrocarbons. *Debaryomyces hansenii* and *Candida guiliermondii* can grow on hydrocarbons and have been isolated from hydrocarbon contaminated sites. There are many reports on the metabolism of hydrocarbons from yeasts, but very few information on the metabolites is produced [50].

The influence of the carbon source in biosurfactant production has been extensively studied in some microorganisms. For the study of yeast, different types of carbon sources have been used, depending on the yeast strain. For example, Silva et al. [51] found that biosurfactants produced using vegetable and mineral oils have different stability properties when incorporated into aqueous solutions, with better stabilization properties when vegetable oil was used. Daverey et al. [52] reported that a *C. bombicola* strain can produce sophorolipids when growth on a mixture of hydrophobic and hydrophilic substrates. Amaral et al. [53] reported that for the production of Yansan by *Y. lipolytica*, it is important to use glucose as carbon source.

Changes in yeast cell hydrophobicity have been related to the ability of the microbial strain to degrade hydrocarbons [54]. Amaral et al. [41] observed that the interaction of *Y. lipolytica* cells with hydrophobic surfaces is mediated by proteins or glycoproteins present in the cell wall. Furthermore, they suggested that van der Waals forces were involved in the interactions between the yeast cell surface and the nonpolar solvent and biosurfactant production improved these interactions. Regarding biosurfactant chemical properties, yeast biosurfactants maintained their functionality at different pH values as well as over a wide range of temperatures [55]. *Pichia anomala* and other yeasts are thermophilic, and so their biosurfactants could have a wide range of industrial applications [39].

5. Role of biosurfactants in bioremediation

Waste or used lubricating oils have caused a serious environmental problem because once in the environment, it can bind to organic matter, mineral particles, and organisms, with the consequent persistence and toxicity of oil components in the environment. Research on the interaction between hydrocarbon and microorganisms has supported the hypothesis that petroleum and its derivates are subjected to microbial degradation. In the environment, with the presence of emulsifying agents, hydrocarbons are more bioavailable for degradation; it has been observed that the greater the oil–water interface of hydrocarbons, the faster the rate of decomposition by the microbial community present [56].

Bioremediation can be done in two different ways: *in situ* and *ex situ*. The *ex situ* process can be carried out in a prepared bed or in a slurry reactor system. *In situ* processes are usually accomplished by the addition of microbial nutrients to the soil, which allows considerable

growth of soil microbial indigenous population [16]. Biodegradation efficiency depends on the ratio of hydrocarbon-degrading microorganisms in soil, the composition and physical state of hydrocarbon mixture and oxygen availability, and the condition of water, temperature, pH, and inorganic nutrients. The physical state of the hydrocarbon can also affect biodegradation. In addition, the biodegradation of hydrocarbon in bioremediation might be enhanced by the addition of surfactant. For use in bioremediation procedures, biosurfactants are more promising than synthetic surfactant because they are produced by microorganisms in soil and are commonly considered as low- or nontoxic compounds [57, 49].

Bioremediation involves the acceleration of natural biodegradative processes in contaminated environments by improving the availability of materials (e.g., nutrients and oxygen), conditions (e.g., pH and moisture content), and prevailing microorganisms [58]. Biosurfactants can improve bioremediation effectiveness by the following two mechanisms. The first mechanism includes the increase of substrate bioavailability for microorganisms; for bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil. When the surface area of microorganisms with hydrophilic solvents like water is limiting, biomass increases arithmetically rather than exponentially. The second mechanism involves interaction with the cell surface, which increases the hydrophobicity of the microbial cell wall, allowing hydrophobic substrates to associate more easily with bacteria [1, 19]. Microbial cell hydrophobicity can be described as the affinity to adhere to hydrophobic substrates, such as hydrocarbons. This capacity can give the microbial cells the ability to better degrade hydrocarbons, and it can be a factor to understand microbial biodegradation rate differences [54]. The increase of microbial adhesion to hydrocarbons is directly related to the ability of such microorganisms to grow in the medium where hydrocarbons or other hydrophobic substrates are present [56]. If the biosurfactant compound is bound to the microbial cell wall, the cell surface will be more hydrophobic. Microorganisms can use their biosurfactants to regulate their cell-surface properties to attach or detach from surfaces accordingly to their needs [1].

There are many research reports dealing with the degradation of hydrocarbons and production of biosurfactants by microorganisms, as stated in Section 3, and there are some *in field* reports on the use of bosurfactants for bioremediation. For example, Thavasir et al. [59] demonstrated the enhanced degradation of hydrocarbons by the addition of biosurfactants to the culture media, as well as the enhancement of degradation by the addition of mineral nutrients (fertilizers). There are also reports on the identification and characterization of biosurfactant-producing microorganisms, including some genera not usually related to bioremediation, such as *Staphylococcus*. Studies include the determination of functional characteristics of the biosurfactants produced and their potential use in bioremediation [60]. Also, there are reports on the production of biosurfactants by microorganisms isolated from particular environments, such as marine sediment, that could be helpful in the bioremediation of those particular sites [61]. Furthermore, the efficiency of different surfactant solutions in removing crude oil from contaminated soil has been tested. Urum et al. [62] demonstrated the efficiency of surfactant solutions used in a soil-washing process. The synthetic surfactant SDS (sodium dodecyl sulfate) was as efficient as a biosurfactant derived from bacteria (rhamnolipid), and both were more efficient than saponins.

There are some recent literature reviews on the production and use of biosurfactants for bioremediation [63–66], but in those revisions, there are only few cases described where biosurfactants have been used on bioremediation processes at pilot-scale or field-scale studies [64]. Calvo et al. [63] focused on the need for the optimization of biosurfactant production and the tools from molecular biology that can be used to obtain hyperproducing microbial strains. This approach leads to the strategy of producing the biosurfactant and then using it to amend contaminated sites [62]. The question remains if it is possible to inoculate biosurfactant-producing microorganisms in contaminated sites and then promote the production of tensioactive agents on site so that it can be a continuous source of biosurfactant.

Sachdev and Cameotra [66] proposed that biosurfactant-producing microorganisms might have different roles in soil, which can help on agricultural production. They described the use of biosurfactants for the recovery of organic pollutant contaminated soil, with the consequent improvement in the plant-microbiota beneficial interactions, but they also suggested that biosurfactants can be used to disperse fertilizers. Considering the antimicrobial effect of some tensioactive molecules, the authors also suggest that biosurfactants can help on the control of phytopathogens.

A recent review [65] has a more critical point of view on the efficiency of biosurfactants on bioremediation. The authors did a critical analysis of reports on the use of biosurfactants and described that there are many cases on the amendment of contaminated soil with biologically synthesized surfactants showing no differences with control experiments or even showing negative results. A question that needs to be addressed is the variability of experiments reported, as well as the actual role of biosurfactants in noncontaminated environments. As with many other biological processes where the microorganisms are taken from their natural habitats and places in restricted and controlled environments, the contribution of a particular metabolite can be misled. This has always been a major concern in environmental microbiology because there are still few methods that can help us on the understanding of the actual interactions of microbiota in their environments. Therefore, questions about the role of a particular metabolite in the microhabitat and the concentration of such compound in nature are still unanswered.

6. Conclusion

This chapter presents a description of biosurfactants and their uses in bioremediation. Biosurfactants are molecules produced by microorganisms that help them on the absorption and degradation of hydrophobic compounds. Bacterial strains have been extensively studied for biosurfactant production, but recent studies have also reported production of biosurfactants by yeasts. Yeasts are considered as GRAS microorganisms and are often used in the food and pharmaceutical industries and also in bioremediation.

The addition of chemical surfactants to enhance biodegradation efficiency in bioremediation processes is not acceptable because of its toxicity and persistence in the environment; hence, it is better to use biosurfactants. However, to use biosurfactants in bioremediation, the

optimization of large-scale production is needed, as well as studies on the use of alternative carbon sources derived from agroindustrial wastes. Also, it is important to evaluate the possible on-site production of biosurfactants in contaminated sites to expedite contaminated soil restoration.

It is then necessary to continue the isolation of biosurfactant-producing microorganisms on the characterization of their metabolites, on the identification of factors and conditions for production optimization, and on their use in field studies. As with any important subject in science, there are more questions than answers; research in biosurfactants for use in bioremediation of contaminated sites is still an innovative subject that needs more reliable scientific data.

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Chapter 6

Explorations and Applications of Enzyme-linked Bioremediation of Synthetic Dyes

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Additional information is available at the end of the chapter

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Abstract

Extensive use of synthetic dyes and their subsequent release in industrial wastewater is a growing environmental problem. These dyes are recalcitrant in nature, and some dyes are also well established to be potentially carcinogenic and mutagenic as well as genotoxic. Research efforts have been devoted to develop new, low-cost, and eco-friendly treatments capable of reducing and even eliminating synthetic dye compounds from the environment. Enzymatic approach has attracted much interest recently in the decolorization of textile and other industrially important dyes from wastewater as an alternative strategy to conventional chemical, physical, and biological treatments, which pose serious limitations. In this chapter, the accumulated research data on the potential of the oxidoreductive enzymes—high redox potential peroxidases (lignin peroxidase [LiP], EC 1.11.1.14; manganese peroxidase [MnP], EC 1.11.1.13; dye decolorizing peroxidase [DyP], EC 1.11.1.19; and versatile peroxidases [VP], EC 1.11.1.16), laccases (benzenediol–oxygen oxidoreductase, EC 1.10.3.2), polyphenol oxidases (EC 1.14.18.1), and azoreductases (azobenzene reductases, EC 1.7.1.6)—that have been exploited in the decolorization and degradation of synthetic dyes are presented. An overview of enzyme technology, including the importance of redox mediators for enhanced range of substrates and efficiency of degradation, current biodegradation applications, and suggestions to overcome the limitations to these proteins' large scale and efficient use, is made. Different strategies currently being used and future prospects for the potential use of genetic engineering techniques to improve the performance of these oxidoreductases in terms of stability, selectivity, and catalytic activity in dye bioremediation technologies are also explored.

Keywords: Dye decolorization, oxidoreductases, enzyme immobilization, genetic engineering, bioremediation

1. Introduction

Dyes are compounds that absorb light with wavelengths in the visible range, i.e., 400 to 700 nm, thereby giving different colors [1]. Generally, dyes contain chromophores, delocalized electron systems with conjugated double bonds responsible for light absorption in dye molecules, and auxochromes, electron-withdrawing or electron-donating substituent that cause or intensify the color of the chromophore by altering the overall energy of the electron system. The chromophores include -C=C-, -C=N-, -C=O, -N=N-, -NO_2, and quinoid rings, whereas the auxochromes include -NH_3, -COOH, -SO_3H, and -OH. On the basis of chemical structure or chromophore, 20 to 30 different groups of dyes can be discerned [2]. Synthetic dyes are therefore named according to the chemical structure of the chromophoric group (azo dyes, anthraquinone dyes, indigoid dyes, xanthene dyes, triarylmethane dyes, etc.) [2, 3] or according to the dyeing method (their mode of binding to the fiber) as reactive dyes, direct dyes, and cationic dyes [4].

Azo (R-N=N-R′), anthraquinone, and triphenylmethane dyes are quantitatively the largest classes of commercially produced colorants (Figure 1). Azo dyes make up approximately 70% of all dyes by weight and account for the majority (more than 3000 different varieties) of all textile dyes produced globally because their synthesis is easy and cost-effective, they are stable, and produce a wide variety of colors [2]. These dyes include at least one or more azo (R-N=N-R′) double bond, with one or more aromatic systems, and classified into two subgroups according to number of their double bond as mono-azo and poly azo types [2]. However, these dyes are recalcitrant in the environment as the breakdown of azo bonds (R-N=N-R) is quite difficult, and they can be stable in acidic and alkaline conditions. They are also resistant to high temperatures and light.

After azo dyes, anthraquinone compounds are the next most important textile dyes. These dyes are known for their good fastness and light fastness [2, 5] and a large range of colors, and they are commonly used to dye cellulosic fabric, wool, and polyamide fibers. Another group of dyes, the triphenylmethane dyes, e.g., malachite green, crystal violet, and pararosaniline, are characterized by the presence of chromogens, which contain three phenyl groups bound by a central carbon atom [2]. These dyes are extensively used for dyeing nylon, polyacrylonitrile-modified nylon, wool, silk, and cotton. They are also used by other dyestuff manufacturing industries as a biological stain and in printing paper [3]. Most of these dyes are stable against light, temperature, and biodegradation and therefore accumulates in the environment as recalcitrant compounds [1, 2, 4].

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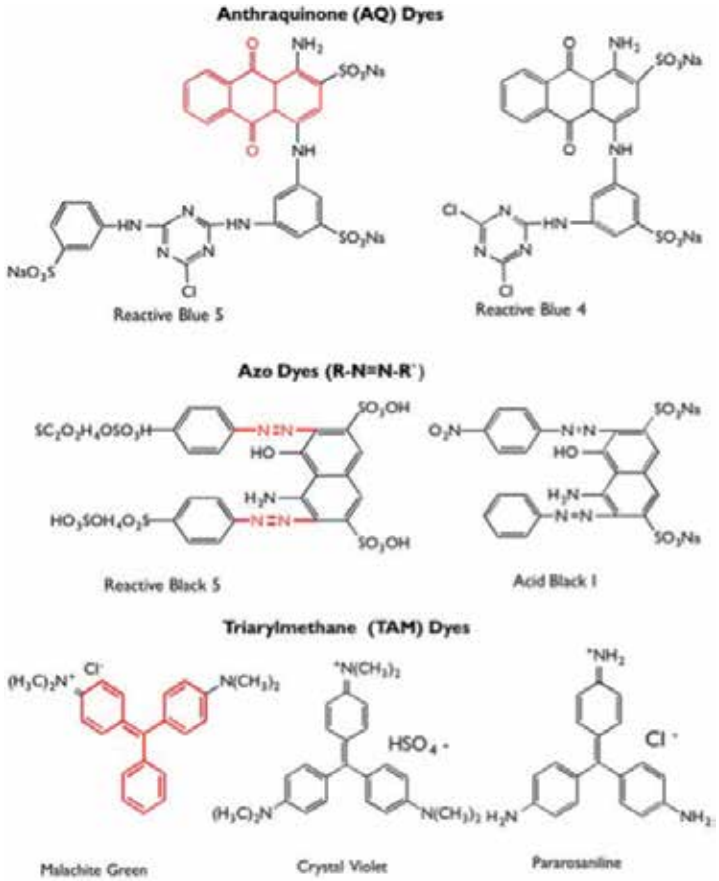

Figure 1. Chemical structure of major synthetic dye compounds showing their chromophoric groups (in red) and different auxochromes.

The increased demand for dyed products such as textiles coupled with the proportional increase in their production and the use of synthetic dyes have together contributed to dye wastewater becoming one of the substantial sources of severe pollution problems in current times [6]. Due to their synthetic origin and complex aromatic molecular structure, some of the dyes are thought to be toxic and mutagenic, resistant to biological degradation, and may accumulate in the food chain [2, 7]. In recent years, increased public concern and ecological awareness regarding the polluting properties of dyes have led to a stricter legislative control of wastewater discharge. This has led to increased interest in various methods of dye decolorization. Dye decolorization using physicochemical processes such as adsorption, adsorption on activated carbon, electrocoagulation, flocculation, froth flotation, ion exchange, membrane filtration, ozonation, and reverse osmosis and oxidation with ozone has proved to be effective [8, 9]. However, these processes are generally expensive, generate large volumes of sludge, and require the addition of environmentally hazardous chemical additives [5-7]. Research efforts have been devoted to develop new, low-cost, innovative, and eco-friendly treatments,

such as biological processes capable of reducing and even eliminating synthetic dye compounds from the environment.

2. Microbial bioremediation of synthetic dyes

Biological decolorization and degradation are an environmentally friendly, cost-competitive, and efficient alternative to physical/chemical decomposition [3, 6, 7, 10]. Decolorization by biological means may take place in either one of three ways: (1) adsorption (or biosorption) on the microbial biomass, (2) biodegradation by cells, and (3) biodegradation by enzymes. Biosorption involves the entrapment of dyes in the matrix of the adsorbent (microbial biomass) without destruction of the pollutant. In contrast, biodegradation involves the fragmentation of the original dye structure into smaller compounds, resulting in the decolorization of synthetic dyes. Several studies have described the use of microorganisms as biosorption agents in the removal of dye pollutants from wastewater [11, 12]. However, relative to the operational simplicity and adaptability of microorganisms to a given set of conditions, the biodegradation mechanism is considered efficacious and hence preferable to biosorption for treatment of dye wastewater [13].

There are numerous reports of microorganisms capable of decolorizing synthetic dyes: bacteria [14-16], fungi [17-19], yeasts [20, 21], actinomycetes [22, 23], and algae [24, 25]. Several fungi are capable of mineralizing pollutant compounds by action of their highly oxidative and nonspecific ligninolytic enzymes, which are also responsible for the decolorization and degradation of many different dyes. There are reports that white rot fungi (WRF), members of the basidiomycetes such as *Funalia trogii* [26], *Phanerochaete chrysosporium* [17, 18], *Trametes versicolor* [19], *Trametes hirsuta* [27], *Irpex lacteus* [28], and *Lentinula edodes* [29], can efficiently degrade xenobiotic textile dye compounds. Lignin-degrading fungi also degrades a wide range of aromatics owing to the relatively nonspecific activity of the extracellular ligninolytic enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and laccase.

Bacterial strains that can aerobically decolorize azo dyes have also been isolated during the past few years. However, there are only very few bacteria that are able to grow on azo compounds as the sole carbon source. The degradation of various azo dyes by mixed aerobic and facultative anaerobic microbial consortia under anoxic conditions has also been reported [30]. *Pseudomonas luteola*, *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas* sp., and *Proteus mirabilis* can also decolorized azo dyes under anoxic conditions [30-32]. These bacteria are specific toward their substrate, cleaving R–N=N–R bonds reductively and using the resultant amines as a source of carbon and energy for their growth.

The efficiency of dye degradation is mainly dependent on the following: (1) type of microorganisms used; (2) process conditions such as temperature, oxygen level, pH, available carbon, and nitrogen sources that affects microbial growth; and (3) dye concentration and chemical structure [10, 33]. Dependence on microbial growth to metabolize the toxicants makes the bioremediation of dyes by these organisms a relatively slow process. Another drawback to the

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microbial anaerobic reduction of synthetic dyes is its production of compounds such as carcinogenic aromatic amines [34, 35].

3. Major enzymes used in bioremediation of synthetic dyes

Over the last two decades, there has been increasing interest in the use of formulated enzymes rather than live bacteria as bioremediation agents owing to their specificity, and the ease with which their robustness can be enhanced with engineering [36]. Other advantages of using enzymes include increased enzyme production, enhanced stability and/or activity, and lower costs by using recombinant DNA technology. However, the high structural complexity of dye molecules means that only a few enzymes can degrade these compounds. These dye-degrading enzymes share one common mechanistic feature—they are all redox-active molecules that exhibit relatively wide substrate specificities. The most important dye-degrading enzymes are azoreductases, laccases, and peroxidases.

In this chapter, the accumulated research data on the potential of the oxidoreductive enzymes —high redox potential peroxidases, polyphenol oxidases, and azoreductases—that have been exploited in the decolorization and degradation of dyes are presented. The current initiatives and future prospects for the potential use of genetic engineering techniques to develop novel enzyme variants that are more durable and versatile biocatalyst, with respect to both the varieties of xenobiotics degraded and the operative conditions of dye bioremediation technologies are also discussed.

3.1. Peroxidases

Peroxidases (oxidoreductases, EC 1.11.1.x) are a diverse group of versatile heme-containing enzymes that utilize hydrogen peroxide or organic hydroperoxides (R-OOH) electron acceptor to catalyze oxidation of numerous substrates. Due to their catalytic versatility and enzymatic stability, peroxidases are one of the most studied groups of enzymes, more so as potential industrial/environmental biocatalysts [37]. Several good reviews have summarized years of peroxidase research and described their potential applications [37-40].

Generally, peroxidases are ubiquitous in nature; found in fungi, plants, animals, and eubacteria; and are classified within different superfamilies on the basis of their sequence homologies; animal and nonanimal peroxidases (former plant peroxidases) form the largest groups. The plant superfamily is further grouped into three subclasses according to cellular localization: class I—intracellular, organelle-associated, and bacterial peroxidases (e.g., cytochrome *c* peroxidase [CCP]); class II—secretory fungal peroxidases, including ligninolytic peroxidases such as LiPs, MnPs, and VPs [41–43]; and class III—secreted plant peroxidases (horseradish peroxidase [HRP]) [37]. Among peroxidases, a new superfamily—"dye-decolorizing peroxidases"—has arisen. These enzymes are known to successfully oxidize a wide range of substrates, but most importantly, they effectively degrade high redox synthetic dyes such as anthraquinone and azo dyes. DyPs form a novel superfamily of peroxidases that are generally

characterized by "atypical" molecular architecture and divergent mechanistic behavior that is not fully understood yet but different from the classical peroxidases [37, 40, 41].

Structurally, peroxidases share several features, including the overall protein fold and the general architecture of the heme pocket with the high-spin ferric iron (Fe^{III}) coordinated to the proximal histidine and the conserved distal histidine and arginine residues [38, 39]. These highly conserved residues in the heme catalytic pocket are critical for peroxidase activity, i.e., in the generation and stabilization of compound I and II. The functional and catalytic diversity of heme peroxidases is thereby attributable to specific structural differences around the heme-binding site, including the nature of the axial ligand, and the environment of the substrate-binding site.

Catalytically, peroxidases share a general reaction mechanism by using hydrogen peroxide (H_2O_2) as the final electron acceptor in the oxidation of a broad range of substrates (AH) to radicals ($AH^{\cdot+}$), which involves a three-step sequential mechanism via compound I and compound II intermediates [37, 38, 40, 42]. Initially, the native enzyme, which is in the ferric form Fe^{III}, undergoes two-electron oxidation by H_2O_2 to produce compound I (oxyferryl porphyrinyl radical [Fe^{IV}=$O^{\cdot+}$]). One electron is removed from the ferric iron Fe^{III} to form the ferryl Fe^{IV}, whereas the second electron is withdrawn from the porphyrin ring to form a porphyrin cation radical [43]. During this reaction step, H_2O_2 is reduced to water. Next, compound I oxidizes substrates by one electron and is reduced to compound II. In this step, the porphyrin ring gains an electron. Thus, compound I is able to oxidize substrates with a higher redox potential than compound II [38, 44]. The reduction of compound II, by a second electron of substrate, brings the enzyme back to the native state and completes the catalytic cycle.

In the absence of substrate and in the presence of excess H_2O_2, compound II is converted to compound III, a ferrous-oxy or ferric superoxide species. In this process, H_2O_2 reduces compound II by one electron to produce a ferric enzyme and a superoxide radical. The latter readily combines with the ferric peroxidase to produce compound III. In the peroxidase catalytic cycle, the generation of highly reactive radicals that undergo a complex series of spontaneous cleavage reactions accounts for the degradation ability of peroxidase toward numerous substrates, e.g., phenolic and nonphenolic aromatics, metal ions, and complex dyestuff molecules [45].

3.1.1. Ligninolytic peroxidases

Lignin-modifying peroxidases (LMPs; LIP, MnP, and VP) refer to a group of glycosylated, heme-containing ligninolytic enzymes produced by the fungi during secondary metabolism in nutrient starved cultures [46]. These enzymes are produced in multiple isoforms and are affected by many external factors, such as nutrient level, mediator compounds, and metal ions. Phylogenetically, they belong to class II extracellular fungal peroxidases in the so-called "plant peroxidase superfamily."

Lignin peroxidases (diarylpropane: oxygen, hydrogen peroxide oxidoreductase, EC 1.11.1.14) were first described in the basidiomycete *P. chrysosporium* Burdsall in 1983 [47] and have

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become the most studied peroxidase since then. They have also been reported in several species of white rot basidiomycetes [48-51], actinomycetes [52, 53], and some bacteria, such as *Brevibacillus laterosporus* MTCC 2298 [54] and *Streptomyces viridosporus* T7A [55]. LiPs are generally dependent on H_2O_2 and have very high redox potential and low optimum pH [56, 57]. Both these characteristics are important for their ability to oxidize a variety of reducing substrates, including polymeric substrates such as complex dye compounds. LiPs have a typical enzymatic cycle, characteristic of other peroxidases. LiP, MnP, and VP share an almost identical heme environment, which is responsible, among other factors for their high redox potential. However, they differ in the substrates that they can oxidize because of the presence of different catalytic sites in their molecular structures. LiP oxidizes nonphenolic lignin model compounds in direct contact with a tryptophan radical exposed to the solvent [58].

Biochemically, LiPs utilize veratryl alcohol (VA) to complete the catalytic cycle by reducing compound II to a resting enzyme to avoid inactivation. VA (3,4-dimethoxybenzyl alcohol) is a secondary metabolite, concomitantly produced by basidiomycetous fungi, along with LiPs usually synthesized from glucose in liquid cultures. Moreover, VA acts as a cation radical redox mediator of remote substrates [58, 59]. Similar to other peroxidases, LiP shows little substrate specificity, and, due to its high redox potential, reacts with a wide variety of lignin model compounds and even unrelated molecules. Interestingly, LiP has the distinction of being able to oxidize methoxylated aromatic rings without a free phenolic group, generating cation radicals that can react further by a variety of pathways, including ring opening, demethylation, and phenol dimerization [60], a typical mechanism also employed in the degradation of dye compounds. Numerous studies have reported the application of LiPs in the decolorization of various synthetic dyes and industrial dye wastewaters [20, 43, 48, 54, 61-63].

MnP (Mn^{II}:hydrogen peroxide oxidoreductase, EC 1.11.1.13), like LiPs, is a heme-containing peroxidase and is the most common ligninolytic peroxidase produced by almost all white rot basidiomycetes [37]. MnP is a glycoprotein with a heme (ferric protoporphyrin) group that shares the mechanistic properties of other peroxidases and the formation of oxidized intermediates, compound I and compound II, in the presence of H_2O_2 for aromatic and nonphenolic substrates oxidation [44]. Catalytically, MnP requires Mn^{II} as an electron donor; Mn^{II} is oxidized to Mn^{III} [45], a deviation from other typical peroxidase reaction mechanisms. This specific oxidation of Mn^{II} by MnP generally occurs in a small channel formed by three acidic residues located directly on the heme internal propionate [64]. MnP depends on Mn^{II} as a substrate for compound I and II formation. In this reaction scheme, MnP oxidizes Mn^{II} to Mn^{III} in the presence of H_2O_2, and the Mn^{III} formed oxidizes a variety of compounds [44]. The chelation of Mn^{II} to Mn^{III} by organic acids, such as oxalate, is necessary for MnP activity [53]. Its unique catalytic activity enables MnPs to biodegrade dyes, as well as decolorize various types of synthetic dyes, indicating their potential application in the environmental bioremediation of dye industry wastewater [65-67].

VPs (EC 1.11.1.16) are also known as hybrid peroxidases or lignin–manganese peroxidases because of their dual LiP and MnP catalytic properties. They are relatively new fungal peroxidases that were first thought to be MnPs but have since been isolated and thoroughly characterized in *Pleurotus* and *Bjerkandera* [68-72]. In contrast to other ligninolytic peroxidases,

VPs possess two catalytic sites, one for the direct oxidation of low- and high-redox potential compounds and the other for oxidation of Mn^{II} in a preferred manner [70-72]. *Pleurotus eryngii* VP (PeVP) possesses three acidic amino acid residues for Mn^{II} binding and a catalytic efficiency (k_{cat}/K_m) for Mn^{II} oxidation that is typical of MnPs. In addition, PeVP has a tryptophan residue, Trp164, which is analogous to the PeLiP Trp171 that participates in electron transfer from aromatic donors and consequently enables the enzyme to oxidize nonphenolic lignin-related structures [64]. The dual catalytic mode of action observed accounts for their ability to catalyze the direct degradation/oxidation of a broad spectrum of persistent substrates (e.g., nonphenolic lignin compounds, dyes, such as RB5 and others) in the absence of mediators [71, 72], an important feature as a potential catalyst for a variety of biotechnological applications.

3.1.2. Dye decolorizing peroxidases

Dye decolorizing peroxidases (DyPs) comprise a novel group of heme-containing enzymes, named for their ability to efficiently oxidize high redox potential trichromatic anthraquinoic (AQ) dyes. They were first reported in the extracellular secretions of a plant pathogenic fungus, *Bjerkandera adusta* Dec 1 (wrongly annotated previously as *Thanetophorus cucumeris*) [73]. The main features of DyP from *B. adusta* Dec1 include the following: (1) a monomeric 60-kDa glycosylated enzyme having higher specificity for AQ than for azo dyes, and different degradation spectra for phenolic compounds such as 2,6-dimethoxy-phenol, guaiacol, and VA; (2) a low pH optima (pH < 3.0); (3) lack of a conserved active site for distal histidine; and (4) structural divergence from classical plant and animal peroxidases (Figure 2) [73, 74]. To date, these enzymes, the physiological function of which is still unclear, have been identified from the genomes of fungi, bacteria, and archaea (http://peroxibase.toulouse.inra.fr/index.php). Interestingly, there is increasing evidence for the key role that microbial DyP peroxidases play in the degradation of lignin (see [37, 40, 41] up-to-date reviews on DyP-type peroxidases and their known biological, chemical, and structural features).

Similar to other peroxidases, DyPs are catalytically bifunctional enzymes displaying both oxidative and hydrolytic activity. They exhibit significant catalytic versatility arising from their ability to oxidize a variety of organic compounds, some of which, including dyes, phenols, β-carotene, lignin model compounds, and aromatic sulfides, are poorly degraded by conventional peroxidases [75-78]. Moreover, studies have demonstrated that DyPs are very robust enzymes in terms of pH [76], temperature, and pressure [78]. Several mediators can also improve DyPs substrate range. For example, we observed a drastic enhancement of azo dye oxidation in presence of a natural mediator syringaldehydeby AnaPX, a bacterial DyP [75], whereas Mn^{II} activates *Rhodococcus jostii* DyP2 activity [79]. The potential utility, as industrial/environmental biocatalysts in the bioremediation of wastewater contaminated with synthetic dyes specifically—the recalcitrant and xenobiotic AQ dyes that are generally not substrates of peroxidases such as HRP—has made DyPs the focus of significant interest.

Biochemically, the physicochemical properties of DyPs such as UV–vis spectral characteristics, molecular masses, or isoelectric points resemble those of classical heme proteins [76, 80, 81]. DyPs are structurally divergent from typical peroxidase; the basic architecture has a dominant α+β-helical secondary structure with extended loop regions (Figure 2). The N-terminal and C-

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terminal domains contain an antiparallel β-sheet that is arranged into a characteristic ferredoxin-like motif on the distal side of the heme moiety [41, 82, 83]. Obviously, structural peculiarities, including the nature of the axial ligands, the environment of the substrate-binding site, and the involvement of intramolecular electron transfer, appear to account for the novel and the varied catalytic differences between DyPs and other peroxidases. Although DyPs possess a heme iron prosthetic group with a conserved proximal Fe–His–Asp triad found in most other peroxidases, the generally conserved distal His is absent in DyPs. Instead, an Asp residue forming the absolutely conserved novel GXXDG motif and an Arg in the distal position of DyPs are present (Figure 2). The distal Asp residue assumes the part of the catalytic base, mediating peroxide cleavage and thus the formation of compound I [84]. Moreover, the distal Asp's "swinging movement" determines heme cavity access for small organic molecules, making it function as the heme cavity's gatekeeper [75, 85]. The observed smaller heme-access channel for DyPs indicates that the oxidation of bulky substrates such as bulkier AQ dyes requires another substrate interactions sites. The existence of substrate interaction sites, involving long-range electron transport (LRET) via either surface-exposed tyrosyl or tryptophan residues, has been demonstrated in several DyPs [77, 82]. A Mn^{II} binding site, which accounts for the oxidase activity of DyP2, in the absence of peroxide, has also been reported in *R. jostii* DyP2 [79].

3.2. Laccases

Laccases belong to the multicopper oxidase family of enzymes that catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water, through a radical-catalyzed reaction mechanism [86]. They are mainly of fungal or plant origin, although a few representatives have been identified and isolated in bacteria and insects [87, 88]. The most studied laccases are fungal in origin, mainly in phyla Ascomycota, Zygomycota, and Basidiomycota (see reviews [87, 88]). The most biotechnologically useful laccases are also of fungal origin. Physiologically, the functions of laccases are diverse, ranging from lignolysis, pigment formation, detoxification, to pathogenesis. All these functions are attributed to the enzymes' ability to oxidize a wide range of aromatic substrates such as polyphenols and diamines and even some inorganic compounds [87, 88].

Compared with fungal laccases, bacterial laccases are generally more stable at high pH and temperatures [89]. Although fungal laccases can be both intra- and extracellular, bacterial laccases are predominantly intracellular. Lacasse have been isolated from the rhizospheric bacterium *Azospirillum lipoferum* [90], the melanogenic marine bacterium *Marinomonas mediterranea*, and the endospore coat component (CotA) of *B. subtilis* [89, 91]. In addition, the optimum pH of bacterial laccases is higher than that of fungal laccases [89, 91], whose optimum pH is acidic [89, 91]. The optimum temperature for most laccases is between 50°C and 70°C, with thermal stability dependent on the microbial source.

The structure of an active holoenzyme laccase molecule is a dimeric or tetrameric glycoprotein containing four copper atoms per monomer, bound to three redox sites (T_1, T_2, and T_3 Cu pair) [87, 88]. The four Cu atoms differ from each other in their specific properties, such as the characteristic electronic paramagnetic resonance (EPR) signals that allow them to play an

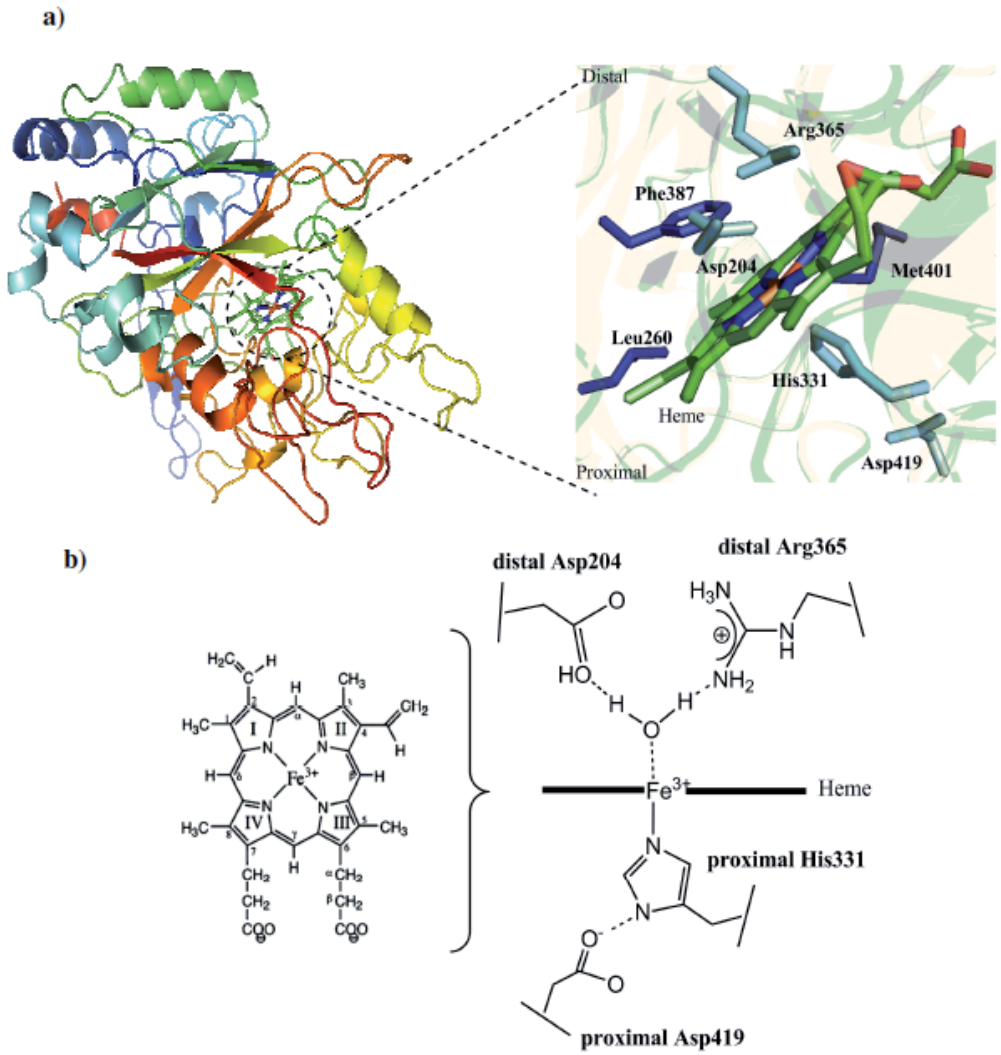

Figure 2. The structural model of DyP showing the heme active site architecture. (a) Typical α+β protein structure of DyP and the heme active site showing the conservation of key catalytic proximal Fe–His–Asp triad residues and substitution of His with Asp in the distal heme active. (b) Possible coordination of distal Asp and other active site residues with a water molecule involving hydrogen-bond network in the heme active site as implicated by the resolved crystal structures of ferric DyP enzymes [82-84].

important role in the catalytic mechanism of the enzyme. For catalytic activity, a minimum of four Cu atoms per active protein unit is needed. The T_2 and T_3 Cu atoms form a trinuclear cluster site, which is responsible for the binding and reduction of oxygen to water. T_2 Cu is coordinated by two His and T_3 Cu pair by six His. The strong antiferromagnetic coupling between the two T_3 Cu atoms is maintained by a hydroxyl bridge [87, 88]. The function of the T_1 site, in this type of enzyme, involves electron abstraction from reducing substrates (electron

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donors), with a subsequent electron transfer to the T_2/T_3 Cu cluster. Generally, laccase catalyzes the four-electron reduction of oxygen to water (at the T_2–T_3 trinuclear Cu centers) by the sequential one-electron uptake from a suitable reducing substrate (at the T_1 mononuclear Cu center) [86]. Lacasses are categorized into high potential lacasses (HPLs) and low potential lacasses (LPLs) on the basis of T_1–T_3 Cu having a redox potential of 0.6–0.8 V and 0.4–0.6 V, respectively,

The catalytic efficiency of laccases (k_{cat}/K_m) depends on the redox potential of T_1 site; consequently, laccases with high redox potential at the T_1 site are of special interest in biotechnology for their potential application in bleaching and bioremediation processes [92]. Compared with LiP, MnP, and VP that exhibit higher redox potentials (1.15–1.25 V), most laccases typically have low redox potential (0.5–0.8 V). Consequently, most laccases lack the ability to degrade nonphenolic aromatic substrates due to their low redox potential. However, in the presence of mediators, particularly small chemical compounds with redox potential higher than 0.9 V, the substrate range of laccases can be expanded to include the oxidation of nonphenolic compounds such as lignin and complex dye compounds [27, 86, 87]. A mediator is a low molecular weight (LMW) chemical compound that is continuously oxidized by the laccase enzyme and subsequently reduced by the substrate. Due to its bulky size, high molecular weight (HMW) substrates cannot enter the laccase active site, and the mediator acts as a carrier of electrons between the enzyme and the substrate, thereby overcoming the steric hindrances that exist between them [86].

Laccases and lacasse-mediator systems (LMSs) have been intensively studied with regard to their degradation of various recalcitrant compounds, such as chlorophenols, polyaromatic hydrocarbons (PAHs), lignin-related structures, organophosphorous compounds, phenols, and synthetic dyes [27, 87]. These enzymes have great potential in various biotechnological processes mainly because of their high nonspecific oxidation capacity, the lack of a requirement for cofactors, and the use of readily available oxygen as an electron acceptor. Laccases and LMSs have found various biotechnological and environmental applications, including as analytical tools/biosensors for phenols, and in the development of oxygen cathodes in biofuel cells, textile dye degradation, organic synthesis, immunoassay labeling, delignification, demethylation, and in bleaching of craft pulp [87, 88, 92].

3.3. Azoreductases

Azoreductases are a group oxidoreductive enzymes that catalyze the NAD(P)H-dependent reduction of azo compounds to the corresponding amines, via cleavage of the azo linkages (R-N=N-R), resulting in azo dye degradation [93]. They are a varied family of enzymes that have been identified in almost all species except in viruses. Physiologically, these enzymes participate in enzymatic detoxifications systems, involving the reduction of quinones, quinone imines, azo dyes, and nitro groups, and protect cells against the toxic effects of free radicals and reactive oxygen species arising from electron reductions [93].

Azoreductase activity has been characterized from a wide variety of bacteria, including *Pigmentiphaga kullae* K24, *Xenophilus azovorans* KF46F, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus* sp. strain OY1-2, *Pseudomonas aeruginosa*, and *Rhodobacter*

sphaeroides [93-101]. There are at least two different types of bacterial azoreductases: those that require flavin and those that do not [94]. Flavin-dependent azoreductases can be further classified into two families according to their amino acid sequences. Azoreductases from *E. coli* and *Bacillus* sp. strain OY1-2 are representative of the two flavin-dependent azoreductases, respectively. *Bacillus* sp. strain OY1-2 azoreductase is a 23-kDa protein with the ability to reduce the azo dyes Rocceline, Sumifix Red B, and Methyl red, producing dimethyl p-phenylenediamine and o-aminobenzoic acid in the presence of β-NADPH [102]. On the other hand, *Xenophilus azovarans* KF46 and *P. kullae* K24 azoreductases are monomeric flavin-free enzymes that use NADPH as a cofactor to degrade the azo dyes carboxy-Orange II and I. These two enzymes exhibit different substrate specificities and sizes (21 and 30 kDa) and require the presence of hydroxyl groups in the aromatic ring of the substrate [95].

3.4. Other dye-degrading enzymes

In addition to peroxidases, laccases, and azoreductases, the bioremediation of synthetic dye compounds with other enzymes such as tyrosinases [103], aryl alcohol oxidases [104], and biosulfidogenic hydrogenases [105] have been demonstrated. Similar to laccases, tyrosinases (monophenol monooxygenase, EC1.14.18.1) are oxidoreductases that can catalyze the oxidation of phenolic and other aromatic compounds, without the use of a cofactor in presence of oxygen. Catalytically, these enzymes possess both cresolase activity (ortho-hydroxylation of monophenols to *o*-diphenols) and catecholase activity (the oxidation of *o*-diphenols to *o*-quinones) [103]. Tyrosinases also degrade aromatic amines and *o*-aminophenols via the similar ortho-hydroxylation and oxidation reactions. The resultant products undergo subsequent polymerization, giving rise to oligomeric products.

Biotechnologically, tyrosinases are used as markers of the oxidative enzymes involved in the degradation of azo dyes. For example, the involvement of tyrosinase in the degradation of Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112 [106], disperse dye brown 3REL by a microbial consortium consisting of *Galactomyces geotrichum* MTCC 1360, and sulfonated azo dyes by *Brevibacterium* sp. strain VN-15 [103] and *Bacillus* sp. VUS [62] has been demonstrated. The activity of aryl alcohol oxidase (AAO) has been reported in *B. adusta* Dec 1; it oxidizes VA to veratraldehyde producing H_2O_2, which is important for supporting the *in vivo* dye-decolorizing activity of fungi [104]. The involvement of a similar enzyme in *Comamonas* sp. UVS during the decolorization of Red HE7B and Direct Blue GL has also been reported [107].

4. Enzyme-linked biotransformation of industrial dyes

4.1. Enzymatic degradation of azo dyes

A general mechanism for the peroxidase-catalyzed oxidation mechanism for azo dye degradation, via either the symmetrical and/or asymmetrical azo bond cleavage, has been proposed. Goszczynski et al. [108] proposed mechanisms in the course of which these enzymes convert dyes to cation radicals and become susceptible to nucleophilic attack by water or hydrogen peroxide. This results in the simultaneous split of the azo linkage, both symmetrically and

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asymmetrically, to produce intermediates that subsequently undergo several redox reactions, such as depolymerization, repolymerization, and demethylation, finally leading to more stable intermediates. In this reaction scheme, two successive one-electron oxidations of the phenolic ring, by the hydrogen peroxide–oxidized forms of the enzyme, produce a carbonium ion. A water molecule then reacts with the phenolic carbon, bearing the azo linkage, and an unstable hydroxyl intermediate, which breaks down into a quinone, and an amidophenyldiazine, is formed. The latter compound is then oxidized, by oxygen, into the corresponding phenyldiazene radical, which, after elimination of nitrogen, gives a phenyl radical that is reoxidized by oxygen. This mechanism leads to the detoxification of azo dyes because no aromatic amines are formed. LIP, MnP, and VP from basidiomycetous fungi are able to oxidize several azo dyes by following this mechanism [20, 43, 48, 54, 61-63]. Although exhibiting a general preference toward trichromatic AQ dyes than azo dyes, it is possible that DyP-type peroxidases also use the same mechanism.

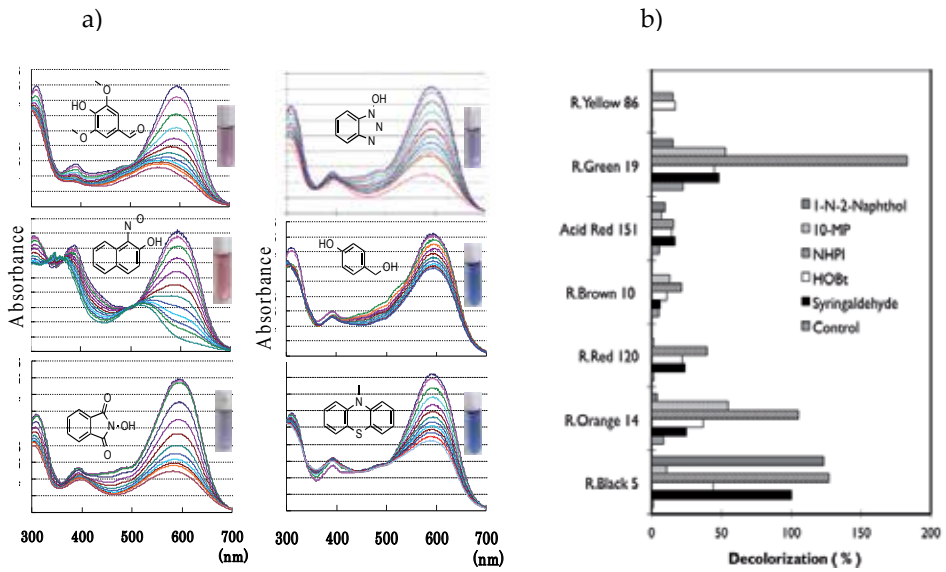

Figure 3. Decolorization of azo dyes in the presence of mediators by AnaPX. (a) Biotransformation of Reactive Black 5 by AnaPX in the presence of various mediators. Inset shows the structures of the mediators and the resultant product upon decolorization. (b) Decolorization profile of several azo dyes by AnaPX in presence of different mediator. compounds.

Some azo dyes are oxidized effectively only in the presence of mediator compounds. In previous studies, we have shown that the azo degradation ability of AnaPX was significantly improved in the presence of redox mediators [75]. The decolorization range and oxidation rates of AnaPX, compared with HRP for azo dyes, increased markedly (2- to 5-fold) in the presence of a lignin-derived phenolic redox mediator, syringaldehyde (SA) (Figure 3). The degradation of Reactive Black 5, in presence of SA, was pH dependent, with the AnaPX-mediator reaction exhibiting maximal activity at pH 6.0, although the enzyme optimum pH is 4.5 [75]. Similar

results have been reported in the presence of NOH-type (1-hydroxy-1H-benzotriazole (HOBt), *N*-hydroxyphthalimide (NHPI), 1-nitroso-2-naphthol), and quinine-containing mediators such as 10-methylphenothiazine (10-MP); however, decolorization efficiencies were different, possibly due to differences in the type of dye structure (Figure 3). Differences in the position of the substituents (-CH_3, -OCH_3) and/or their substitution with -Cl or -NO_2 groups on the phenolic ring affect the electronic character of dye compounds and may render the azo dye more or less susceptible to oxidation by enzymes, resulting in the observed dye-structure-related effects on decolorization efficiencies [109]. Our earlier work also showed that the transformation of Reactive Black 5 resulted in a decrease in the intensity of the dye absorption band, at λ_{max} = 600, 400, and 310 nm, indicating the degradation of the dye. The complete decolorization of the dye resulted in the formation of light brownish products within 1 min in the presence of SA and 1-nitroso-2-naphthol. In contrast, other mediators showed varied decolorization efficiency for Reactive Black 5, illustrating the importance of mediator specificity toward different functional groups. It has been postulated that phenoxy radicals from SA act similarly to the -NO- radicals from -NOH- compounds, i.e., they extract a hydrogen atom from the substrate [110, 111]. It is very likely that the varied oxidative ability observed for the different AnaPX mediators is governed by the dissociation energy of the corresponding bond, which consequently affects the azo dye degradation.

The laccase-catalyzed oxidation of azo dyes has also been postulated to follow a similar mechanism, albeit with slight modifications. In this reaction scheme, fungal laccases oxidize azo dyes through a highly nonspecific free radical mechanism to form phenolic type compounds without the cleavage of the azo bond [89, 109, 112]. A similar mechanism has been reported in the biotransformation of the azo dye, Sudan Orange G by bacterial Cot-laccase from *B. subtilis*, that exhibits an optimal pH of around 8–9 for dye decolorization [89]. According to this mechanism, lacasses initially catalyze a one-electron transfer reaction of the dye to generate a phenoxyl radical that is sequentially oxidized to various unstable radical molecules, with the concomitant destruction of the chromophoric structure of the dye. The resultant biotransformation radical species undergo coupling reactions to form less toxic oligomeric or polymeric condensation products. This laccase-catalyzed oxidation of phenolic azo dyes is however dependent on the electronic character and position of the substituent on the phenolic ring [89, 109]. For example, azo dyes with electron-donating 2-methyl or 2-methoxy substituents are more easily oxidized than compounds containing a methyl group in position 3, or those unsubstituted or substituted with 2-chloro and 2-nitrogroups. Generally, the laccase-catalyzed oxidation of azo dyes excludes the formation of toxic aromatic amines obtained under reductive conditions, making these enzymes important in azo dye bioremediation.

All azoreductases reduces azo compounds via a Ping Pong Bi Bi mechanism [94, 101]. In the proposed mechanism, azo compound reduction undergoes two cycles of NAD(P)H-dependent reduction; the azo substrate is reduced to a hydrazine in the first cycle, and the hydrazine is further reduced to two amines in the second cycle. In this reaction, FMN serves as a redox center in the electron-transferring system by mediating the electron transfer from NAD(P)H to the azo substrate [94, 113]. However, corresponding aromatic amines, formed during anaerobic azo reduction, are generally more toxic, mutagenic, and carcinogenic than azo

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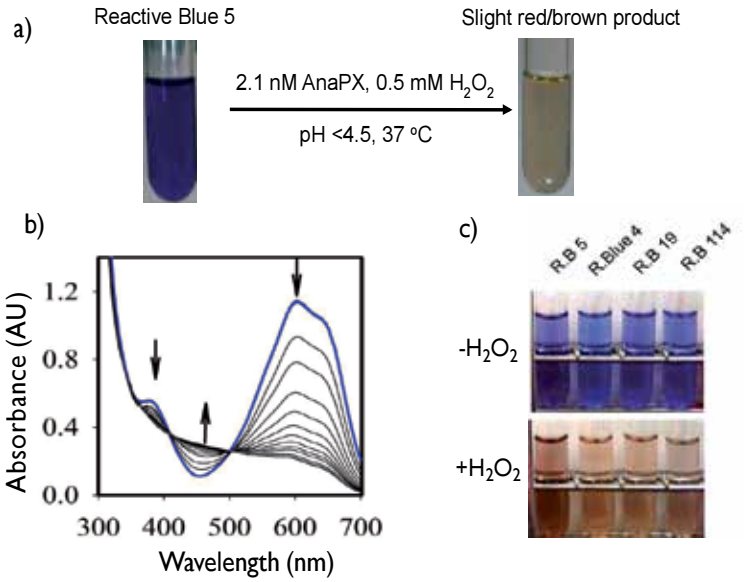

Figure 4. Biotransformation of AQ dye Reactive Blue 5 by AnaPX. The formation of characteristic light reddish brown product (2,2-disulfonyl azobenzene) upon enzymatic degradation of (a) Reactive Blue 5 and (c) other AQ dyes, Reactive Blue 4, Reactive Blue 19, and Reactive Blue 114. (b) UV absorption spectra of the degradation process showing the decrease in absorbance at 600 nm and an increase in absorbance at 400–500 nm. Each spectrum was taken after 4-s intervals.

substrates [32, 94, 113]. Moreover, the requirement for expensive cofactors is a barrier to the wider utilization of azoreductases in bioremediation.

4.2. Enzymatic biotransformation of anthraquinone dyes

Although there are many reports on the involvement of peroxidases in the biodegradation of azo dyes as described above, very few studies have reported the degradation of anthraquinoic (AQ) dyes by these peroxidases. Since the first report on the DyP peroxidases' high specificity to AQ dyes [73], several proteins have been isolated and characterized, and their ability to decolorize synthetic dyes was demonstrated. In our study, AnaPX decolorized over 90% of the AQ dyes—Reactive Blue 5 (262 U mg^{-1}), Reactive Blue 4 (167 U mg^{-1}), Reactive Blue 114 (491 U mg^{-1}), and Reactive Blue 19 (401 U mg^{-1})—within 5 min [75]. These dyes have a vinyl sulfonic reactive moiety in their structure; their aromatic anthracene-9,10-dione structure is highly stabilized by resonance, accounting for their general resistance to both chemical and enzymatic oxidation. The enzyme also decolorized over 70% of Reactive Blue 4 and the triazine dyes, Procion Blue H-ERD and Procion Blue H-EXL, within 2 h. The kinetic parameters determined for AnaPX clearly revealed that it has a higher affinity and greater redox potential for H_2O_2 and RB5 than HRP and other peroxidases. This may explain the higher decolorization activity of AnaPX toward RB5. The decolorization of RB5 and Acid Blue 45 by AnaPX results in a decrease in absorbance at 600 nm and an increase in absorbance at 400–500 nm, accompanied

by the formation of a light reddish-brown product (Figure 4). Similar results have been reported in other DyP peroxidases [75-78, 82, 83, 114-117].

The biotransformation of AB62 (an AQ), by *B. subtilis* Cot-laccase, also results in a decrease in the intensity of the dye absorption bands, at λ_{max} = 600 and 630 nm, along with an increase in absorption around 500 nm, due to the formation of reddish products [118]. Thus, it is probable that both laccase and peroxidase utilize similar mechanism for AQ degradation.

In the transformation of Reactive Blue 5 by *B. adusta* Dec 1 DyP, analysis of the final enzymatic reaction mixtures, by NMR and MS techniques, showed that dye degradation results in three reaction products: (1) phthalic acid, (2) product 2 (m/z = 472) lacking the anthraquinone frame, and (3) product 3 (m/z = 305), formed from the loss a 2,5-diaminobenzene sulfonic acid (ABS) molecule from product 2 [119]. In the proposed reaction mechanism, the anthraquinone frame undergoes initial oxidative ring opening due to attack of the carbonyl group by the H_2O molecule. This oxygenase/hydrolase-like activity leads to the production of phthalic acid. In contrast, the formation of products 2 and 3 proceeds via cationic radical catalysis, typical of peroxidases, followed by the subsequent dimerization and polymerization of the intermediates to form final products with high molecular weights, such as 2,2-disulfonyl azobenzene. The formation of 2,2-disulfonyl azobenzene resulted in the characteristic reddish-brown product observed during the DyP-catalyzed degradation of Reactive Blue 5 (Figure 4) [75, 119]. Further treatment of the final product with TcVP1, a VP from *B. adusta* Dec 1, decolorized these colored intermediates to colorless by products [74]. The concerted action of these two enzymes, for the complete decolorization of Reactive Blue 5, illustrates the potential utility of DyPs in dual-enzyme systems as a novel strategy in the treatment of dye wastewater.

4.3. Biodegradation of other synthetic dyes by enzymes

Dye-degrading enzymes can also be applied in the degradation of other synthetic dyes such as indigoid, triarylmethane, and phthalocyanine dyes. Similar to the laccase-catalyzed degradation mechanism for azo dyes, the initial hydrolytic attack by the water molecule, coupled with laccase-catalyzed electron transfer, causes the cleavage of the indigoid frame, forming an intermediate isatin. The subsequent decarboxylation of isatin leads to the formation of anthranilic acid as a final stable oxidation product [120]. This process is used industrially to achieve the stonewashed effect of indigo-dyed denim fabric via mild enzymatic decolorization. This process can be used to treat textile wastewaters containing indigoid dyes. *P. chrysosporium* extracellular lignolytic enzymes such as MnP and LiP have been demonstrated to successfully decolorize indigoid dyes [121, 122]. In the peroxidase-catalyzed decolorization of indigo carmine, isatin sulfonic acid is formed as a final yellowish product when LiP is used. In MnP-catalyzed oxidation of indigo carmine, a stable reddish product, probably a dimeric condensation product, is formed instead. *P. chrysosporium* cultures, extracellular fluid, and purified peroxidases have been reported to degrade generally recalcitrant crystal violet and six other triphenylmethane dyes [123, 124]. The degradation of these dyes follows N-demethylation reactions. For example, the decolorization of crystal violet has been shown to form Michler's ketone, a metabolic dead-end product [125].

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The removal of phthalocyanine dyes in aqueous solution by peroxidase has been widely reported, especially by white rot fungi [51, 67]. Phthalocyanine dyes are reactive dyes containing metallic complexes, mostly Cu, used to produce blue and green shades in textile dyes. The peroxidase-catalyzed degradation of these dyes involves cleavage of the nitrogen bonds in the inner ring of the phthalocyanine molecule and release of Cu^{2+} from the metal complex [126]. However, the resultant products tend to be more toxic in the environment [126].

5. Evolutionary issues and scope for improvement of dye-degrading enzymes

Enzymes are capable of carrying out a tremendous range of biochemical functions, including dye bioremediation. However, their efficiency, stability, and costs often do not correspond to the needs of industrial operation [127]. In dye bioremediation, the choice of enzymes also depends on the effluent characteristics, operational requirements, and costs. Although some peroxidases and laccases are being employed successfully in industry, there is still no enzyme that combines the desired attributes of being stable and active over a range of temperatures and pH values, with high reduction potential [128].

To overcome this limitation, tailor-made biocatalysts can be created from wild-type enzymes by protein engineering using either rational design via computer-aided molecular modeling and site-directed mutagenesis, or by directed evolution techniques. These techniques can be used to successfully modify protein activity, stability, enantioselectivity, soluble expression, and binding affinity. In this regard, the availability of the structure of the enzyme and knowledge about the relationships between structure and function is requisite to undertake rational design and is consequently very information intensive [129]. Rapid progress in solving protein structures, and the enormously increasing number of sequences stored in public data bases have significantly eased access to data and structures, making rational protein engineering possible.

To overcome challenges faced by rational design, directed evolution has emerged as a key technology for protein engineering, generating impressive results [129]. Direct evolution involves four key steps: (1) selecting a starting gene sequence, (2) creating a library of variants, (3) selecting variants by high-throughput screening with improved function, and (4) repeating the process until the improvement or function is achieved [130]. The most common mutagenesis method used includes error-prone PCR (ep-PCR), saturation mutagenesis, and DNA shuffling [131]. For both approaches to protein engineering, the gene(s) encoding the enzyme(s) of interest, a suitable expression system, and a sensitive detection system are prerequisites.

5.1. Engineering for specificity

There have been many attempts to use rational approaches to engineer laccases over the last couple of decades. Using site-directed mutagenesis, Xu et al. [132] developed a collection of variants with structural perturbations at the T_1 Cu center to determine what parameters define the catalytic activity and the redox potentials of laccase. In the study, F463M mutation resulted

in a T_1 Cu site with an EPR signal intermediate between that of the wild-type laccase and plastocyanin, an altered UV-visible spectrum, and a decreased redox potential (by 0.1 V). In oxidizing phenolic substrate, the mutation also led to a more basic optimal pH as well as an increase in k_{cat} and K_m. Similarly, triple mutations V509L/S510E/G511A and L466V/E467S/A468G near T_1 Cu center of *Myceliophthora* and *Rhizoctonia* laccase, respectively, resulted in a phenol-oxidase activity with an altered K_m, k_{cat}, fluoride inhibition, and pH optimum shifted 1 unit lower and higher, respectively [133]. These observations were attributed to mutation-induced structural perturbations on the molecular recognition between the reducing substrate and laccase and on the electron transfer from the substrate to the T_1 Cu center. Modifications in the amino acid composition in the enzyme active site of *Tinea versicolor* laccase also improved enzyme activity and affinity toward larger phenolic substrates [134].

Random mutagenesis experiments on *Pleurotus ostreatus* laccase POXC and POXA1B cDNAs, using ep-PCR, have been reported to result to variant library with altered enzyme properties [135]. In this study, two variants 2L4A and 3L7H showed a higher specific activity than the wild-type enzyme toward typical aromatic substrates and expanded dye degradation specificities [136]. Several directed evolution studies of bacterial laccase CotA have also been used to successfully improve enzyme substrate specificity and functional expression [137-140]. Gupta and Farinas [138] reported a variant of CotA having 120-fold more specificity for ABTS with unexpectedly enhanced thermal stability with the half-life for the heat inactivation ($t_{1/2}$) at 80°C increased by 62 min. This newly generated laccase variant represents a helpful "evolved form" of the enzyme that is more durable and versatile as a biocatalyst, with respect to both the varieties of xenobiotics degraded and the operative conditions.

Similar to laccases, several attempts have been made to engineer peroxidases specificity using rational approaches [68, 141-143]. Using a combination of site-directed mutagenesis and *in vivo* shuffling, Garcia-Ruiz et al. [68] developed VPL2 variants of *P. eryngii* with enhanced VP activity (~129-fold) compared with the parental VPL2. Engineering of the cavity of cytochrome c peroxidase (CCP) via W191G mutation has been shown to alter the specificity of the enzyme toward substrates 2-aminothiazole [144]. Two mutations (A147M and A147Y) in CCP have also been reported to exhibit unique specificities toward oxidation of small substrates [142].

5.2. Engineering for properties of enzymes

Protein thermostability is a crucial issue in the practical application of enzymes in dye bioremediation applications. Several studies have reported the application of protein engineering techniques to improve thermal stabilities of peroxidases [68, 145, 146], azoreductase [147], and laccases [148, 149]. *In silico* design and site-directed mutagenesis of thermo-labile residues of *Coprinus cinereus* peroxidase (CiP) resulted in two variants (S323Y and E328D) with increased thermostability over the wild-type enzyme in addition to conserved catalytic activity [145]. Similarly, five rounds of mutagenesis/recombination followed by high-throughput screening yielded a variant 1B6, showing 300-fold higher half-life at 50°C than that exhibited by the homodimeric wild-type PpAzoR azoreductase from *Pseudomonas putida* [147]. In *P. ostreatus* VPL2, directed evolution involving six rounds of DNA shuffling cycle was used to improve enzyme secretion, activity, and stability [68]. The generated variant had a higher T_{50} of 8°C and increased enzyme stability at alkaline pH. In addition, the K_m for H_2O_2 was enhanced

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15-fold with the catalytic efficiency maintained, accompanied by an improvement in peroxide stability.

In our study, we have reported the stabilization of bacterial DyP AnaPX against H_2O_2-induced inactivation by replacing the Met residues in the heme pocket with high redox residues Ile, Leu, and Phe [80]. The heme cavity variants M401L, M401I, M401F, and M451I had significantly increased H_2O_2 stabilities of 2.4-, 3.7-, 8.2-, and 5.2-fold, respectively. Surprisingly, M401F and M451I variants retained 16% and 5% activity at 100 mM H_2O_2, respectively. In addition, the two mutants maintained high dye decolorization activity toward AQ and azo dyes at 5 mM H_2O_2 and exhibited a slower rate of heme degradation than the wild-type enzyme (Figure 5). The observed stabilization of AnaPX was attributed to (1) the replacement of potentially oxidizable Met residues, (2) the increased local stability of the heme pocket, or (3) the alteration of the self-inactivation electron transfer pathways due structural perturbations of the heme pocket by the above mutations. The observed increased stabilities and broad substrate specificity can be potentially useful for the further practical application of these AnaPX mutants in bioremediation of wastewater contaminated with recalcitrant AQ, under conditions of higher peroxide concentrations.

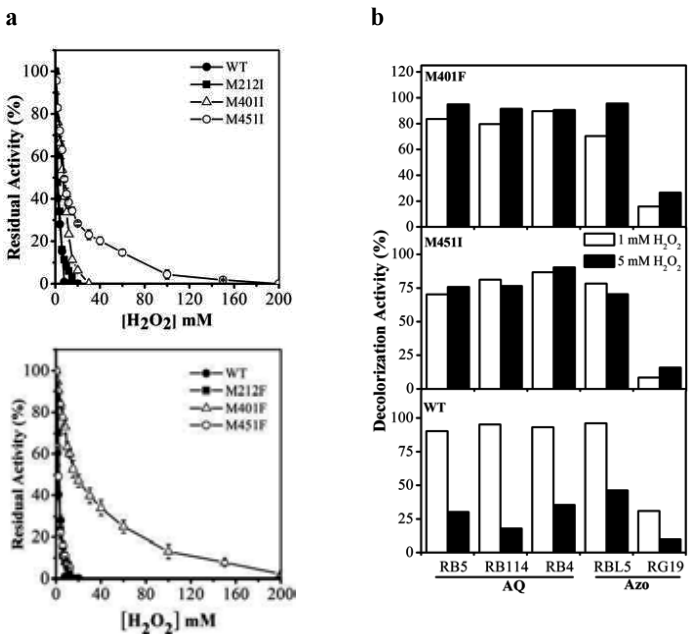

Figure 5. (a) H_2O_2 stability of wild-type AnaPX and Met-substituted variants. (b) Dye decolorization activity of AnaPX and two improved variants (M401F and M451I) on AQ and azo dyes at two different H_2O_2 concentrations (1 and 5 mM) [80].

Strategies for further improvements of laccase through genetic, metabolic, and protein engineering in suitable heterologous hosts for enzyme overproduction and enhanced enzyme kinetics and substrate binding, improving enzyme activity and stability, have been reported

[128]. The substitution of the aromatic amino acids residues with nonaromatic residues of *T. versicolor* laccase resulted in increased resistance to inactivation by free radicals [150]. Directed evolution has been used to increase laccase activity by 170- to 32,000-fold, pH, and temperature stability [151, 152]. The resultant mutants also exhibited increased tolerance to organic solvents such as ethanol and acetonitrile by 30% and 20%, respectively [153]. In addition, the directed evolution of high redox potential laccases has been used to overcome the obstacles associated with their functional expression in host suitable for *in vitro* evolution experiments [151, 153].

6. Current and potential synthetic dye biodegradation applications

6.1. Free enzyme biodegradation of industrial dyes

Enzymes, as potent biocatalysts, have been employed in numerous fields primarily for their immense catalytic potential [36]. In dye bioremediation, cell-free or isolated enzymes are preferred over the intact organism, especially when the effluent to be treated contains pollutants that cannot support growth. The key to successful application of enzymes for dye decolorization is the selection of appropriate enzyme cocktail that will exhibit versatility and efficiency, even under mild reaction conditions. The delivery system selected must be well suited to the purpose, simple, efficient, and cost-effective. Enzymes may be delivered to the target effluent in different ways in either cell-free or immobilized form.

Currently, there are commercial preparation of peroxidases and laccases used for different applications. DeniLite II S®, a commercial laccase formulation containing laccase, a mediator, and a nonionic surfactant, is used in the finishing process for indigo stained clothes [154]. A laccase from ascomycete fungus *Myceliophthora thermophila*, sold commercially by Novozymes as Suberase®, is used in the removal of astringency of cork stoppers for wine bottles. Ligno-Zym® system (GmbH), a mixture containing *T. versicolor* laccase and a group of mediators (N-OH-, N-oxide-, oxime-, or hydroxamic acid–functional groups), is used to remove 50% to 70% of lignin from pulp within 1 to 4 h [154]. However, the potential of these commercial laccase preparations for treatment of effluents containing dyes has yet to be demonstrated.

In the industrial scale of operation, the use of pure enzymes in effluent treatment is not economically feasible due to high start-up and running costs. The use of free enzymes as compared with their immobilized forms also show some significant drawbacks such as (1) thermal instability, (2) susceptibility to attack by proteases, (3) activity inhibition, (4) high sensitivity to several denaturing agents, and (5) difficulty of separating or reusing the free catalyst at the end of the reaction from the reaction mixture [155].

6.2. Immobilized enzyme bioremediation of synthetic dyes

In recent years, it has been shown that many industrial dyes can be decolorized by laccases and peroxidases immobilized on different supports. In contrast to soluble enzymes, immobilization offers higher enzyme stability, reusability, and capability to work in aqueous as well as in organic solvents due to protection against denaturants and proteolysis and reduced susceptibility to microbial contamination. This may be partly due to enzyme stabilization effect

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on immobilization leading to restricting the protein unfolding process, as a result of the introduction of random intra- and intermolecular cross-links [155]. The development of immobilization methods has consequently caused a significant increase in the application of oxidoreductases in various technological processes [156].

Laccases have been immobilized on various supports such as glass-ceramic materials, imidazole-modified silica, montmorillonite, alginate-gelatin mixed gel, hydrophobic sol-gel, and green coconut fiber and applied in decolorization/degradation of various textile and nontextile dyes and phenolic compounds [27, 157-160]. For example, *Trametes modesta* laccase immobilized on alumina decolorized 41 commercial azo, triphenylmethane, indigoid, and heterocyclic dyes. The immobilization of laccase on alginate mixed gels or hydrophobic sol-gel also led to it improved pH stability, thermostability, and reusability of the enzymes, although a slight decrease in enzyme activity and dye affinity was observed. The entrapment of laccase in alginate-gelatin, alginate-chitosan mixed gels, or hydrophobic sol-gel matrix of trimethoxysilane and proplytetramethoxysilane has been reported to lead to significant lacasse stability toward heat denaturation [161]. The reported improved decolorization of wastewaters by immobilized laccases is attributed to both enzymatic catalysis and support adsorption [112].

There are comparatively fewer investigations on dye decolorization by immobilized peroxidases, probably due to their requirement of H_2O_2 for activity. For peroxidase catalysis, H_2O_2 must be added or generated *in situ* to avoid enzyme deactivation and to achieve a stable decolorization process [158, 162]. For example, the half-life of *Saccharum spontaneum* peroxidase immobilized on polyethylene was favored by careful addition of H_2O_2 to the reactor to decolorized 15 batches of Procion green HE-4BD [158]. Higher loading rates of H_2O_2 resulted in 50% loss in decolorization activity of Orange II within 2 h by *Bjerkandera* sp MnP in a membrane reactor; however, the enzyme maintained 96% efficiency under optimized H_2O_2 and enzyme feeding rates [163]. Similar to laccases, the immobilization of peroxidases into a sol-gel matrix of tetramethoxysilane and propyltrimethoxysilane or in alginate gel and mixed alginate-pectin gel improves their storage stability, pH stability, and thermostability, in addition increased enzymes reusability and decolorization efficiency. The above-mentioned examples illustrates importance of immobilization as a powerful technique in expanding the application of oxidoreductases in bioremediation, particularly in those circumstances where the enzyme can be reused in the application many times to reduce operation costs.

7. Future prospects and conclusion

In this chapter, we have discussed the descriptive information on the oxidoreductases from various microorganisms, including their discovery, biochemistry, current biodegradation applications, and limitations to their large scale and efficient use. An ideal enzyme for dye bioremediation application should have the following properties: (i) broad substrate specificity; (ii) high redox potential; (iii) high tolerance to inactivation by radicals, organic solvents, and shearing forces; (iv) ability to work with a large number of mediators; (v) broad pH and temperature optima; (vi) high enzyme activity and stability; and (vii) low production costs.

The current oxidoreductases such as peroxidases and laccases are not well suited for industrial applications that require particular substrate specificities and application conditions (pH and temperatures) in addition to high expression levels. Consequently, effluent treatment using enzymes on a large scale is still not economically viable.

Within the last decade, there is increased research interest in the application of genetic engineering techniques to develop "designer" enzyme cocktails for large-scale dye bioremediation applications in different industrial sectors. The availability of high-throughput screening assays and functional expression systems plus the crystal structures of the enzymes has helped these efforts enormously. Breakthroughs through protein engineering involving combination of directed evolution with both hybrid and rational approaches, including computational studies, will permit the conversion of the current array of oxidoreductases into versatile biotechnological products for dye bioremediation. In addition, an interdisciplinary approach to wastewater treatment involving nanotechnology and enzyme technology will enable the utilization of peroxidases, laccases, and azoreductases to their full potential. These studies need to be conducted in the context of dye mixtures simulating real dyeing baths, as real bioremediation process is affected by all the factors involved in the dyeing processes, such as components and auxiliaries present in the wastewater, that markedly affects dye decolorization and/or the enzyme.

In conclusion, the promise of the concerted research efforts evident thus far and the potential of modern microbial and enzyme technologies to make radical improvements in the oxidoreductases give confidence that the development of successful technologies for industrial synthetic dye bioremediation will be possible in the near future.

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Chapter 7

Use of Additives in Bioremediation of Contaminated Groundwater and Soil

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Additional information is available at the end of the chapter

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Abstract

This chapter reviews the application of additives used in bioremediation of chlorinated solvents and fuels for groundwater and soil remediation. Soluble carbon substrates are applicable to most site conditions except aquifers with very high or very low groundwater flow. Slow-release and solid substrates are intended to be long-lasting in supplying carbon for microbial growth thereby minimizing operation and maintenance requirements. Microbes as special additives can be used to enhance bioremediation (bioaugmentation) where such microbes are lacking. Oxygen gas can be added to increase aerobic biodegradation, and nutrients addition may be needed to stimulate and maintain sufficient microbial population. pH modifiers to control acidity for optimal microbial growth and degradation can also be added. Delivery of additives to the subsurface can be accomplished through permanent injection wells, direct-push methods, or permeable reactive barriers (biowall). Potential issues with additive use include biofouling, stalling, short circuiting, displacement, reduced hydraulic conductivity, and secondary water quality deterioration. Methods and techniques to deal with these issues are provided and future research needs are identified.

Keywords: Organic contaminants, carbon substrates, additives, bioremediation implementation, potential issues

1. Introduction

Bioremediation is an important type of remediation technology that uses microorganisms (mainly bacteria) to destroy hazardous contaminants or transform them to less harmful forms.

Microorganisms, through their enzymatic pathways, act as biocatalysts and facilitate the progress of biochemical reactions that degrade the targeted contaminants. Bioremediation has been used in the cleanup of organic (e.g., chlorinated solvents, petroleum hydrocarbons, and pesticides), inorganic (e.g., perchlorate and nitrate), metals, and radionuclides contaminated sites [1-3].

Because of the role microorganisms play in bioremediation, any factors impacting survival and growth of these organisms will impact bioremediation. Although microorganisms have been isolated even under extreme conditions, most of them grow optimally over a narrow physical/chemical/biological range. Thus, to effectively and efficiently cleanup a contaminated site through bioremediation, it is important to achieve optimal biogeochemical conditions for microbial communities [4]. The conditions to consider include site hydrogeological characteristics, contaminant concentration, pH, redox potential, nutrients, moisture, and temperature [5-7].

In some cases, natural conditions at a contaminated site can provide all essential materials in large enough quantities that bioremediation can occur without human intervention. This process is often referred as intrinsic bioremediation. This is the primary degradation mechanism behind natural attenuation. However, under the natural conditions of most sites, microorganisms that degrade contaminants may be naturally present in the subsurface, but may not necessarily be there in sufficient quantities required for optimal bioremediation of the site. In these cases, engineered bioremediation (also termed as biostimulation) is needed. Engineered bioremediation relies on accelerating desired biodegradation reactions by encouraging growth of target microorganisms, as well as by optimizing the environment in which the organisms must carry out the detoxification reactions [8]. Engineered bioremediation involves the addition of substrates (electron donors), nutrients, and/or other materials (e.g., pH buffers) into the subsurface to stimulate microbial growth and activity or establish supportive geochemical conditions.

This chapter focuses on the use of additives to stimulate bioremediation of organic contaminants (e.g., chlorinated solvents, petroleum hydrocarbons, and pesticides). Following the introduction, the second section of the chapter discusses major reaction pathways in bioremdiation. The third section focuses on the major types of additives and the fourth section discusses in detail how the additives are implemented. The fifth section deals with potential issues associated with additives use in bioremediation. The final section summarizes the chapter and identifies future research needs.

2. Fundamental reactions in bioremediation

Bioremediation can be generally classified into two major types: bioaugmentation and biostimulation. Bioaugmentation is a type of bioremediation that adds microorganisms to enhance degradation of contaminants at contaminated sites. Bioaugmentation involves the delivery of selective and enriched microbial cultures into the subsurface to accelerate biodegradation reactions to achieve rapid and complete degradation of contaminants. The objective

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of bioaugmentation is to increase the overall degradation rate in cases where the indigenous microbial populations cannot completely degrade a contaminant or degradation rates are too low to meet the remedial goals in an acceptable time frame. The introduced microorganisms augment, but do not replace, the resident microbial population [9-10].

Biostimulation, the other type of bioremediation, involves stimulating growth of existing bacteria at sites to enhance degradation of contaminants. This can be done by adding various forms of rate limiting nutrients (such as phosphorus and nitrogen) and electron acceptors (organic carbon), and geochemical conditions modifiers (e.g., oxygen, pH modifiers).

Fundamentally, contaminants at bioremediation sites are degraded through a number of biochemical reactions that include aerobic reactions, anaerobic oxidative reactions, anaerobic reductive reactions, and cometabolic reactions. Aerobic bioremediation uses molecular oxygen (O_2) as an electron acceptor in remediation through direct microbial metabolic oxidation of a contaminant. Aerobic bioremediation is most effective in degrading non-halogenated organic compounds (e.g., BTEX, diesel) to carbon dioxide and water [3, 11]. Similar to aerobic bioremediation, anaerobic oxidative bioremediation also relies on direct microbial metabolic oxidation of a contaminant and is an alternative to aerobic bioremediation in anaerobic aquifers [3]. Contaminants that can be anaerobically oxidized include aromatic hydrocarbons, BTEX, fuels, and some chloroethenes [12].

Oxygen needs to be depleted before anaerobic reductive bioremediation can proceed. Anaerobic reductive dechlorination may be the most important type of anaerobic reductive bioremediation at contaminated sites. In this reaction, bacteria gain energy and grow as one or more chlorine atoms on chlorinated aliphatic hydrocarbons (CAH) molecule are replaced with hydrogen in an anaerobic environment [13-14]. The chlorinated compound serves as electron acceptor and hydrogen serves as the direct electron donor. Hydrogen in the form of H_2 gas used in this reaction is typically supplied by fermentation of organic substrates [15-17].

Cometabolic bioremediation is a reaction in which contaminants are reduced by a non-specific enzyme produced during microbial metabolism of another compound (i.e., the primary substrate) in an anaerobic environment. Cometabolism occurs when microorganisms using one compound as an energy source fortuitously produce an enzyme that chemically transforms another compound. Organisms thus can degrade a contaminant without gaining any energy from the reaction [3, 15, 18].

3. Common substrates and additives used for bioremediation

To stimulate microbial growth and degradation of contaminants, supplemental amendments including those that directly support microbiological growth (C, N, P) and those that maintain or create favorable geochemistry (pH buffering, dissolved O_2) are used. In some cases, surfactants are also used to enhance solubility and bioavailability of contaminants from soil and sediments in order to improve treatment efficiency [19-21].

3.1. Organic carbon substrates

As the major building block for microorganisms, organic carbon may be the most important and prominent additive used in bioremediation. Under anaerobic conditions, many microorganisms are capable of fermentation of organic matter, and some bacteria can produce hydrogen gas. Thus, almost any fermentable substrate can be a potential source of carbon and hydrogen to stimulate bioremediation. These include naturally occurring dissolved organic carbon (DOC), accidental releases of anthropogenic carbon (e.g., fuels), carbohydrates (sugars), alcohols, oils, solids (e.g., bark mulch, chitin), and complex compounds (e.g., whey and cellulose) [22-23]. Table 1 summarizes the attributes of several common substrate types. These substrates are generally classified into three types (soluble, slow release, and solid substrates), and each type will be discussed in more detail in the following sections.

Substrate	Typical delivery techniques	Form of application	Frequency of application
Soluble substrate			
Methanol, ethanol, sodium benzoate	Injection wells or recirculation systems	Dilute in water	Continuous to monthly
Lactate and butyrate	Injection wells or recirculation systems	Dilute acids or salts in water	Continuous to monthly
Molasses, high fructose corn syrup	Injection wells	Dissolved in water	Continuous to monthly
Whey	Direct injection or injection wells	Dissolved in water or slurry	Monthly to annually
Slow release substrates			
HRC® or HRC-X®	Direct injection	Straight injection	Annually to biennially for HRC®, every 3-4 years for HRC-X®, potential for one-time application
Vegetable oils	Direct injection or injection wells	Straight oil injection with water push or high oil/water content (>20%) emulsions	Typically one-time application
Vegetable oil emulsions	Direct injection or injection wells	Low oil/water content (>10%) microemulsions suspended in water	Typically every 2-3 years
Solid substrates			
Mulch and compost	Trenching or excavation	Trenches, excavations, or surface amendments	One-time application
Chitin (solid)	Trenching or injection of a chitin slurry	Solid or slurry	Annually or biennially, potential for one-time application

Table 1. Substrates used in bioremediation (modified from [24-25])

3.1.1. Soluble carbon substrates

As shown in Table 1, sodium lactate, molasses, ethanol, methanol, butyrate, and sodium benzoate have been used as soluble substrates, and sodium lactate and molasses are among the most widely used in bioremediation. Soluble substrates are applicable to most site conditions with the exception of aquifers with very high (> 30 cm per day) or very low (<30 cm per year) groundwater velocities. Soluble substrates applied as dissolved or "aqueous" phase offer the greatest potential for uniform distribution throughout the aquifer matrix relative to other substrates. Soluble substrates are easy to handle, mix, and inject. Advection helps soluble substrate distribution in the subsurface. As a result, it is possible to increase the radius of influence (ROI) and reduce the number of injection points, as a larger volume of substrate can be dispersed from a single injection point. Soluble substrates are best suited for remediation of deep aquifers where drilling costs are high.

The following disadvantages associated with the use of soluble substrates need to be recognized:

1. In order to achieve target total organic carbon (TOC) levels, and at the same time avoid adverse impacts to pH and maximize ROI, it is often necessary to test and adjust substrate loading rates and mixing ratios during the initial phase of injection. The need for optimization of loading rates increases costs of operation and maintenance (O&M) during startup. In general, the life-cycle cost of O&M for soluble substrate systems is high relative to other substrates [23].

2. In high-flow aquifers, soluble substrates readily mix with groundwater. And also because of rapid replenishment of competing electron acceptors from the groundwater, soluble substrates are rapidly degraded and it is difficult to maintain sufficient reducing conditions [24] for bioremediation. Thus, soluble substrates may not be suitable for these conditions.

3. For low flow groundwater, insufficient mixing and contact time of the substrate with the groundwater plume is also an issue. Degradation of substrate can occur before the processes of advection and dispersion allow for distribution of the dissolved organic carbon. Biofouling is also a concern if too much substrate is injected into low flow aquifer without adequate dispersion.

4. Due to the rapid degradation of soluble substrate, repetitive injection may be required, and frequent high concentration injections could lead to biofouling and low pH in groundwater.

3.1.2. Slow-release substrates

The common slow-release carbon substrates used to stimulate anaerobic bioremediation include HRC® (Hydrogen release compounds) and vegetable (edible) oils. These substrates are intended to be long-lasting in their ability to supply carbon for microbial growth. They are relatively immobile in the subsurface, and rely on advection and dispersion of soluble

compounds from the slow-release substrates (e.g., lactic acid for HRC®) for effective delivery throughout the aquifer matrix.

The primary benefit of slow-release substrates is that they require infrequent injection (often only once) with no O&M requirements other than performance monitoring; however, uneven distribution may be an issue for slow-release substrates because of the viscous characteristics of these fluid substrates.

To improve the distribution of slow-release substrates in the subsurface, while still providing a long-lasting source of organic carbon, vegetable oil emulsions have been developed. Microemulsions consisting of 5% to 10% vegetable oil in water (by volume) are relatively low-viscosity mixtures. The use of microemulsions is the result of lessons learned in early vegetable oil field trials. In earlier tests using coarse viscous emulsions or neat vegetable oil, high injection back pressures limited ROI, and reductions in hydraulic conductivity were observed [24-25].

3.1.3. Solid substrates

Solid substrates that have been used in bioremediation include tree mulch, compost, as well as other agricultural byproducts such as cottonseed hulls. Mulch used in bioremediation is usually obtained from shredding and chipping tree and shrub trimmings. To provide a source of nitrogen for microbial growth and also provide a source of more readily degradable organic carbon, green plant material or compost is often incorporated into solid substrates in these applications. Degradation of the solid substrates by microbial processes in the subsurface provides a number of breakdown products (e.g., humic acids). Solid substrates are intended to be long-term sources of organic carbon, with anticipated lifespans exceeding 5 to 10 years [26-27]. The drawback with the solid substrates also lies in the fact that it is hard to be degraded and used by the microbes as readily as the soluble substrates.

3.2. Other additives used in bioremediation

3.2.1. Oxygen gas

In aerobic reactions, microorganisms extract energy via electron transfer during oxidation of contaminants and reduction of oxygen gas. Electrons are removed from contaminants and transferred to oxygen during the process. The major kinetic limitation on aerobic bioremediation is often the availability of molecular oxygen due to low solubility of oxygen gas in water. In the absence of any external supply of oxygen, concentration of dissolved oxygen in water quickly decreases to very low levels, resulting in anoxic conditions and disruption of aerobic metabolism.

To promote aerobic biodegradation, air, oxygen, or other oxygen sources (e.g., hydrogen peroxide, ozone, sodium nitrate, and perchlorate) may need to be added in some systems. Depending on their physical properties, site hydrogeology, and the desired delivery efficiency, oxygen and oxygen-releasing compounds can be delivered to groundwater via different methods. There are two methods to introduce oxygen to aquifers: one is direct supply of air into groundwater through aeration wells; the other is through addition of hydrogen peroxide.

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Dissolved oxygen is released from hydrogen peroxide as the hydrogen peroxide rapidly degrades into water and oxygen gas through hydrolysis [28].

3.2.2. Nutrients

An aquifer normally contains sufficient amounts of nutrients for microbial growth. In engineered bioremediation, however, due to the addition of organic substrate, the nutritional demand imposed by rapid microbial growth may exceed the capacity of the aquifer system [29]. In addition to a readily degradable carbon source, microorganisms also require nutrients such as nitrogen, phosphorous, and potassium (N, P, and K) for cellular metabolism and therefore successful growth [28, 30].

Commonly used nutrients include mineral salts (e.g., KNO_3, $NaNO_3$, $Ca(NO_3)_2$, NH_4NO_3, $(NH_4)_2SO_4$, K_2HPO_4, $(NH_4)_2HPO_4$, $MgNH_4PO_4$,), anhydrous ammonia (NH_3), urea $(NH_2)_2CO$, and many commercial inorganic fertilizers [8]. In practice, nitrogen and phosphorus requirements are often estimated by calculating a carbon to nitrogen to phosphorus ratio C/N/P close to 100/(10 to 5)/1. Many authors report optimum experimental results with a C/N/P of ~70/3/0.6 [31], 8/1/0.07 [32] for crude oil bioremediation.

3.2.3. pH modifiers

The pH range within which bioremediation processes operate most efficiently is approximately 5.5 to 8 [8], as this is also the optimal pH range for many heterotrophic bacteria, the major microorganisms active in most bioremediation technologies; however, the optimal pH range for a particular situation is site-specific.

At a field site, pH is influenced by a complex relationship between organisms, contaminant chemistry, and physical and chemical properties of the local subsurface environment. For example, in low-alkalinity systems, fermentation of complex substrates generates acids, and hydrochloric acid (HCl) is formed during anaerobic dechlorination. These processes may significantly decrease groundwater pH. Reducing groundwater pH to below 5 will likely inhibit microbial growth (e.g., sulfate reducers, methanogens, and some dechlorinating microbes) [33]. Normally, the natural buffering capacity of the aquifer matrix is adequate to prevent the development of acidic groundwater pH; however, at some sites, pH buffer amendments such as sodium bicarbonate may be required to maintain near-neutral pH in groundwater systems with insufficient natural buffering capacity. The maintenance of near-neutral groundwater pH is not only important for microbial growth, but also for secondary groundwater geochemistry.

4. Additives implementation in bioremediation

4.1. Organic carbon substrate selection

The choice of electron donor (substrate) and the delivery methods are essential components of bioremediation. Substrates differ in rates at which they are degraded and become available

for biodegradation. They also differ in the complexity of their composition, in their physical form, and in their cost [24, 34].

The selected organic substrate should be suitable for the biogeochemical and hydrodynamic characteristics of the aquifer to be treated. Selection of an appropriate substrate should take into account expected performance in developing appropriate anaerobic reactive zones, the rate at which the substrate is used (efficiency of use), site infrastructure or land use, substrate availability, application configuration, delivery and distribution requirements, system O&M requirements, and cost of implementation (life-cycle cost, including cost of O&M).

The most commonly added substrates in bioremediation include lactate, molasses, hydrogen release compound (HRC®), and vegetable oils. Ethanol, methanol, benzoate, butyrate, high-fructose corn syrup (HFCS), whey, mulch, compost, chitin, and gaseous hydrogen are less frequently used. The physical and chemical characteristics of a substrate (e.g., phase and solubility) may make certain substrates more suitable than others in a particular application. Sometimes a single substrate is not sufficient, and in fact, combinations of various substrates are becoming more common in contaminated site remediation. For example, an easily distributed and rapidly degraded soluble substrate, such as lactate, may be combined with a slow-release substrate, such as vegetable oil, as it provides longer term supply of organic carbon. In aquifer systems that are naturally aerobic, an easily distributed and highly degradable soluble substrate (e.g., ethanol or lactate) can be used to rapidly induce anaerobic, reducing conditions, thus reduce lag phase of anaerobic bacteria. Then a longer lasting, "slow-release" substrate (e.g., vegetable oil, chitin, or whey) is used to sustain the reaction zone. The combination use of these two types of substrates will minimize the cost of maintaining the treatment system [23-24].

In practice, however, selection of a substrate is often based on contractor experience or familiarity, or as a result of commercial marketing. Substrates are usually selected from a wide variety of available low cost food-grade products such as molasses, vegetable oils, and whey. The potential pitfall of this strategy lies in that ineffective, even inappropriate substrate, may have been selected in some cases, leading to poor treatment results.

4.2. Organic carbon dosage

The underlying principle in determining substrate loading rate is maintaining the balance in a remediation system so that native electron acceptors are fully utilized, while at the same time sufficient electron donor is left in the system to degrade the contaminant mass. To calculate substrate mass required to deplete available electron acceptors flux, substrate composition, stoichiometry, and utilization efficiency of the anticipated degradation reactions need to be known. However, the exact stoichiometric reactions and electron acceptor flux that occur in a site is difficult, if not impractical, to determine [34]. Calculations for substrate demand can be obtained from theoretical hydrogen equivalents resulted from a known mass of substrate versus estimated electron acceptor demand at a site. The electron acceptor demand typically comprises the following three components:

1. Contaminant electron acceptor demand. During anaerobic dechlorination, the contaminant is the electron acceptor, and there is a stoichiometric relationship between electron donor (e.g., hydrogen) and electron acceptor (contaminant mass). The stoichiometric relationships for direct anaerobic dechlorination of CAHs are favorable. For example, on a mass basis, 1 mg of H_2 will dechlorinate PCE (21 mg), TCE (22 mg), DCE (24 mg), and VC (31 mg), assuming 100% use of H_2 by the dechlorinating microorganisms [25, 35].

2. Native electron acceptor demand. The flux of groundwater and minerals in aquifer matrix include electron acceptors that in many cases are preferentially used over target contaminants. In general, bacteria using oxygen, iron, and sulfate generally outcompete *Dehalococcoides* for available hydrogen gas. Therefore, their presence exerts a demand on the electron donor required to satisfy removal of more energetically favorable electron acceptors, these alternate electron acceptors must be depleted before efficient reductive dechlorination to ethene will occur [23, 25].

3. Non-specific demand. In reality, a large percentage of injected substrate, resultant organic acids, hydrogen gas, and other byproducts will be used by opportunistic microbes for different life processes including cell growth, instead of being used by targeting microorganism for remediation. In addition, other factors such as groundwater flow rate, and concentrations of electron acceptors introduced as part of prior remediation efforts (e.g., dissolved oxygen gas, persulfate from in situ chemical oxidation (ISCO)) also impact dosage [25].

Therefore, it is not easy to have a good estimate on the substrate dosage for a remediation site. On one hand, sufficient carbon is required for contaminant degradation; on the other hand, excessive substrate will not only increase cost, but also may cause secondary problems. In practice, a safety factor of 5 to 20 times of pre-tests may be used to account for the uncertainties at a specific site and to provide for a design contingency. The uncertainties may arise from estimating substrate utilization for alternate electron accepting processes (e.g., methanogenesis or solid-phase alternate electron acceptors), and it may also be caused by the presence of DNAPL or sorbed contaminant mass. Nevertheless, care should be taken to use a loading rate that is not excessive (i.e., use of excessive safety factors) to avoid the creation of low pH conditions or secondary impacts to groundwater quality [24, 34].

Alternatively, the substrate loading rate for soluble substrates can be based on achieving an empirical TOC concentration in groundwater. The volume and strength of substrate are estimated to achieve a particular target level in the treatment area after mixing and dilution. For example, Suthersan et al. [36] suggested that a loading rate between 0.1 and 1 gram of organic carbon per liter of groundwater flux per day is sufficient to create and maintain a reducing reactive zone.

4.3. Additives implementation techniques in bioremediation

There are a number of system configurations and delivery strategies that can be used to distribute organic substrates in the subsurface. The appropriate technique depends not only on application goal (mass removal or plume containment) but also on the substrates used. The

physical nature of substrates dictates the addition technique and potential system configurations. The frequency of addition is determined by the longevity of the substrate and its ability to supply required substrate and nutrients.

Liquid substrates can be deployed through direct-push or permanent injection wells. Solid substrates are typically placed in trenches or in excavations as backfill in a one-time event using conventional construction techniques. In addition, groundwater recirculation systems, infiltration galleries, and trenches may also be used to deliver substrates to impacted aquifers.

4.3.1. Injection

Direct injection refers to the process of adding substrates, microorganisms, nutrients, oxidants, or reductants directly into the aquifer at injection points. Installed injection wells or direct-push well points are commonly used injection methods to deliver liquid substrates (Figure 1). Injection wells and injection point locations and spacing depend on site geology and hydrogeology, aquifer and plume characteristics, and volume of substrates or additives to be injected.

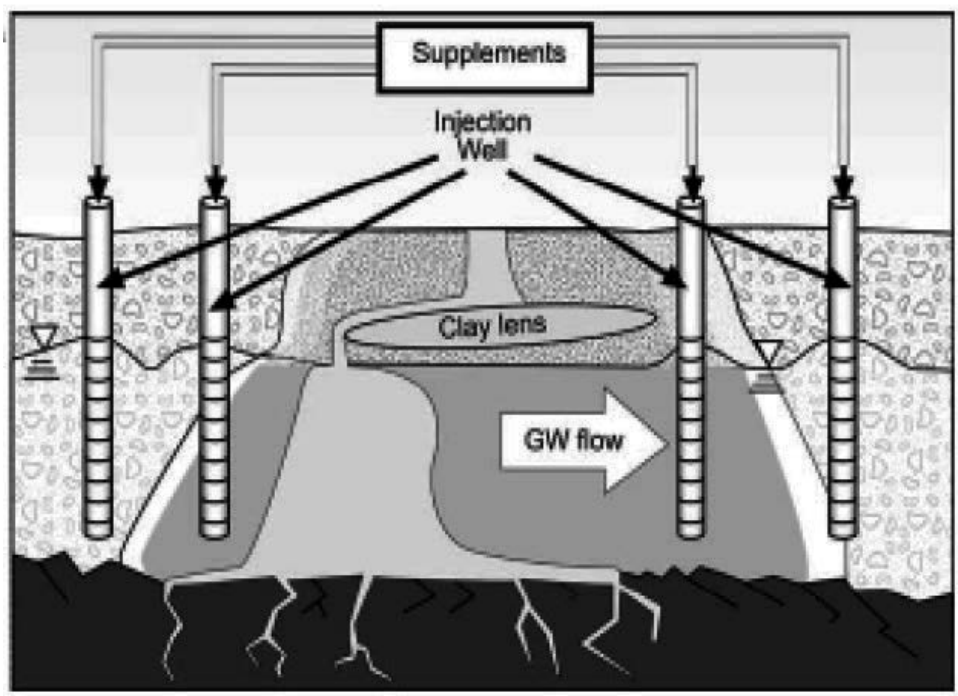

Figure 1. Schematic of the use of direct injection system in bioremediation (Adapted from [15])

Direct-push injection is suitable for both soluble and viscous liquid substrates, and is commonly used for shallow groundwater remediation in unconsolidated formations. The application of this technique is constrained by soil characteristics such as grain size or degree of

cementation. Gravel and cobbles in sediment inhibit use of direct-push technology. Direct-push (e.g., Geoprobe®) injection does not leave well points in place after injection (as opposed to permanent injection wells), and is only practical for slow-release substrates such as HRC®, vegetable oil emulsions, or whey slurries. Direct-push methods have also been used to install semi-permanent well points for short-term injections [23-24].

Permanent injection wells are generally used for soluble substrates where continuous or multiple injections of substrate or recirculation are required. Permanent injection wells are also necessary for sites that cannot use direct-push technology due to depth or soil lithology issues. If screened at appropriate depths and located within appropriate portions of the plume, existing monitoring or extraction wells from previous investigation or remediation activities can also be used for injection [23-24].

Direct injection may be performed through frequent, single-well injections or less-frequent, multiple-well injections with properly spaced injection points. Direct injection is effective at sites with moderate groundwater flow. Sites with very high groundwater flow can be problematic due to low cross-gradient distribution of the substrate within the plume. Direct-injection approaches are not suitable for highly heterogeneous aquifers because the substrate is not distributed evenly around individual injection points and the contaminant plume.

4.3.2. Recirculation

For sites with low groundwater flow rate, recirculation techniques may be required to obtain effective mixing of substrates and contaminated groundwater. Recirculation systems consist of a closed network of extraction wells and injection wells (Figure 2). The recirculation system is designed to hydraulically control substrate transport through the treatment zone. The distance between injection and extraction wells is dictated by groundwater flow velocities, plume size, and bioremediation process kinetics. Excess amendment that is not consumed is extracted at the recovery well and recycled to the injection well. Recirculation approaches may be the only effective method to achieve more uniform distribution of substrates and amendments at sites that lack significant natural hydraulic gradients [37-38].

Recirculation increases retention time of contaminated groundwater in the treatment zone. Substrate and amendments applied in recirculation systems are more readily controlled and they are distributed through the treatment zone. Recirculation systems can influence a much greater volume of the aquifer, and allow much greater distances between injection and extraction wells; however, continuous operation of a recirculation system requires dedicated equipment and maintenance and thus can be very expensive. In addition, continuous operation creates a potential for biofouling. Alternatively, a recirculation system can be operated in an intermittent fashion, i.e., recirculate for a short time (days to weeks), then shut off for several months, during which time electron donor is consumed and used for contaminant degradation. Intermittent operation of a groundwater recirculation system may be considerably less expensive than continuous recirculation. Periodic operation of a recirculation system will also result in less biofouling of injection wells compared to systems that require continuous recirculation of groundwater and injection of substrates [25].

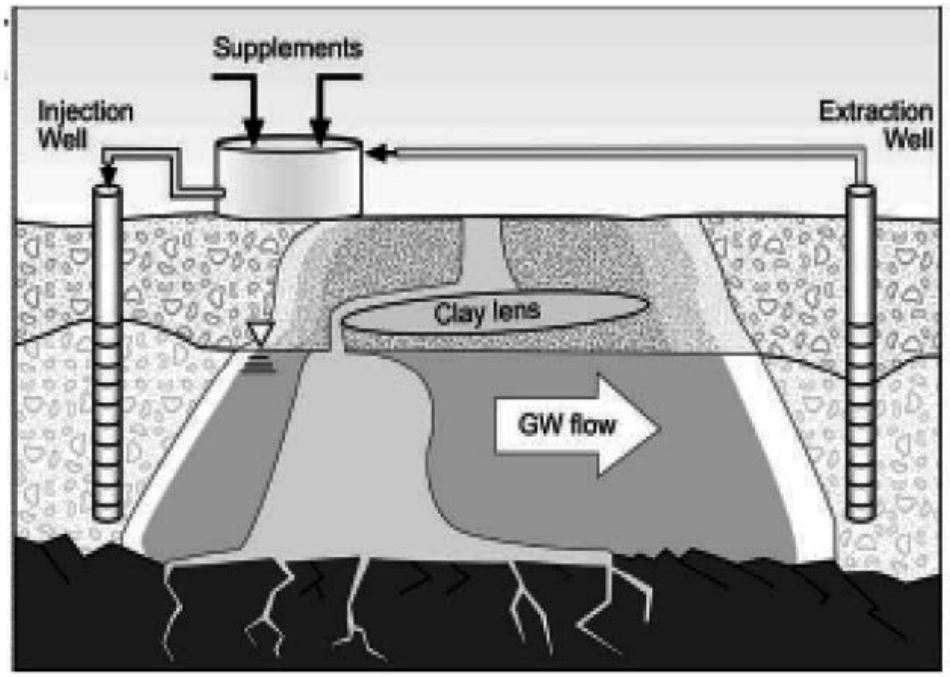

Figure 2. Schematic of a recirculation system in bioremediation (Adapted from [15])

4.3.3. Trenching

Solid substrate treatment systems are typically deployed in an excavated trench in the form of a permeable reactive barrier (PRB, e.g., biowall) (Figure 3). This treatment method relies on the natural flow of groundwater through solid substrates within the PRB to promote contact with slowly released soluble organic matter. This method is particularly suitable for sites with low permeability. Sometimes, perforated pipes can be laid on the top or bottom of the organic fill material to amend the organic fill material with liquid substrates or other amendments to improve performance [23-24].

Trenches may be installed using either continuous one-pass trenchers designed for installing subsurface utilities or hydraulic excavators. Trench depths are limited by type of equipment used, stability of the formation, and ability of the equipment to excavate the formation. Continuous trenching is not practical in hard and consolidated bedrock [23, 27].

5. Potential issues with additives use in bioremediation

Bioremediation has been demonstrated to be an effective technology for degrading chlorinated solvents and fuel hydrocarbons and has been used widely in groundwater and soil remedia-

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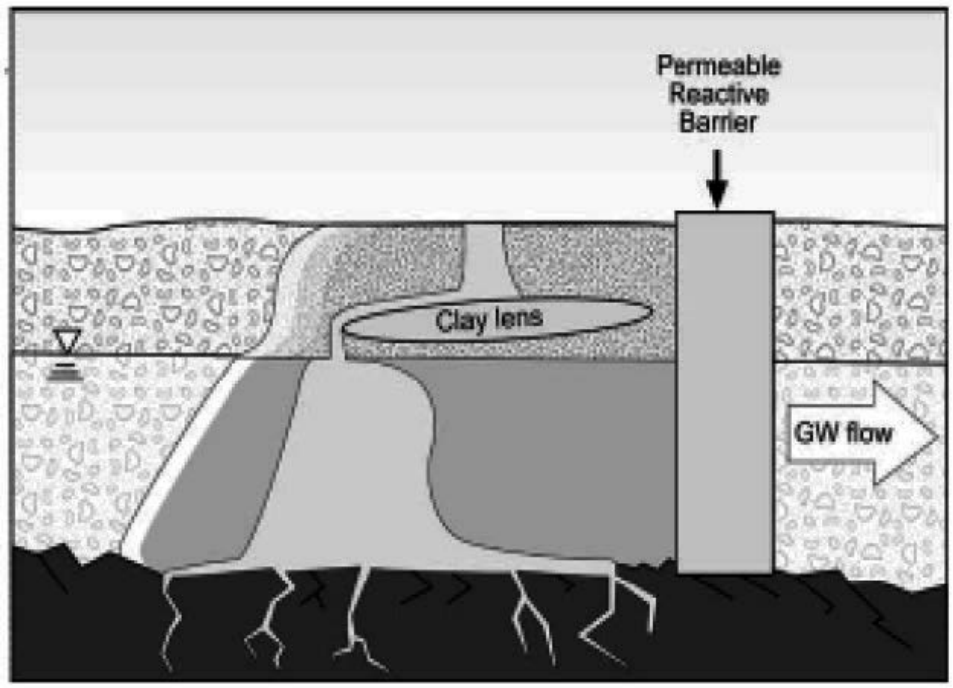

Figure 3. Schematic of a permeable reactive barrier (PRB) composed of organic carbon based materials (biowall) for bioremediation of contaminated groundwater (Adapted from [15])

tion. A number of issues associated with additives use in bioremediation have been encountered and need to be taken into consideration when considering bioremediation at a site.

5.1. Biofouling

Biofouling refers to failing or decreasing effectiveness of a remediation system due to excessive growth of biomass of non-targeting microorganisms. Biomass buildup in wells could decrease fluid injection rates. Biofouling of injection or recirculation wells has been observed at several sites due to growth of biomass or biofilms within well screens and surrounding sand packs. Biofouling in injection wells may impact the ability to effectively inject and distribute substrate.

Several approaches have been used to prevent or mitigate the biofouling issue. Preventive measures typically include well rehabilitation techniques such as surging and pumping, high pressure jetting, pulsed injection, injection of carbon dioxide under pressure, use of a clean water push to remove substrate residue, chemical methods such as surging and scrubbing with hydrogen peroxide or use of non-oxidizing biocides (e.g., Tolcide®) [16,25,39].

5.2. Stalling

Stalling in bioremediation refers to the incomplete degradation of targeted compounds due to accumulation of degradation daughter products. Anaerobic reductive bioremediation of PCE

and TCE, for example, may undergo incomplete degradation to DCE or VC resulting in the accumulation of these daughter products. Stalling has been observed in a number of scenarios:

1. Reductive dechlorination is most effective and efficient under sulfate-reducing to methanogenic conditions. In attempts to increase substrate utilization and reduce competition for hydrogen (e.g., by methanogenesis), hydrogen concentrations are controlled in some remediation systems. This may result in significant portions of the treatment zone remaining insufficiently reducing for complete dechlorination to occur, leading to "stalling" at intermediate degradation products such as *cis*-DCE or VC [24, 40].

2. Microorganisms generally gain more energy from dechlorination of more highly chlorinated contaminants such as PCE and TCE. Dechlorination of daughter products such as DCE and VC may not start until after parent products are sufficiently depleted [23].

3. *Dehalococcoides mccartyi* is the only known bacteria that can degrade chlorinated ethenes completely [41-42]. However, this bacterium may not be present at populations required to achieve complete dechlorination. Limitations in microbial populations create the potential for incomplete degradation and the buildup of daughter products such as *cis*-DCE or VC.

4. Co-contaminants or other geochemical conditions such as low pH, inadequate electron donor availability, or unfavorable geochemical conditions may inhibit microbial populations and lead to incomplete degradation of chlorinated solvents.

5.3. System bypassing or short circuiting

System bypassing or short circuiting is a common issue with in situ remedial technologies that rely on injection and distribution of amendments within the subsurface. Aquifer permeability and preferential pathways are two major factors that may impact distribution of substrates throughout a DNAPL source zone.

Short circuiting of substrates in the vadose zone may occur during the injection of liquid substrates. Whether injections are conducted through permanent wells or by direct-push methods, injection pressures must remain relatively low to avoid unintentionally fracturing the formation. Injection pressures greater than the overburden pressure may cause fracturing of the aquifer formation. This may lead to preferential flow of substrates along open fractures resulting in nonuniform distribution and short circuiting.

Another factor that can result in substrate bypassing is aquifer heterogeneity. Any liquid substrate, including aqueous substrate mixtures, will migrate along the pathway of least resistance (highest permeability). In heterogeneous systems, substrate distribution may bypass lower permeability aquifer zones. In these situations, multiple well points screened in each lithologic layer or zone may be required to avoid short circuiting of substrate to higher permeability zones.

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5.4. Reduction of hydraulic conductivity

Reduction in hydraulic conductivity may cause contaminated groundwater to flow around the treatment zone, impacting the ability of the targeted compound(s) to come into contact with the organic substrate and also impacting the ability to effectively further distribute soluble organic substrates. During enhanced bioremediation, hydraulic conductivity may be adversely impacted by several factors:

1. Biofouling of the aquifer due to excessive biomass growth, especially in low flow aquifers.
2. Gas clogging from generation of excessive amounts of dissolved gases including carbon dioxide, methane, and hydrogen sulfide. Gas clogging in the formation may occur when excessive amounts of gases are produced by biological activity. The formation of gas bubbles in the aquifer matrix lowers the aquifer permeability, reducing hydraulic conductivity [24].
3. Physical reduction in hydraulic conductivity and permeability due to the presence of viscous or non-aqueous substrates (e.g., vegetable oils). A significant reduction in hydraulic conductivity has been observed at saturations as low as 10% to 15% of viscous substrate. When reduction in hydraulic conductivity is a concern, oil-in-water saturations of 10% or less are typically recommended [24].

In the case of solid substrates used in trenches, permeability of solid substrate mixtures must remain equal to or higher than that of the surrounding formation. In practice, coarse sand and pea gravel are usually mixed with the substrate to maintain a sufficiently high permeability.

5.5. Displacement and dilution

In groundwater remediation, some mixing of injected substrate with contaminated groundwater will occur due to advection and dispersion. In order to achieve widespread distribution of substrates, injecting large volumes of soluble substrate mixed with potable water is necessary in some cases. However, injection of large volumes of substrate may cause significant displacement and dilution of the contaminant plume. One way to address the displacement and dilution issue is to inject a low volume/high concentration substrate mixture and to rely on advection and dispersion for mixing, although this requires relatively high rates of advection and dispersion to occur. The use of recirculation systems can aid in avoiding this issue.

5.6. pH and secondary water quality issues

When applying organic carbon in bioremediation, caution is required to not supply too much substrate to the subsurface because excess organic substrate generates organic acids and causes decreases in the pH of groundwater. In addition, anaerobic reductive dechlorination generates HCl that could also decrease groundwater pH.

A decrease in pH to the acidic range could potentially mobilize metals (notably iron, manganese) and metalloids (arsenic), creating secondary water quality issues at a site. The release of

these metals is also impacted by the prevailing redox conditions at a site. A decrease in pH could also inhibit growth of bacteria communities such as *Dehalococcoides*, thereby stopping the bioremediation process.

Changes in redox conditions can also enhance solubilization of metals and promote the formation of the following undesirable products (e.g., hydrogen sulfide and methane gases):

1. If nitrate is used, byproducts including nitrite, nitric oxide, nitrous oxide, and nitrogen gas could be generated. The predominant byproduct depends on the enzymes possessed by the microbes present.
2. Iron(II) is more soluble than iron(III), so iron reduction could lead to exceedance of iron water quality criteria.
3. Sulfate is reduced to sulfide under anaerobic conditions. If there are not enough dissolved metals to precipitate metal sulfides, free sulfide and hydrogen sulfide will be generated. Sulfide is toxic to microbial communities and could inhibit degradation of contaminants.

6. Conclusions

1. Bioremediation is widely used in groundwater and soil remediation of organic contaminants such as chlorinated solvents and petroleum hydrocarbons. Additives play an essential role in stimulating microorganism growth and in accelerating contaminant degradation in engineered bioremediation systems.
2. Common additives used in engineered bioremediation include organic carbon, oxygen, nutrients, and pH modifiers. Organic carbon substrate is the most important and widely used additives. A wide-range of materials can be used as carbon substrates. These substrates can be generally categorized into soluble, slow-release, and solid substrate subgroups.
3. The use of additives in engineered bioremediation systems depends on the physical properties of additives, site characteristics, treatment goals, and other factors. Additives are usually added to the subsurface environment through direct injection, recirculation, and trenching.
4. Despite the success of bioremediation technology applications in groundwater remediation, a number of issues have been identified with the use of additives in bioremediation. These include biofouling, stalling, system bypassing or short circuiting, reduction in hydraulic conductivity, contaminant plume displacement and dilution, and pH and secondary water quality issues. Taking these issues into consideration during remediation design and properly addressing these issues during implementation is essential for efficient and cost-effective site cleanup.
5. To enhance the efficiency and promote the application of bioremediation technology in contaminant remediation at contaminated sites, a number of research needs related to

additive use have been identified. These include: (I) Developing new and improved additives to allow for better distribution, less background consumption, and increased effectiveness of target organisms; (II) Developing new technologies for cost-effective delivery of additives, especially for low permeability aquifer systems; (III) Developing innovative ways to address issues encountered in additive use, such as secondary water quality issues and reduction in hydraulic conductivity.

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Chapter 8

Hexavalent Chromium (VI) Removal by *Penicillium* sp. IA-01

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Additional information is available at the end of the chapter

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Abstract

The objective of this work was to study the removal of chromium (VI) in aqueous solution by the fungus *Penicillium* sp. IA-01, isolated from polluted air with industrial vapors. To obtain the fungal biomass, pre-inoculums were performed in thioglycolate broth from a strain isolated from vapours contaminated with Cr (VI). The fungus was incubated for four weeks at ambient temperature, filtered, and washed three times with trideionized water. In preparing cellullar fractions, it was necessary to break the fungal cells with glass beads using a homogenizer being careful to keep the samples in frosty cold ice. To obtain the fungal biomass, the fungus was filtered and stored in an oven at 80°C, allowing it to dry for 48 h. Removal of Cr (VI) in vitro was evaluated using different cellular fractions and dead fungal biomass. We determine the optimal characteristics for metal removal in the reaction mixture. Concluding that the ideal conditions for the removal of Cr (VI) in the cell extracts were 37°C and pH 7.0, also we observ that the highest enzyme activity was in the mixed membrane fraction. In dead fungal biomass, the ideal conditions for removal of metal are 60°C and pH 1.0.

Keywords: *Penicillium*, Fugal biomass, Cellular fractions, Chromium (VI), Biosorption, Removal

1. Introduction

Environmental pollution with heavy metals is caused by anthropogenic and natural actions. Discharges of wastewater from various industrial activities such as electroplating, mining, paint factory, plastics, coating metal cables, and automotive radiators, and certain industries producing energy, metal engineering industry and producers of welding materials contain high concentrations of metals. Several heavy metals are highly toxic and ingestion of these metals by drinking contaminated water or breathing polluted air can cause serious health problems in human beings. Several metals are considered toxic at certain levels of concentrations in wastewater, such as arsenic, cadmium, cobalt, copper, chromium, nickel, lead and mercury. Unlike the organic compounds, heavy metals cannot be biodegraded or destroyd, therefore they must be removed. There are several methods for removal of heavy metals: ion exchange, membrane separation, and separation and electrochemical adsorption on various adsorbents. [1].

Chromium (Cr) is one of the major environmental pollutants coming from industrial effluents and tannery. It is considered the major pollutant cataloged by the United States Environmental Protection Agency (EPA: www.epa.gov), since it is stable in aqueous solution and hence high in mobility in different environments. Chromium is a metal element in the periodic table. It is odorless and tasteless; is found in rocks, plants, soil, and volcanic dust, humans and animals; and exists in the environment most commonly as the trivalent [(chromium (III))], hexavalent [(chromium (VI))] and metallic [(chromium (0))]. Chromium (III) is generally contained in many vegetables, fruits, meats, grains and yeast. Industrial processes generally produce chromium (VI) and chromium (0). The main sources of chromium (VI) in drinking water are discharges from steel and pulp, and erosion of natural deposits of chromium (III). In many places, chromium compounds have been scattered to the environment through leaks, poor storage or improper disposal practices. The chromium compounds are very persistent in water and sediment [2].

Chromium is regarded as an environmental pollutant due to its wide use in various industrial activities, such as electrolytic plating, leather tanning, explosives manufacturing etc. The stable forms of chromium in the environment are trivalent (Cr (III)) and hexavalent chromium (Cr (VI)). Further, Cr (VI) is highly soluble, making it mobile in soil and aquatic environments, with consequent toxicity ecosystems. Chromium in their different forms can be use in the production of steel alloys and other metals chromed, for dyes and pigments, and the preservation of leather and wood. It can also be find naturally in the soil. The primary forms of chromium found in nature are chromium (III) and chromium (VI) and these forms are converted to each other depending on environmental conditions [2]. Cr (VI) is consider the most toxic form of chromium, and is usually associated with oxygen as chromates (CrO_4^{-2}) and dichromates ($Cr_2O_7^{-2}$) [3], which due to its high solubility are highly mobile in soil environments and water [4]. Moreover, Cr (III) is in the form of oxides, hydroxides or poorly soluble sulfates, by which it is much less mobile, and there joined organic matter in the soil and aquatic environments [5, 6]. Cr (VI) is a strong oxidizing agent, and in the presence of organic matter is reduced to Cr (III); this transformation is faster in acidic environments [3]. However, high

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levels of Cr (VI) may exceed the reducing capacity of the environment and thus can persist as a contaminant. It has been established now that various chromium compounds as oxides, chromates and dichromates, are environmental contaminants in water, soil, and industrial effluents, because this metal is widely used in various manufacturing, such as electrolytic plating, explosives manufacturing, leather tanning, metal alloy, dyes and pigments manufacturing, etc. [1, 5].

There are studies of many methods for removal of chromium ion present in water industrial waste, for example: ion exchange on resins, coagulation-flocculation, adsorption on activated carbon, reduction, chemical precipitation, sedimentation, etc., [7], which in most cases are expensive or inefficient, especially when the concentration of these ions is very low [8]. Therefore arise emerging technologies such as biosorption, the process of attracting various chemical species by biomass (live or dead), by physicochemical mechanisms as adsorption or ion exchange [9].

Fungal cells interact with chromium at different levels from the cell wall and, from the periplasm to the cytoplasm and cell organelles. These microorganisms require detecting and regulating intracellular levels of chromium through homeostasis systems that maintain a balance between the incorporation, expulsion, and arrest of metal [1]. It is common for native microorganisms of sites contaminated with chromate ion, show resistance because they have asset or liability mechanisms that allow them to remove from detoxification. In certain species these mechanisms are know in detail, some of which are of basic interest and biotechnological importance, the latter in the context of developing new technologies for the treatment of industrial effluents and for bioremediation of contaminated sites. These mechanisms generally include biotransformation of Cr (VI) reduced species (chemical reduction), which may be direct (enzymatic) or indirect (enzyme); incorporation and bioaccumulation; biosorption of Cr (III), and Cr (VI); and immobilization [1, 9]. Some filamentous fungi reduce Cr (VI) to Cr (III), by different mechanisms of Cr (VI) detoxification, like reducing power generated by carbon metabolism [10, 11, and 12]. *Aspergillus niger* var. *tubingensis* Ed8, has demonstrated the ability to both biotransform Cr (VI) and accumulate it in the biomass, by a reduction and a sorption processes, using electron microscopy techniques [13].

Aspergillus niger strains have been described as coping with chromium mainly via the biosorption of the metal into the cells, rather than via the use of reducing activity [14]. Extracellular reduction of Cr (VI) to Cr (III) was observed during the growth of *Candida utilis* by mechanisms independent from the intensity of culture growth or initial chromium concentration [15], and they hypothesized that Cr (VI) reduction in *C. utilis* could be partly dependent on pH changes of broth during the exponential phase or on exo-enzymatic activities during stationary phase. Also, the biosorption of this metal has has been investigated in different fungi and yeast: *Cyberlindnera fabianii, Wickerhamomyces anomalus* and *Candida tropicalis* in aqueous solutions at different pH conditions. Cr (VI), and pH range between 2 and 4 where the most effective for the three species [16]. Secondly, *Candida maltosa*, isolated from tanning liquors from a leather factory has the capacity to reduce Cr (VI) both in the presence of viable intact cells and in cell-free extracts [17]. This ability was related to NADH-dependent chromate reductase activity associated with soluble proteins and, to a lesser extent, with the membrane fraction [17].

Recently, the reduction of Cr (VI) to Cr (III) through an enzymatic mechanism has been observed in *Pichia*. Both in intact cells and in cell-free extracts of *P. jadinii* M9 and *P. anomala* M10 strains, chromate was reduced, suggesting the presence of a chromate reductase activity possibly associated with the cytosolic or membrane proteins [18]. In the bacteria *Pseudomonas putida* F1, challenged with Cr (VI) in minimal médium (instead of in the complex LB medium), an ATPase involved in DNA repair-like protein (Pput 2963) was overexpressed compared with untreated cultures, suggesting that DNA damage occurs [19], and a non-enzymatic mechanism of Cr (VI) reduction has been described for *A. niger* [20]. The purpose of this chapter is to elucidate the characteristics of removal of chromium (VI) by *Penicillium* sp. IA-01cells.

2. Materials and methods

2.1. Screening of the microorganism showing the resistant to Chromium (VI) and chromate resistance test

We isolate a chromate resistant mycelial fungus from polluted air near the Faculty of Chemical Science, UASLP (San Luis Potosí, México), and this was used for the screening. The chromate resistant filamentous fungus contained in the air was grown on the Petri dish containing modified Lee's minimal medium (LMM) (with 0.25% KH_2PO_4, 0.20% $MgSO_4$, 0.50% $(NH_4)_2SO_4$, 0.50% NaCl, 0.25% glucose, and 2% agar) supplemented with 500 mg/L K_2CrO_4; the pH of the medium was adjusted and maintained at 5.3 with 100 mmol/L citrate-phosphate buffer. The plates were incubated at 28°C for seven days. The strain was identified based on characteristic macroscopic and microscopic observation [21]. Fungal cultures grown in thioglycolate broth were used as primary inoculums. Chromate-resistant tests of the isolated strain, filamentous fungus *Penicillium* sp IA-01, were perform on liquid LMM containing the appropriate nutritional requirements and different concentrations of Cr (VI) (as potassium chromate), and the dry weight was determined.

2.2. Biosorption tests by using dry cells

The fungal cells was grown at 28°C in an stirred and aerated liquid media containing thioglycolate broth at a concentration of 8g/L (p/v). After five days of incubation, the cells were recovered by centrifugation (3000 rpm/10 min), and washed twice in the same conditions with deionized wáter, and subsequently it was dry (80°C/24 h) in an oven. Solutions of Cr (VI) for analysis, were prepared by diluting 71.86mg/L of stock metal solution. The concentration range of chromium (VI) solutions was 50-1000mg/L. The pH of each solution was adjusted to the required value by adding 1M H_2SO_4 solution before mixing with the microorganism. The biosorption of the metal by fungal dry cells was determined at different concentrations (50–1,000mg/L) of 100 mL Cr (VI) solution, with 1g of fungal biomass, at 120 rpm, and the sample was filtered. The filtrate containing the residual concentration of Cr (VI) was determined spectrophotometrically. For the determination of rate of metal biosorption, 200, 400, 600, 800, and 1,000mg/L of Cr (VI) solution was used. The supernatant was analyzed for residual Cr (VI) after the contact period at different times. For determination of the effects of pH and

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temperature, four solutions (pH 1, 2, 3, and 4) and temperatures (28, 40, 50, and 60℃) were respectively used.

Moreover, biosorption to the contaminated soil and water was examined. Four Erlenmeyer glass flasks containing 5g of fungal biomass and 20g of contaminated soil and 20 mL of water (297mg Cr (VI)/g soil or 155mg Cr(VI)/L water), of tannery (Celaya, Guanajuato, México), was completed to 100 mL with trideionized water, were incubated during seven days at 120 rpm, and filtered in Whatman filter paper No. 1, and the concentration of Cr (VI) of the filtrate analyzed with 1, 5 diphenylcarbazide [22].

2.3. Reduction of Cr (VI) by living cells

Reduction efficiency of Cr (VI) by living, resting, and permeabilized cells was examined. To examine the living cells, cultures in 100 mL of LMM were inoculated with 5×10^5 spores/mL (28°C, and 48 h), the cells were centrifugated (2000 rpm, at 4°C/10 min), and washed twice with sterile trideionized water and the pellet was resuspended in 3 mL of the same solution, and was transferred at a fresh LMM (100 mL with 50mg/L Cr (VI)). At different times, 1 mL aliquots were removed and centrifuged (5000 rpm/10 min), and we determine the concentration of Cr (VI) or total Cr in the supernatant.

Reduction efficiency of Cr (VI) was examined by the resting cells. 5×10^5 spores/mL of *Penicillium* sp. was inoculated and incubated in 100 mL thioglycolate broth (pH 7.0) for five days, and was harvested (3000 ×g at 4°C); cell pellets obtained were washed by centrifugation twice with 100 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. The suspended cell pellets were added in 2-10mg/100 mL of Cr (VI) solution, mixed for 30 min, and incubated at 30°C for 6 h. Heat-killed culture pellets (2 mL), which were treated at 100°C for 10 min were used as control. After the incubation, the tubes were centrifuged, and 100 μL aliquots were withdrawn from each sample to estimate the remaining Cr (VI).

Reduction efficiency of Cr (VI) was also examined by the permeable fungal cells. Culture of *Penicillium* sp. IA-01 was grown for five days, harvested, and washed with potassium phosphate buffer (pH 7.0) as described above. The suspended culture pellets were treated with 0.2% (w/v) sodium dodecyl sulphate, 0.2% Tween 80, (v/v), 0.2% Triton X-100 (v/v), and 0.2% toluene (v/v), by vortexing for 30 min to achieve cell permeabilization. Permeabilized cell suspensions (0.5 mL) were then added with 2–10mg/100 mL of Cr (VI) as final concentrations and incubated for 6 h at 30°C.

2.4. Activity of chromate reductase

Cell-free extracts (CFE) of *Penicillium* sp IA-01 were prepared by modifying the previously published protocols. The pellets were resuspended in 5% (v/v) of the original culture volume in 100 mM potassium phosphate buffer (pH 7.0). These cell suspensions were placed to an ice bath and disrupted using an Ultrasonic Mini Bead Beater (Densply) with 15 cycles of 60 sec for each one. The sonicate thus obtained was then centrifuged at 3000 x g for 10 min at 4°C. The pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.0), and this is the CFE.

Enzymatic chromate reduction was estimated as described previously using a standard curve of Cr (VI) 0–30 mM. The assay was as follows: The reaction system (1.0 mL) was made up of varying Cr (VI) final concentrations (5–30 mM) in 700 μL of 100 mM potassium phosphate buffer (pH 7.0) added with 250 μL aliquots of CFE for chromate reduction and 50 μL of NADH. The system volume of 1.0 mL was kept constant for all experiments. Chromate reductase activity was measured at 37°C at different pH values using several buffers (100 mM phosphate citrate, pH 5.0; 50 mM phosphate, pH 6.0–8.0, and 50 mM Tris-HCl, pH 8-9). The effect of temperature was studied by measuring chromate reductase activity at different incubation temperatures between 20 and 60°C, at optimum pH. The CFE samples were also treated with several metal ions to a final concentration of 1mM at optimal pH and temperature; Na^{+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Cd^{2+}, and Fe^{3+} were tested by using 10 mM solutions of Na_2SO_4, $CaCl_2$, $CuCl_2$, $HgCl_2$, $MgCl_2$, $CdCl_2$, and $FeCl_3$. The electron donors tested were NADH, glucose, sodium acetate, formic acid, citrate, cystin, lactic acid, and ascorbic acid in a final concentration of 1mM, and the inhibitors were EDTA, KCN, NaN_3, and β-mercaptoethanol at the same concentration. For chromate reductase activity, one unit was defined as enzyme that reduces 1mmol of Cr (VI)/min/37°C, and the specific activity was defined as unit chromate reductase activity/min/mg protein in the CFE. Protein concentrations were determined by the Lowry method [23].

2.5. Determination of hexavalent, trivalent, and total amount of chromium

Hexavalent and trivalent chromium were quantified employing diphenylcarbazide [22] and chromazurol S [24], respectively, the total amount of Chromium was determined by electro-thermal atomic absorption spectroscopy [22]. Tree dependent experiments were carried out and the mean value was shown

3. Results and discussion

3.1. Isolation and identification of a fungal strain tolerant to Cr (VI)

Microorganism was grown on the LMM agar plates containing 500 mg/L of K_2CrO_4, and the largest colony of fungi was isolated. Colonies isolated grew rapidly within three days. Colonies are usually fast growing, in shades of green, sometimes white, mostly consisting a dense conidiophores. Microscopically, chains of single-celled conidia (ameroconidia) are produced in basipetal succession from a specialized conidiogenous cell called phialide. In *Penicillium*, the phialides may be produced singly, in groups or from branched metulae, giving a brush-like appearance known as a penicillus (Figure 1) [25].

The cells of the isolated strain grew on LMM supplemented with 2 g/L of Cr (VI) about 50% of growth relative to control (85mg of dry weight without metal) was obtained (Figure 2) and, therefore probably is resistant to the metal. Different microorganisms that are Cr (VI) resistant have been isolated from different contaminated sites [1, 16, 26, and 27], and Chromate tolerance has been described in the mutants of stocked culture, and in native isolates of contaminated sites, as in this work; in several cases, both yeast and filamentous fungi showed that tolerance

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to Cr (VI) is due to transport of sulfate disturbance that leads to reduced incorporation of chromate [28] in other cases, phenotypes of hypersensitivity to Cr (VI) are produced as a result of alteration of the vacuolar ATPase and vacuolar structures [29] or by alteration of proteins that protect the oxidative effect of Cr (VI) as the alkyl hydroperoxide reductase [30] or Cu-Zn-superoxide dismutase and methionine sulfoxide peptide reductase [31]. However, the mechanism of tolerance in *Penicillium* sp IA-01fungus are not investigated. Thus, we precisely examined the characteristics of the *Penicillium* strain to estimate the mechanism in the following experiments.

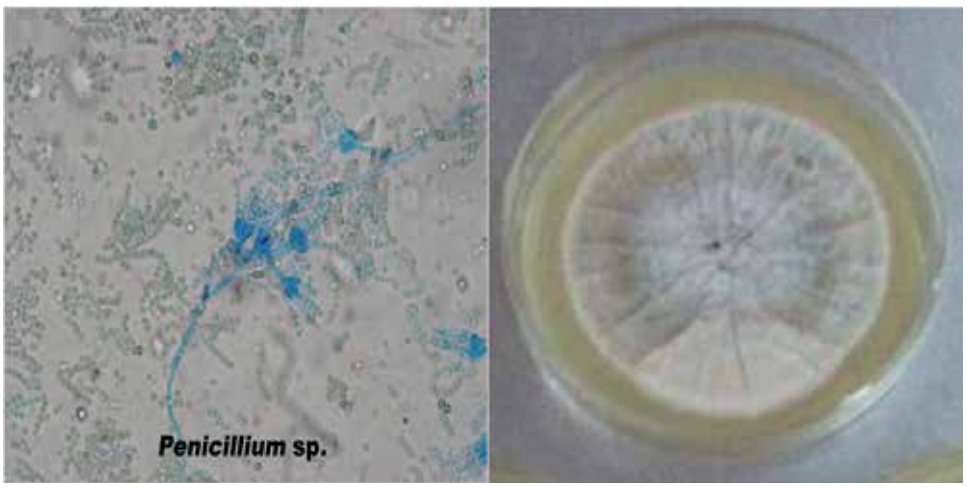

Figure 1. Macroscopic and microscopic morphology of the fungus *Penicillium* sp IA-01

3.2. Absorption of Cr (VI) by the dry cells of *Penicillium* sp. IA-01

First, the ability of absorption was examined by using *Penicillum* sp. IA-01 cells to clarify the mechanism of Cr (VI) tolerance. Figure 3 shows the effect of the incubation time on Cr (VI) removal by *Penicillium* sp biomass. The optimum time for Cr (VI) removal was 150 min at constant values of pH (1.0), biosorbent dosage (1g 100/mL), initial metal concentration (50mg/L) and temperature (28°C). Some studies [32], report an incubation time of 48 h at pH 1.0 by fruiting bodies of the jelly fungus *Auricularia polytricha,* 24 h for *C. fabianii, W. anomalus* and *C. tropicalis*, at pH range between 2 and 4 for the three species [16], to pH of 2.0 to five days for *Aspergillus niger* [20], the latter with 10 g/L of biomass, and at the same pH of 2.0. Changes in the permeability of the fungal cell wall, of unknown origin, could partly explain the differences found in the incubation time, providing greater or lesser exposure of the functional groups of the cell wall of the biomass tested [33].

With respect to the influence of initial pH on removal efficiency, it was found that the highest activity was evident at pH 1.0, at 150 min the metal is removed, while at pHs 2, 3, and 4, the authors did not observe significant differences (20% of removal), and at neutral or alkaline pH ´s, there was no removal (Figure 4). A pH optimum has been reported of 1.0 to removal Cr (VI)

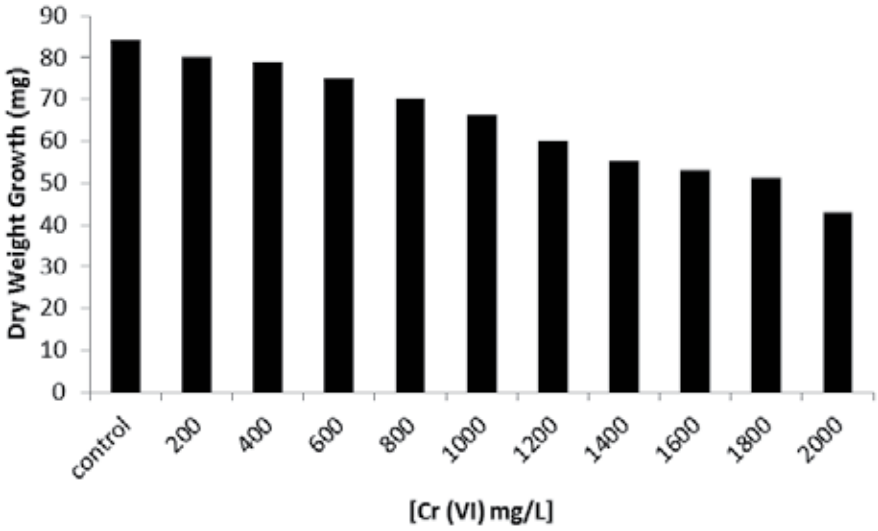

Figure 2. Growth in dry weight of *Penicillium* sp., IA-01with different concentrations of Cr (VI), 1 × 10^5 spores/mL, 28°C, seven days of incubation, 100 rpm.

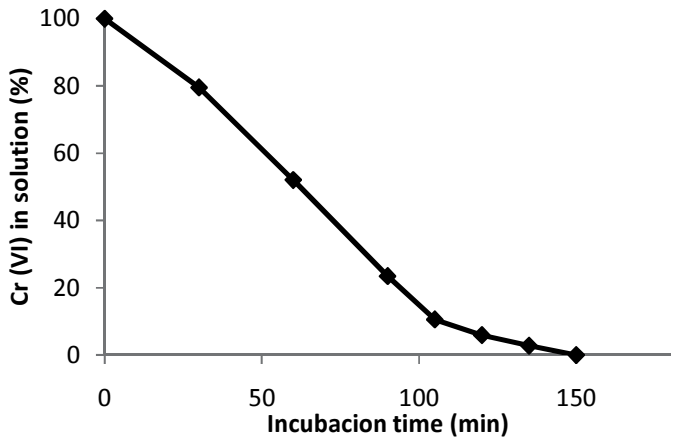

Figure 3. Effect of incubation time on Cr (VI) removal by *Penicillium* sp. 50mg/L Cr (VI), 100 rpm, 28°C, pH 1.0, and 1.0 g of fungal biomass.

by fruiting bodies of the jelly fungus *A. polytricha* [32] and for the yeast *Saccharomyces cerevisiae* and the fungi *Rhizopus arrhizus* a pH range of 1.5-2.5, at 4 h [34], although most indicate a pH optimum range of 2.0 to 4.0 with the yeasts *C. fabianii*, *W. anomalus* and *C. tropicalis*, isolated from sediments in Morocco [16], both *Mucor hiemalis* [35] and *R. arrhizus* [36], at 24 h, *Rhizopus nigricans* in 8 h [37]. The Cr (VI) is found as $HCrO_4^-$, $Cr_2O_7^{2-}$, CrO_4^{2-}, $Cr_4O_{13}^{2-}$, and $Cr_3O_{10}^{2-}$ [12]. A decrease in pH causes protonation of the surface of the adsorbent, which induces a strong attraction for the Cr (VI) ions from solution negatively charged, so that the biosorption increases with increasing acidity of the solution. However, as the pH increases, the concen-

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tration of OH- ions increases, causing changes in the surface of the adsorbent and preventing the biosorption negatively chargen of Cr (VI) ions, thereby decreasing the adsorption of metal to high pH values. It has been reported [11] that while Cr (VI) is obtain by eliminating indigenous strains of filamentous fungi, most of the cations may be reduced to Cr (III).

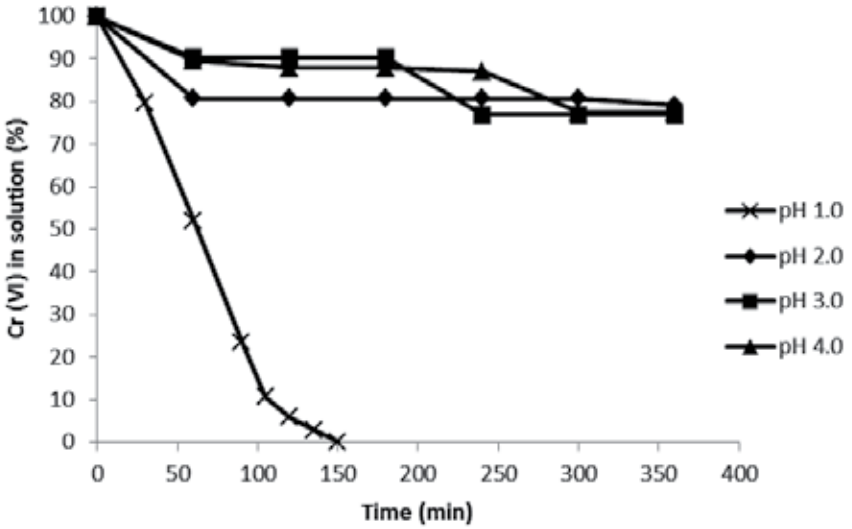

Figure 4. Effect of pH on Cr (VI) removal by *Penicillium* sp. 50mg/L Cr (VI), 100 rpm, 28°C, and 1g of fungal biomass.

Temperature is found to be a critical parameter in the bioadsorption of Cr (VI) (Figure 5). The highest removal was observed at 50°C and 60°C. At this point the total removal of the metal is carried out at 100%, at 40 min. These results are likely for *Paecilomyces* sp [10] and *A. niger* [20] at 45°C and 24 h, but are different for *R. arrhizus* [36]. The increase in temperature increases the rate of removal of Cr (VI) and decreases the contact time required for complete removal of the metal, to increase the redox reaction rate [38].

At different metal concentrations (200, 400, 600, 800, and 1000mg/L), biomass studied, shows the same results for removal, adsorbing 100% between 210 and 240 min while 1000mg/L of metal is removed 100% up to 90 min of incubation (Figure 6) with respect to other fungal biomasses, some reports argue that the amount of metal increases in direct proportion with the increase in concentration of the metal ion in solution [35, 37], and others author report lower removal efficiencies of metal, for example 25 and 250mg/L of chitin and chitosan [36], and 1mg/L for cellulose acetate [39]. This was due probably to the increase in the number of ions found competing for the available functions groups on the surface of biomass [38].

The influence of the biomass on the removal capacity of Cr (VI) was depicted in Figure 7. If we increase the amount of biomass, we also increase the removal of Cr (VI) in the solution (although there is a 100% of remotion, with 3, 4, and 5g of biomass, 60 min), perhaps due to increased of biosorption sites of the same, because the amount of added biosorbent determines the number of binding sites available for metal biosorption [30]. Similar results have been

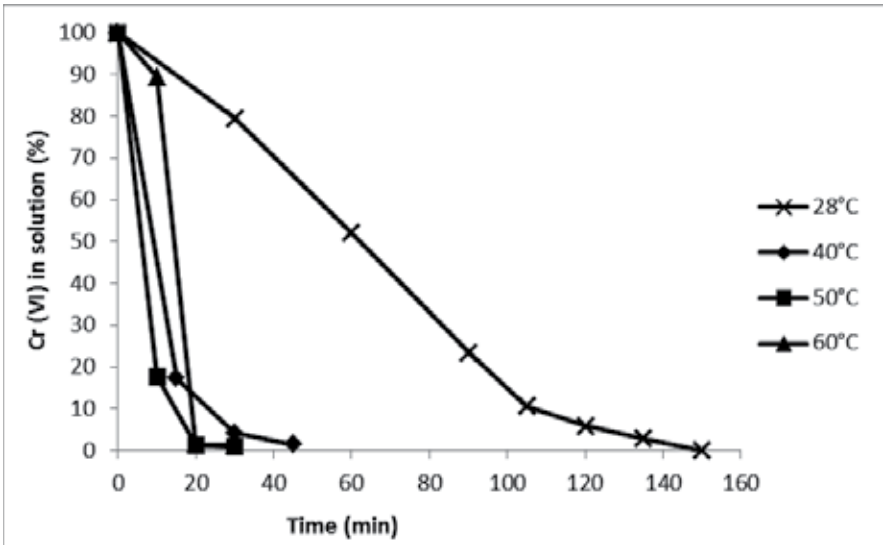

Figure 5. Effect of temperature on Cr (VI) removal by *Penicillium* sp. IA-01. 50mg/L Cr (VI), 100 rpm, pH 1.0, and 1g of fungal biomass.

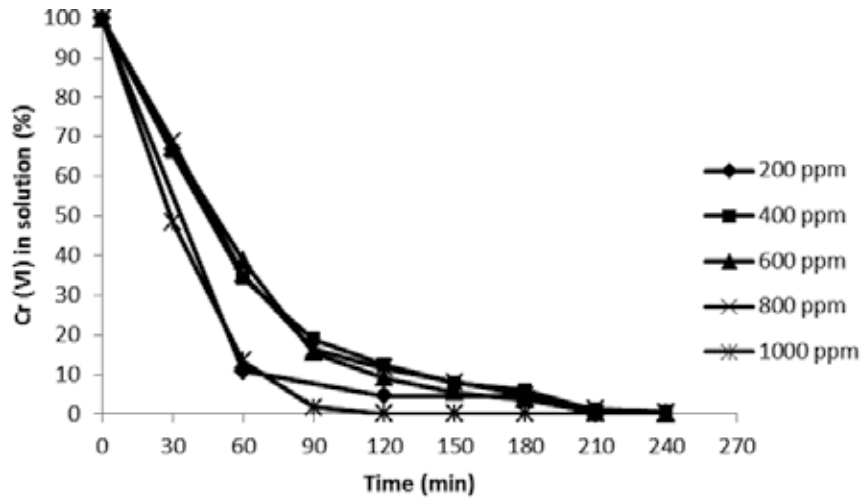

Figure 6. Effect of initial metal concentration on chromium (VI) removal by *Penicillium* sp. IA-01. 28°C. pH 1.0, 100 rpm, and 1g of fungal biomass.

reported for *M. hiemalis* and *R. nigricans*, although the latter has 10g of biomass [35, 37], but different from those reported for biomass wastes from the mandarin (gabassa), whit an optimal concentration of biomass of 100mg/L [40]. Consequently, we found out the following results: *Penicillium* sp. IA-01 has the ability of absorption to Cr (VI) and the value of adsorption is as high as in the fruiting bodies of the jelly fungus *A. polytricha* [32], *C. fabianii, W. anomalus* and *C. tropicalis* [16], *A. niger* [20], *M. hiemalis* and *R. nigricans* [35, 37], and *Paecilomyces* sp. [10]. The

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adsorption rate was affected by pH, temperature, initial concentration of Cr (VI) and dry cells. In the case of polluted soil and water, around 40% of Cr (VI) could not be removed. Therefore, absorption to the organic compounds contained in polluted soil and water may occurr. The results suggest that the reduction of Cr (VI) is necessary to the bioremediation of soil and air.

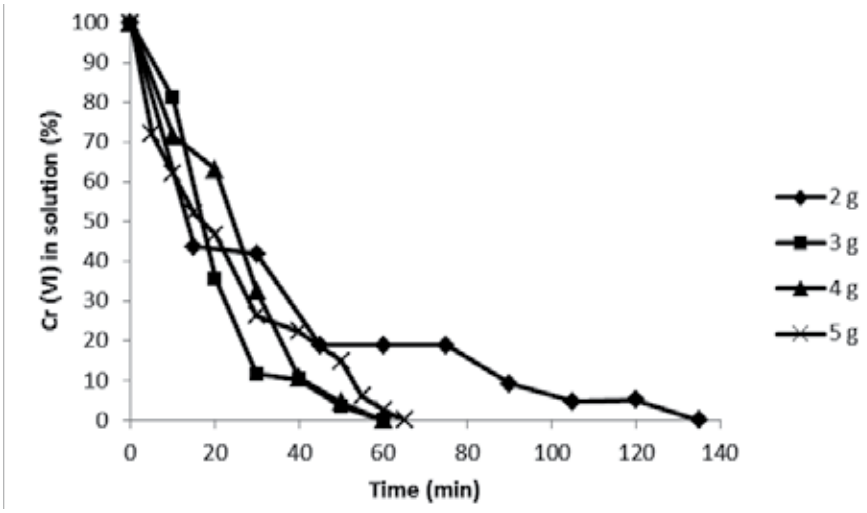

Figure 7. Effect of biomass concentration on chromium (VI) removal by *Penicillium* sp. IA-01. 50mg/L Cr (VI). 28°C, and pH 1.0, 100 rpm.

3.3. Removal of Cr (VI) in industrial wastes with fungal biomass

For the removal of the metal from industrial wastes, we incubate the fungal biomass (5g) with non-sterile oil and contaminated water (297mg Cr (VI)/g, and 155mg Cr (VI)/L), suspended in trideionized water. It was observ that after seven days of incubation with the biomass, the Cr (VI) concentration from soil and water samples decreased to 63.24% and 43%, respectively (Figure 8), without significant change in total chromium (not shown). In the absence of the biomass, the metal concentration of the soil samples decreased slightly (18%, not shown), maybe caused by indigenous microflora and (or) reducing components present in the soil [10, 11, and 18]. The capacity of *Penicillium* sp., biomass to remove the metal are lower to those reported for other biomasses, like *Litchi chinensis,* [41] tamarind shell [42], *Mammea americana* [43], and *C. reticulata* [44], and equal or better than that of *C. maltose* RR1 [17]. The *Penicillium* biomass was more efficient for the chromium removal under acidic conditions. Some studies were carried out at neutral pH [45]. A. *niger* mycelium removal 8.9mg/g dry weight at seven days of incubation (500ppm of Cr (VI)). Otherwise, *Paecilomyces* sp. biomass was incubate with non-sterilized contaminated soil containing 50mg Cr (VI)/g, suspended in LMM, pH 4.0, and it was observed that after eight days of incubation with the biomass, the Cr (VI) concentration of soil sample decreased fully [46]. We found out the following results: *Penicillium* sp, IA-01, has the ability to absorb Cr (VI) and the value of adsorption is as high as the fruiting bodies of the jelly fungus *A. polytricha* [32]; *C. fabianii, W. anomalus* and *C. tropicalis* [16]; *A. niger* [20], *M.*

hiemalis and *R. nigricans* [35, 37] and *Paecilomyces* sp. [10]. The adsorption rate was affected by pH, temperature, initial concentration of Cr (VI) and dry cells. In the case of the polluted soil and water, around 40% of Cr (VI) could not be removed. Therefore, absorption to the organic compounds contained in polluted soil and water may occurr. The results suggest that the reduction of Cr (VI) is necessary to the bioremediation of soil and air.

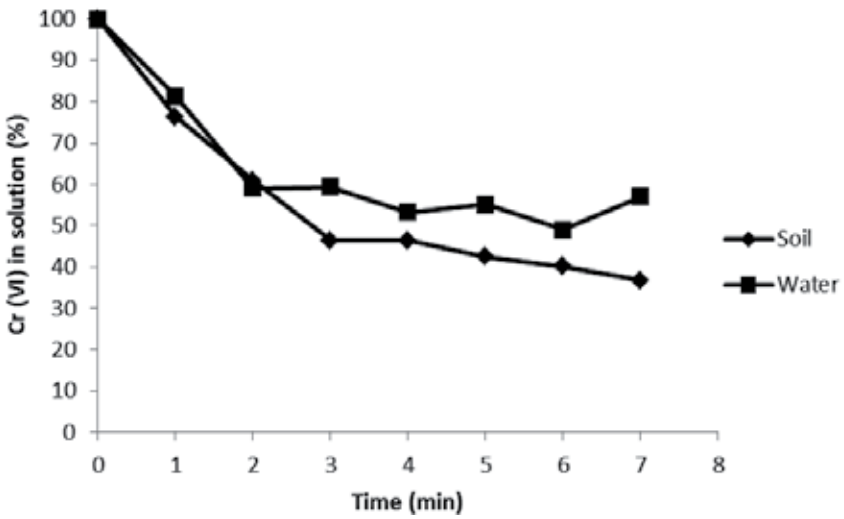

Figure 8. Removal of Cr (VI) in industrial wastes incubated with 5g of fungal biomass. 100 rpm, 28°C, 20g and 100 mL of contaminated soil and water, respectively (297mg Cr (VI)/g soil and 155mg Cr (VI)/L).

3.4. Removal of Cr (VI) by living cells of *Penicillum* sp. IA-01

Next, the reduction of Cr (VI) by *Penicillium* sp. IA-0 were examined by using living cells. The fungal cells, which were cultured in 100 mL LMM containing 50mg/L Cr (VI), under various pH, inoculated amount, Cr (VI) concentration and carbon sources. The amount of Cr (VI) was determined and the percentage of decreased amount to total amount was calculated. The effect of different pHs (4.0, 5.3, and 6.2), show a pH optimum of 5.5 (77% at four days, 28°C, and 100 rpm), while at pH of 4.0 and 6.2 were of 43% and 65%, respectively (Figure 9). About, Coreño-Alonso et. al. [13], reported a 95% of removal at pH of 5.3 and 24 h with *A. niger* var *tubingensis* strain Ed8, and also, at pH 5.0 for Cr (VI) removal with *A. niger* MTCC 2594 [14] and at pH 3.0–5.0 for Pb^{+2}, Cd^{+2} and Cr^{+3} with the yeast *Saccharomyces cerevisiae* [47]. In contrast to our observations, the máximum adsorption capacities by both living yeasts were found at pH 4.0 for *C. fabianii* HE650139 and *W. anomalus* HE648168, and 3.0 for *C. tropicalis* HE650140, with a percentage of removal of 100%, by all living microorganisms [16], also, a maximum uptake of Cr (VI) at pH 7.0 with *Aspergillus foetidus* [48]. On the other hand, using a *Citrobacter* strain, it has been reported that an enhanced uptake of different heavy metals, is increased if pH is from 2.0 to 7.0 and also a decrease in the removal at higher pH values [49]. Al-Asheh and Duvnjak

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[50] also reported most removal increasing pH in the range 4.0–7.0 on Cr (III) uptake using *Aspergillus carbonarius*. At low values of pH, the low efficiency of removal of the metal, may be due to the competition between hydrogen (H+) and metal ions [36], and at higher pH values (7.0), the efficient metal removal may be due to the ionization of functional groups and the increase in the negative charge density on the cell surface. At alkaline pH values (8.0 or higher), a reduction in the solubility of metals may contribute to lower uptake rates [1].

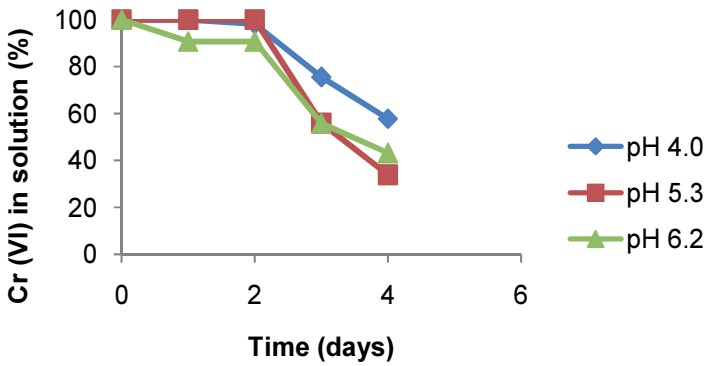

Figure 9. Effect of pH on chromium (VI) removal by biomass of *Penicillium* sp IA-01 50mg/L Cr (VI), 100 rpm, 28°C

In Figure 10, the effect of the biomass concentration (72, 141, and 169 mg of dry weight) on Cr (VI) removal, with percentages of removal of 35%, 49%, and 60%, respectively, is shown. Similarly, most of the reports in the literature observe at higher biomass dose resulting in an increase in the percentage removal [3, 7, 8, 13, 16, 47, and 52]. With higher biomass dose, there are more binding sites for complex of Cr (VI) (e.g., $HCrO_4^-$ and $Cr_2O_7^{-2}$ ions) [3, 28].

Figure 11, shows the effect of Cr (VI) concentration (50 to 200mg/L) on the removal of the same. If we increase the concentration of the metal, the removal of metal decreases (60%, 50%, 28%, and 11%, respectively. This is probably because, if we increase initial metal concentration, we increase the number of ions competing for the available functions group on the surface of biomass. Our observations are like most of the reports in the literature [3, 7, 8, 37, 47, 48, 53].

With different carbon sources, like fermentable: glucose, sucrose, and citrate, and oxidable (glycerol). With glucose, sucrose, and citrate, the decrease in Cr (VI) levels occurred at a different rate, at six days of incubation (52%, 47%, and 27%, respectively), and the other carbon sugars were less effective (glycerol 7% of removal). With another inexpensive commercial carbon sources like unrefined sugar and brown sugar, the decrease in Cr (VI) levels occurred at a similar rate (96% and 100%, respectively) (Figures 12(a), (b)). If we incubate the fungal biomass without a carbon source, there are no changes in the initial Cr (VI) concentration during the experiment (data not shown), suggesting that a carbon source is required to decrease Cr (VI) concentration in the growth medium. Our results are similar to some reports: how in chromate-resistant strains of filamentous fungi indigenous to contaminated wastes,

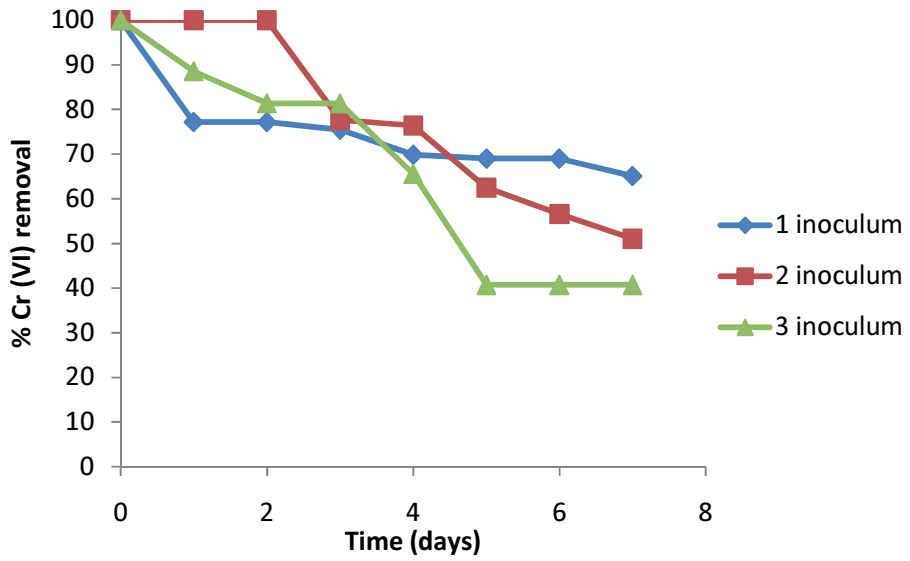

Figure 10. The effect of cell concentration on the removal of Cr (VI). 50mg/L Cr (VI), 100 rpm, 28°C, and pH 5.3

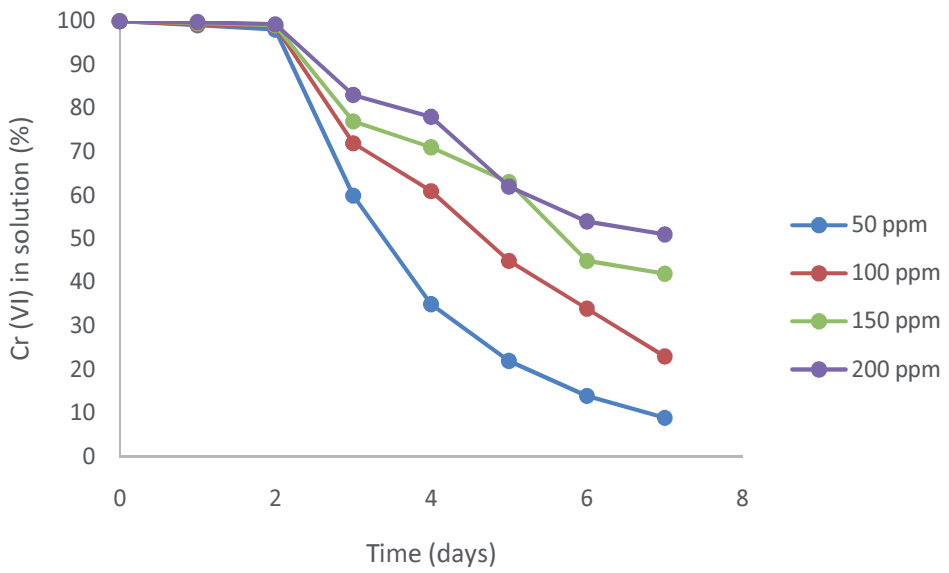

Figure 11. The effect of the concentration of Cr (VI) in the solution on the removal. 100 rpm, 28°C, pH 5.3

with *A. foetidus*, *A. niger* and *A. parasiticus* [11, 48, and 54] with glucose like carbon source, and other carbon sources like sucrose and citrate by a *Paecilomyces* sp fungal strain isolated from environment. [10], but are different from the observations of Srivasta and Thakur [55] with *Aspergillus* sp and *Acinetobacter* sp, who observed that the main carbon source is the sodium

acetate. Consequently, we found out the following results in this section: As shown in Figure 4, the difference between dry cells and living cells were clear. *Penicillium* sp. IA-01 cells could remove Cr (VI) at pH 4.0-6.2, although combining by absorption did not occurr (Figure 4). The result suggests that *Penicillium* sp. cells can absorb and/or reduce Cr (VI) as well as adsorption. Additionally, as shown in Figure 12, the removal of Cr (VI) by adding glucose was higher than that of water (without glucose), and some carbon sources such as brown sugar and piloncillo enhanced the removal. There results suggest that the carbon sources induce the absorption rate of Cr (VI) or increase the amount of chromate reductase activity.

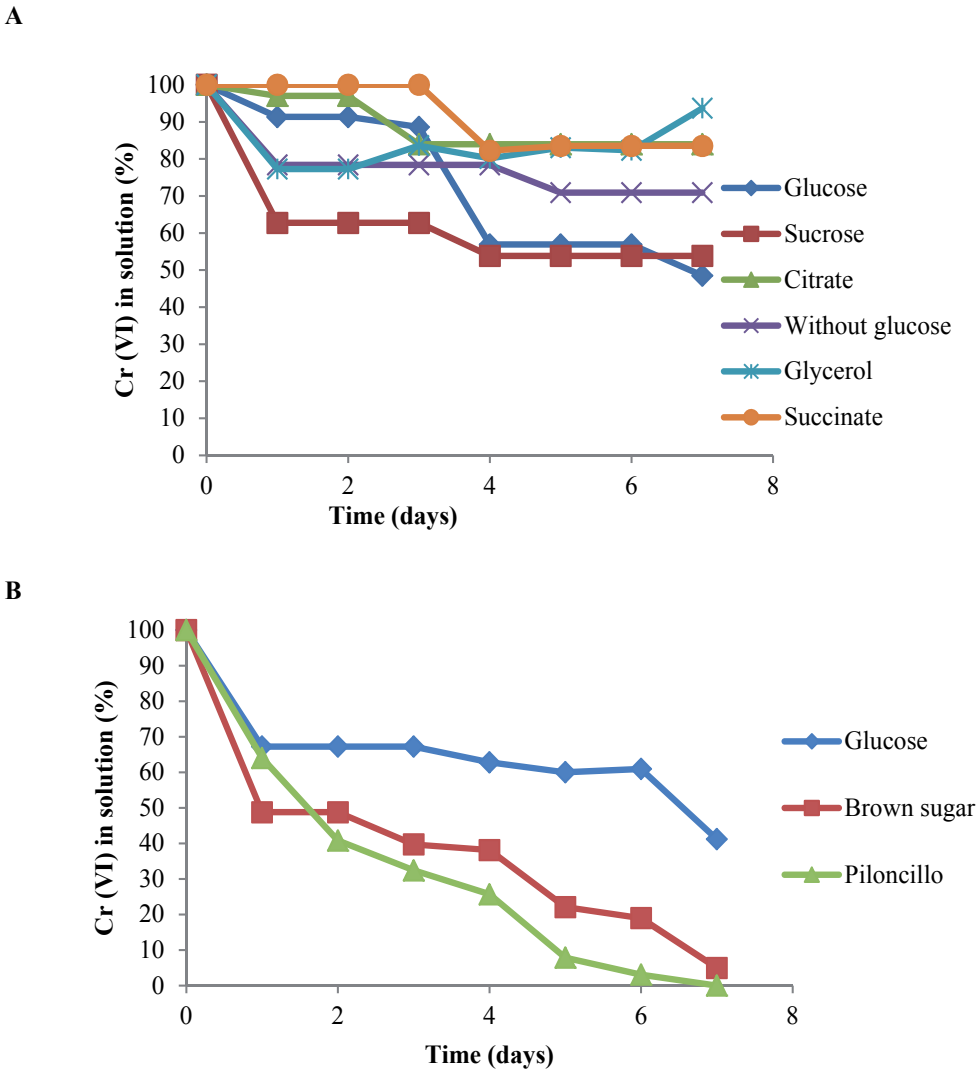

Figure 12. Influence of carbon source on the capability of *Penicillium* sp., IA-01 to decrease Cr (VI) levels in the growth medium. 100 rpm, 28°C, pH 5.3

3.5. Adsorption and reduction by resting and permeable cells

We also estimated the ratio of absorption and/or reduction to adsorption, as we found that the fungi *Penicillium* sp. IA-01, has these abilities as well as adsorption from the results in Section in 3.3. The resting cells and permeabilized cells were used for the examination, and heat-killed cells were used to examine the amount of adsorption. The removal was calculated as value of Cr (VI) in resting cells to the value from total value minus the value of Cr (VI) in heat-killed cells (0% of removal). First, the removal of the metal by resting cells was examined. The cell pellets of *Penicillium* sp, which were cultured in 100 mL thioglycolate broth, were incubated in 100 mM potassium phosphate buffer (pH 7.0) for 6h at 30℃. The resting cells of the fungus were expedient in reducing 0–10mg/100 mL Cr (VI) concentrations in 8 h as shown in Figure 13. The fungus removal was between 53% and 70% (2–10 mg/100/mL) of the metal, and these results resemble those reported by *A. niger* and *A. parasiticus* [54], *Fusarium solani* [56], *Paecilomyces lilacinus* [57], and the bacteria *Pseudomonas* sp. [58] and *Paecilomyces* sp. [46]. Structural properties of the biosorbent including the cellular support and other several factors are known to affect the biosorption rate [59].

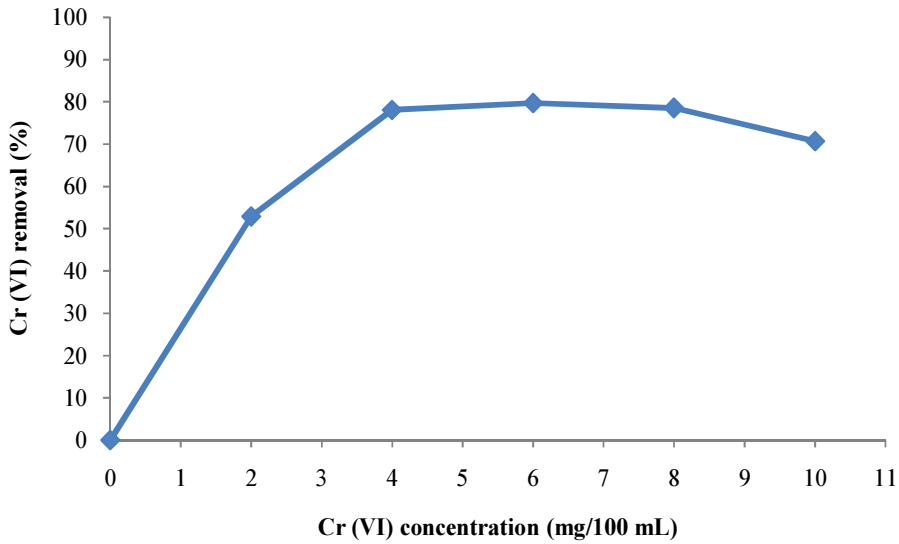

Figure 13. Resting cell assays for Cr (VI) reduction by *Penicillium* sp. IA-01 performed at initial concentrations of 0–10mg/100 mL of Cr (VI), pH 7.0, and 37°C in 8 h

The cell permeabilization increased the Cr (VI) reduction by the resting cells, as the permeabilized cells with Triton X-100 which could reduce 57%, toluene 52%, SDS 47.4%, and Tween 80 40.4% (Figure 14) of 30 mM Cr (VI) within 6 h, suggesting an efficient intracellular mechanism of chromate reduction. The Cr (VI) reductase activity in CFE of cells grown in the absence of Cr (VI) was 94.07 μmoles/min/mg protein.These results indicate that the Cr (VI) reductase was associated with the CFE. Fungal, yeast, and bacteria chromate reductases have been localized either in CFE of *A. niger* and *A. parasiticus* [54], *Pichia jadinii* M9, *Pichia anómala* M10

[60], *Pichia* sp. [61], *and Bacillus* sp. [62], cytosolic fraction of *C.maltosa* [17], *Pichia* sp. [62], and *Pannonibacter phragmitetus* [63] of membrane fraction of *Pseudomonas* sp. G1DM21 [58], *Bacillus megaterium* [64], and *Enterobacter cloacae* [65]. The results by resting and permeable cells suggest as follows: As shown in Figure 13, 70-80% of Cr (VI) could be removed by resting cells, and the result suggests that absorption of Cr (VI) occurrs without energy of carbon sources or ATP. Additionally, as shown in Figure 14, the ratios of the removal of Cr (VI) in case of the pre-treatment by Triton X-100, toluene and SDS of glucose were 2-2.5 times higher. Therefore, the transport through cell membrane is the rate-limiting steps.

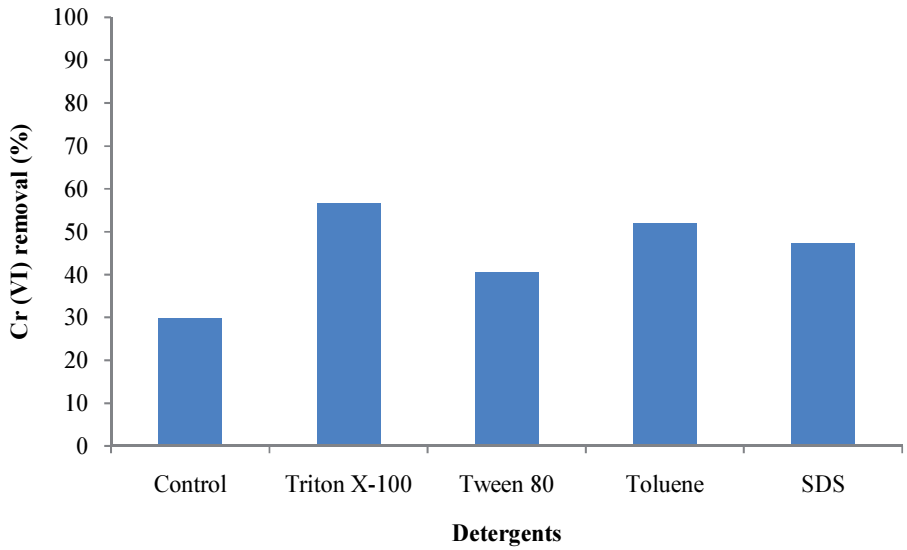

Figure 14. Permeabilized cell assays for Cr (VI) reduction by *Penicillium* sp. IA-01 performed at initial concentrations of 28mM of Cr (VI), pH 7.0, and 37°C

3.6. Chromate reductase activity

The result of permeable cells (Figure 14) suggests that *Penicillium* sp. IA-01 has the enzymatic ability of reduction to Cr (VI). Thus, we investigated the reduction of Cr (VI) by *Penicillium* sp. IA-01. The activity of chromate reductase is examined in the cell-free extract. The function of the chromate reductase of *Penicillium* sp., was characterized in different *in vitro* conditions. In determining the optimal pH for the chromate reductase activity, we used the following buffers at different pH ranges: potassium phosphate, citrate phosphate, and Tris-HCl; and we found the máximum enzymatic activity to be at an optimum pH of 7.0, with potassium phosphate buffer, as depicted in Figure 15, and these results resemble those reported by the fungi *A. niger* and *A. parasiticus* [54], the yeast *P. jadini* M9 [60], and cell-free extract of *Arthrobacter* sp. SUK 1201 [66]. Other authors reported stability between 7.0 and 7.4 for the bacteria *Pseudomonas* sp. G1DM21 [58], 6.5 and 7.5 for *E. coli* CFE [67], and in the range of 5.0 to 8.0 for *Bacillus* sp. [68].

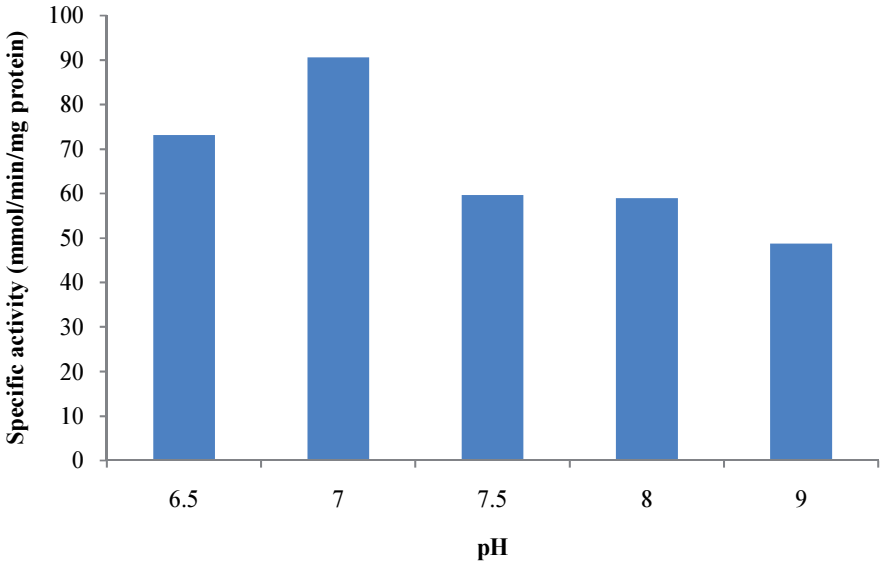

Figure 15. Effect of pH on Cr (VI) reductase activity in cell-free-extracts of *Penicillium* sp. IA-01 determined in different buffers (pH 6.5–9.0) with an initial concentration of 5.6mM Cr (VI), at 37°C

The optimal temperature for the Cr (VI) reductase activity was 37°C, but the reductase activity was altered significantly at 20°C (39% of inhibition); but when the assays were performed at 50°C the reductase activity showed 14.2% of inhibition (Figure 16). For *P. jadinii* M9, incubation at 55°C produced a reduction in activity of 55% [60]. In *P. anómala* M10, when incubated at 8°C, a decrease in activity of 25% was observed, and at 50°C the activity was at 50%. [69]. For *A. niger* and *A. parasiticus* [54], *Pseudomonas* sp. G1DM21 [58], *E. coli* [66], and *Bacillus* sp. CFEs [67], the thermal stability was of 30°C [66, 67], and for the activity in cell-free extract of *Arthrobacter* sp. SUK 1201, it was 32°C [66]. On the contrary, *Pseudomonas putida* CFE probed to be more resistant, keeping its stability up to 50°C [69].

The effect of different metal cations on the chromate reductase activity of *Penicillium* sp. was determined in Figure 17. All the metal ions tested inhibit the Cr (VI) reductase activity of the CFE of 12% with Cu^{2+} and 40.2% with Na^{+}, and these results are different than those reported for the yeast *P. jadinii* M9 chromate reductase because only Cu^{2+} and Na^{+} produced an augmentation in the activity of 63 and 30%, respectively [60]; in *Arthrobacter* sp. SUK 1201, Cu^{2+} also increases the activity [66], and all other ions tested had an inhibitory effect but in different levels. A decrease of 56.5% was observed with Hg^{2+}, while addition of Mg^{2+}, Fe^{3+}, Ca^{2+}, and Cd^{2+} resulted in a decrease of activity between 40% and 51%. In *P. anomala* M10 chromate reductase, only Cu^{2+} produced a rise in activity of 31% [69]. Inhibition by Hg^{2+} was higher in *P. anomala* and *Pseudomonas* sp., than in *Penicillium* sp., with a decrease in activity of 85% and 90%, respectively [58, 60]. Inhibition by Ca^{2+} and Mg^{2+} was approximately 50%, while Fe^{3+} reduced the activity to 32%. These results agree with those reported for *Arthrobacter crystallopoietes* [44] and *Bacillus* sp. [67]. On the other hand, inhibition by Hg^{2+} can be related

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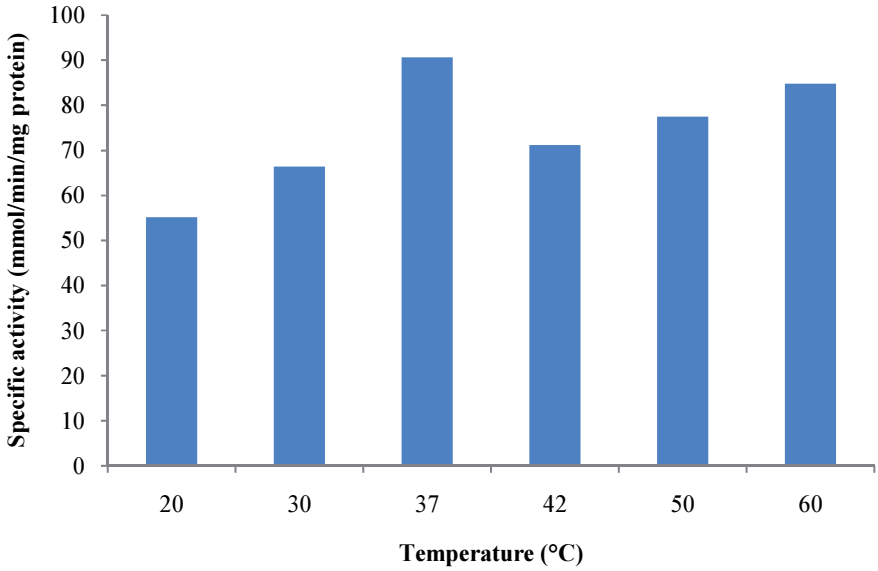

Figure 16. Effect of temperature on Cr (VI) reductase activity in cell-free extracts of *Penicillium* sp. IA-01 with initial concentrations of 28mM Cr (VI) at pH 7.0

to its affinity to –SH ligands, then suspecting the presence of this chemical group in the active site of the enzyme related to chromate reductase activity [70].

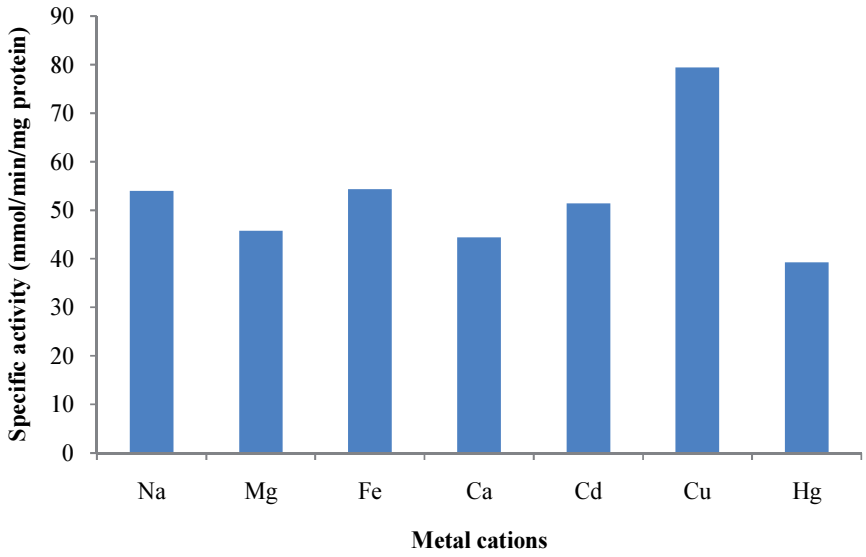

Figure 17. Effect of different metal cations on Cr (VI) reductase activity in cell-free extracts of *Penicillium* sp. IA-01 at pH 7.0 and 37°C

The reductase activity increased on supplementation in the reaction mixtures with electron donors. All the electron donors analyzed increased the activity, and the most efficient were ascorbic acid, NADH, glucose, and citrate by 4.4, 4.0, 2.9, and 2.87 times, respectively (Figure 18), and these results are like those reported for the yeasts *P. jadinii* M9 and *P. anomala* chromate reductase with NADH [60] and *Pseudomonas* sp. with citrate, acetate, glucose, and formate [58]. In previous reports of *Bacillus* sp., glucose has been reported to act as an electron donor and has been demonstrated to increase Cr (VI) reduction [72, 73], and also formate-dependent Cr (VI) reductases have been reported in *Shewanella putrefaciens* MR-1 [74]. Our work supports other studies reporting NADH or NADPH-dependent enzymatic reduction of Cr (VI) under aerobic conditions [58, 60, 67, 69, and 70]. Ramirez-Díaz et al. [75], report the oxidation of NADH donates an electron to the chromate reductase enzyme, and then the electron is transferred to Cr (VI) reducing it to the intermediate form Cr (V) which finally accepts two electrons from other organic substances to produce Cr (III).

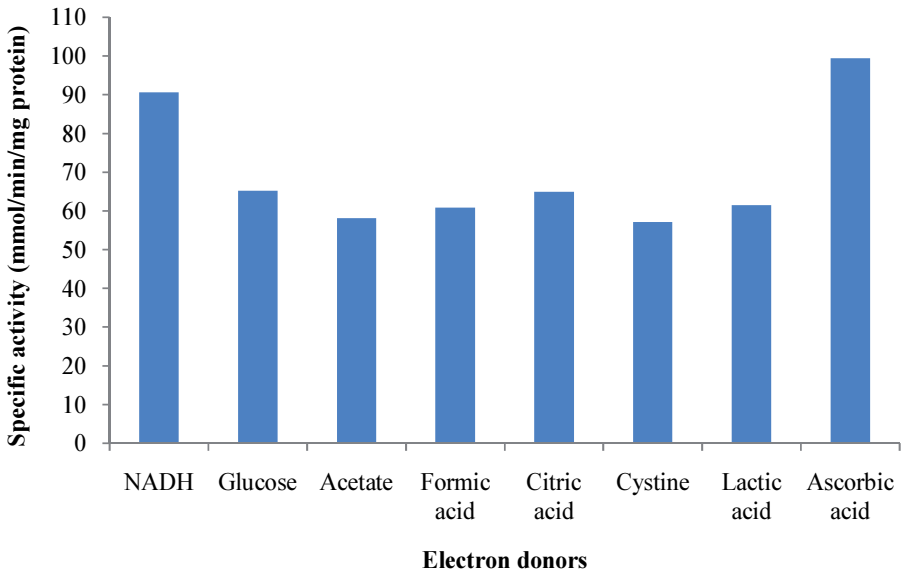

Figure 18. Effect of different electron donors on Cr (VI) reductase activity in cell-free extracts of *Penicillium* sp. IA-01 at pH 7.0 and 37°C

Respiratory inhibitors like azide (1mM), EDTA (1mM), and cyanide (1mM) caused inhibitions of 48%, 47%, and 32%, respectively (Figure 19), in the Cr (VI) reductase activity; these results agree with those obtained in previous studies [66], and it has been observed that cyanide and azide partially inhibited purified chromate reductase of *E. coli* ATCC 33456 19 [67] and aerobic chromate reduction by *Bacillus subtilis* [71] and inhibited more than 50% of membrane-associated chromate reductase activity of *S. putrefaciens* MR-1 [74], while no inhibition was observed in CFE of *Bacillus* sp. ES29 [70]. Respiratory inhibitors act on de novo protein synthesis or affect the respiratory chain intermediates responsible for Cr (VI) reduction,

wherein Cr (VI) serves as a terminal electron acceptor [69]. As shown in Figures 15 and 16, the optimal pH and temperature of chromate reductase in *Penicillium* sp. IA-01 were pH 7 and 37℃, and the results were supported by the results of living cells. Therefore, the reduction is mainly occurred to remove Cr (VI) and to show resistance to high concentration of Cr (VI). Whereas, addition of electron donors caused the decrease of the activity, and therefore, these compounds may be inhibitor of the enzyme.

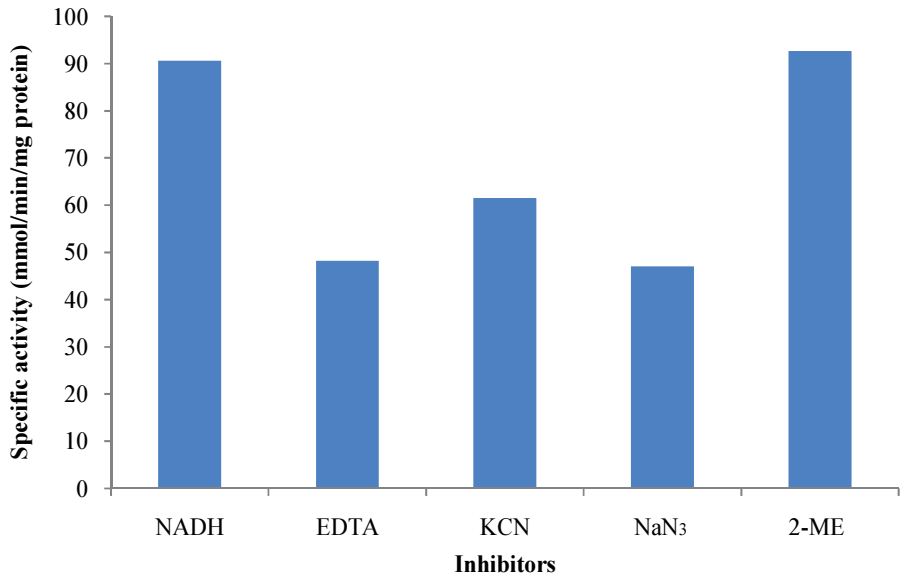

Figure 19. Effect of different inhibitors on Cr (VI) reductase activity in cell-free extracts of *Penicillium* sp. IA-01 at pH 7.0 and 37°C

4. Conclusion

We isolated a *Penicillium* sp. IA-01 strain, which grow about 50% relative to control (85 mg of dry weight without metal) in LMM, probably is resistant to the metal, and also removes efficiently 1g/100 mL of Cr (VI) after 90 min of incubation, and removes 63.2% and 43% from soil and water samples contaminated, respectively. This strain showed an efficient capacity of reduction (91%) of 50mg/L Cr (VI) in the growth medium after seven days of incubation, at 28°C, pH 5.3, 100 rpm and with an inoculum of 169mg of dry weight. The Cr (VI) reduction potential of the resting cells was increased by cell permeabilization. The optimum temperature and pH of chromate reductase activity of the CFE, were found to be 37°C and 7.0, respectively, and activity was enhanced in the presence of 0.1mM NADH and other electron donors. 1mMol of metal ions like Cu^{2+}, Na^{+}, Hg^{2+}, Mg^{2+}, Fe^{3+}, Ca^{2+}, and Cd^{2+} and respiratory inhibitors resulted in a decrease of the activity. Finally, these results suggest the potential applicability of *Penicillium* sp for the remediation of Cr (VI) from polluted soils and waters.

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Chapter 9

Screening of Marine-derived Fungi Isolated from the sponge *Didemnun ligulum* for Biodegradation of Pentachlorophenol

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Additional information is available at the end of the chapter

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Abstract

Contamination by pesticides employed in agriculture has caused serious environmental harm. Pentachlorophenol (PCP) is a phenolic organochlorine compound and a dangerous pollutant which was banned from Brazil since 1985; however, there are still many contaminated areas. This pesticide is a serious problem because it has high toxicity and persistence at the environment due to its resistance to biotic and abiotic degradation. The use of microorganisms as degrading agents is considered an efficient method to reduce the adverse effects of environmental contaminants. It is noteworthy that fungi from marine environment are adapted to extreme conditions, including high chlorine concentrations, and can produce unique enzymes with interesting properties. Therefore, marine-derived fungi have an excellent enzymatic potential for the biotransformation of xenobiotics such as organochlorine pesticides. In this work, fifteen fungi strains isolated from a marine invertebrate, the ascidian *Didemnun ligulum*, were evaluated according to their ability to grow in solid culture media (3% malt extract agar) in the presence of different concentrations (10, 25, 30, 40, and 50 mg L^{-1}) of PCP. Among the tested strains, nine could grow in at least one concentration, and *Trichoderma harzianum* CBMAI 1677 showed optimal growth at the higher evaluated concentration (50 mg L^{-1}), showing toxicity resistance and suggesting its potential for biodegradation of PCP. In a later work, it was observed that *T. harzia-*

num CBMAI 1677 was able to degrade PCP. These results confirmed the efficiency of marine-derived fungi to biodegrade persistent compounds and could enable the development of bioremediation methodologies using these microorganism.

Keywords: Organochlorine pesticide, Agrochemicals, Marine Microorganisms, Biotransformation

1. Introduction

1.1. Pesticides

Pesticides are pure substances or mixtures of chemicals used to control undesired organisms during production, harvest, and food storage. These compounds can be organic or inorganic molecules classified according to their chemical structure or type of the target organism [1]. They can be introduced into the environment during their manufacturing, application, or subsequent leaching affecting target and nontarget organisms [2]. The term pesticide, used in this chapter, is a synonym of biocide, agrotoxic, and agrochemical, though, there are more specific definitions that include and exclude different chemicals groups [3]. Regardless of the term used, these compounds act by blocking a vital metabolic process of the target organism [4].

The use of different toxic substances against pests and diseases is dated from antiquity. Different natural products such as nicotine, pyrethrum, tobacco plants extracts (*Nicotiana tabacum* L.) [5] and inorganic compounds such as mercury and sulfur were employed in ancient times [6]. The modern use of pesticides is dated from the twentieth century with the intensive use of inorganic substances like sodium aceto arsenite, calcium fluoride, white arsenic, and others [7]. Since the 1930s, the increased agricultural production demanded the formulation and use of substances with best biocide action [7]. Intensive development of the chemical industry occurred with the Industrial Revolution, which led to an increase in the research, and consequently, the production of new pesticides, which was expanded on a global scale after 1940s [8].

The cultivated area increasing and need for higher agricultural productivity stimulated the use of pesticides, mainly in Brazil. In this sense, the use of pesticides in Brazilian agriculture began in the 1970s encouraged by the National Development Plan (in Portuguese, Plano Nacional de Desenvolvimento) [9]. In 2011, the pesticide market in this country was considered the largest in the world, representing 16% of the global market according to the National Health Surveillance Agency (in Portuguese, Agência Nacional de Vigilância Sanitária, ANVISA) [10].

Over the past 50 years, pesticides had been used to increase the food quantity and quality for a growing world population. While worries about their adverse effects in nontarget organisms, including humans, had been also increased [11]. These chemicals, while having a beneficial effect toward agricultural production, are alien to nature and can produce changes and imbalances [12]. Many of them are toxic not only to insects and harmful pests but also to other

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living beings that are essential to several environmental processes [6]. Different reactions may act in these chemicals affecting their fate and behavior during natural processes [13]. Therefore, pesticides may be one of the most dangerous contaminants to the environment, since they are very toxic, can bioaccumulate, and be part of chemical, physical, and biological processes in nature.

Pesticides used in agriculture remain in the soil at the application site, or are transported to different parts of the environment, such as sediments, plants, surface and ground waters, marine environments and even volatilized into the atmosphere, depending on their physical-chemical properties [14-16]. The metabolic fate of the pesticides also depends on the abiotic environmental conditions (temperature, pH, soil moisture), the microbial community, the pesticide characteristics (hydrophilicity, degree of solubility, molecular weight), and the chemical and biological reactions [18]. Once they entered in the soil, pesticides are transferred or degraded by evaporation, leching, infiltration, adsorption, absorption in inorganic matter and biotic and abiotic degradation [17]. The abiotic degradation occurs through physical and chemical transformations in reactions of hydrolysis, oxidation, reduction, photolysis, and rearrangement [18]. However, the enzymatic transformations performed by microorganisms and plants are the major detoxification pathways [11].

Pesticides are used in several products involving herbicides, fungicides, nematicides, insecticides, fumigants, and substances used as desiccants, defoliants, and growth regulators [19]. Based on the chemical functional groups of the active ingredients, pesticides may be classified as organochlorines, organophosphates, carbamates, and pyrethroids [20]. Organochlorines, which shows high toxicity and persistence because of their resistance to biotic and abiotic degradations, are especially worrisome [21].

1.2. Organochlorine pesticides

The age of the organochlorine compounds was started in 1948 with the Nobel Prize in Physiology or Medicine delivered to Paul Müller, who condensed chlorobenzene to synthesize *p*-dichlorodiphenyltrichloroethane (DDT), a high effective insecticide [22]. Since then, new types of organochlorines compounds had been developed and extensively used (Figure 1). However, the harmful effects of those compounds, such as persistence, toxicity, and bioaccumulation had been also reported [23].

In 1962, the American biologist Rachel Carson published the book "Silent Spring" alerting for the damage that insecticides, especially the DDT, could cause. Despite having been the target of much criticism, the publication was fundamental for the prohibition of organochlorine pesticides in the United States in the early 1970s [17]. Although the use of organochlorine pesticides in agriculture was banned, elimination methods are still studied since these compounds had been widely used from 1960 to 1980, and thus, a toxic waste accumulation occurred in various ecosystems around the world [24].

The organochlorine pesticides are highly thermostable compounds with cyclic structures [26] mainly formed by hydrogen, carbon, and chlorine [27] and recognized as the most toxic and persistent pollutants among organic compounds [28-29]. These compounds dissolve well in

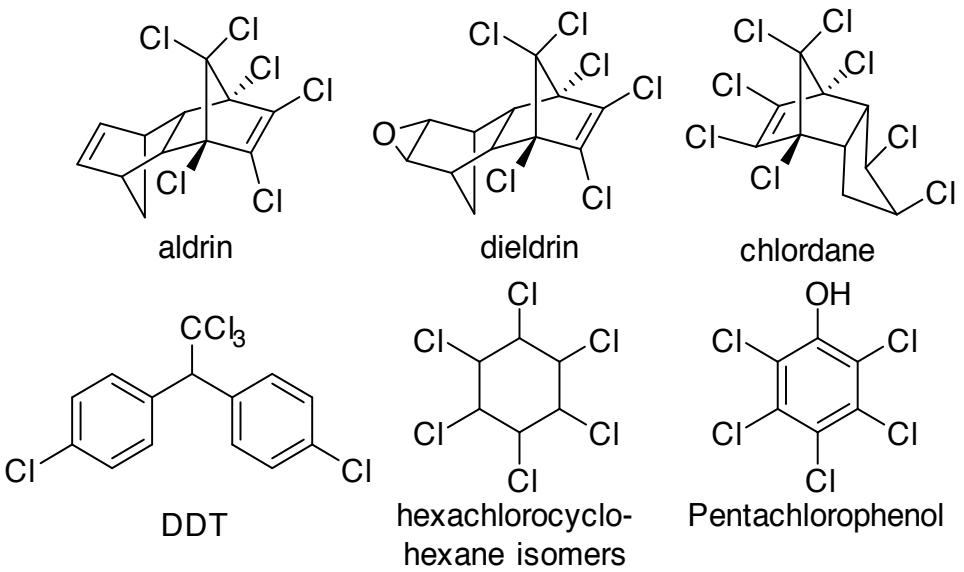
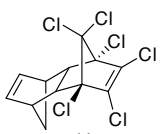
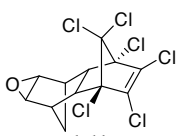


Figure 1. Synthetic organochlorines used as insecticides in the early days (Adapted from Santos et al. [25]).

lipids (fat-soluble), and favors its accumulation in adipose tissues of animals [23]. Thus, they are biomagnified through the biological chain [30], affecting the health of the top predators, including humans [31]. Additionally, organochlorine compounds may interfere in the normal functions of the endocrine system and disturb the reproduction in animals, since they show estrogenic and carcinogenic activity [32-33].

Organochlorine pesticides and some of their physical and chemical characteristics are described in Table 1. Among them, pentachlorophenol (PCP) is one of the most studied organochlorine compounds, because it slightly dissolves in water and has strong solubility, toxicity [34], volatility, ability to release dioxin (and its derivatives), and resistance to biodegradation [35].

Compound	CAS number	Solubility in water	Steam pressure
 aldrin	**309-00-2**	**27 μg L^{-1} at 25°C**	**2.31 × 10 mm Hg at 20°C**
 dieldrin	**60-57-1**	**140 μg L^{-1} at 20°C**	**1.78 × 10 mm Hg at 20°C**

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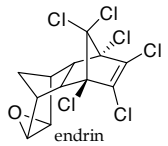
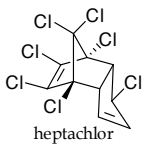
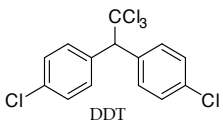
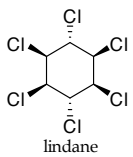
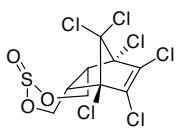
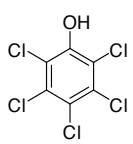
Compound	CAS number	Solubility in water	Steam pressure
 endrin	**72-20-8**	**220-260 μg L^{-1} at 25°C**	**7 × 10 mm Hg at 25°C**
 heptachlor	**76-44-8**	**180 μg L^{-1} at 25°C**	**0.3 × 10 mm Hg at 20°C**
 DDT	**50-29-2**	**1.25.5 μg L^{-1} at 25° C**	**0.02 × 10 mm Hg at 20° C**
 lindane	**58-89-9**	**7 mg L^{-1} at 20° C**	**3.3 × 10 mm Hg at 20° C**
 endosulfan	**115-29-7**	**320 μg L^{-1} at 25° C**	**0.17 × 10 mm Hg at 25° C**
	**87-86-5**	**14 mg L^{-1} at 25° C**	**16 × 10 mm Hg at 20° C**

Table 1. Physical and chemical characteristics of the main organochlorine pesticides (Adapted from Almeida *et al*. [36]).

1.3. Pentachlorophenol

PCP is used as an insecticide, fungicide, herbicide, and wood preservative [37]. Moreover, PCP is a by-product of the paper bleaching, disinfection of water containing phenols with chlorine or sodium hypochlorite, incineration of municipal solid waste and other processes [38-39]. PCP can be found in the air in the form of steam, adsorbed in soil and sediments, in surfaces and groundwater in its ionized salt form [40]. Table 2 shows some physical and chemical properties of PCP.

CAS number	87-86-5
Molecular formula	$C_6H\ Cl_5O$
Molar mass	266.34 g mol^{-1}
Melting point	190–191° C
Boiling Point	309–310° C (dec.)
Appearence	White crystalline solid
Density	1.978 g cm^{-3}
Vapor density	9.20 (air = 10)
Solubility in water	0.020 g.L^{-1} at 30° C
Henry's Law constant	2.45×10^{-8} atm-m^3 mol^{-1}

Table 2. Physical and chemical properties of the PCP (Source: adapted from EPA [41]).

The PCP is produced by two different routes, i.e., the gradual chlorination of phenols in the presence of catalysts (ferric chloride or anhydrous aluminum chloride) and by dechlorination of hexachlorobenzene [42]. According to the Environmental Sanitation Technology Company of São Paulo State (in Portuguese, Companhia de Tecnologia e Saneamento Ambiental do Estado de São Paulo, Cetesb), PCP is a white solid insoluble in water, but highly soluble in oils and fat compounds. The commercial reagent of PCP contains about 85% of active ingredient, 6% of tetrachlorophenol, 6% of other chlorinated phenolic compounds and inert materials [43]. Other impurities are dioxins (tetra-, hexa-, and octachlorodibenzene-*p*-dioxin) and hexachlorobenzene as by-products of manufacture, which can be easily released to the environment. PCP is no longer marketed in Brazil, but pentachlorophenate, which is a water-soluble persistent product formed by the neutralization with sodium hydroxide, can be easily obtained because it is still used as wood preservative [44-45]. The high solubility of the sodium salt in water enables the persistence for long periods in water bodies, increasing the intoxication level [46]. Fish absorb PCP thorough their gills and alimentation, and then contaminate humans through the food chain [47]. According to Ondarza et al. [48], this accumulation in fish reflects the environment contamination degree.

According to the United States Environmental Protection Agency [41], several studies have provided data on PCP levels in human blood and urine (samples from general population or those with known PCP exposure), indicating that the main route of PCP absorption is inhalation during production and handling [49]. It can be easily absorbed by skin and gastrointestinal tract, and then dissipated throughout the body. Consequently, PCP is concentrated in heart, brain, adrenal glands, adipose tissue, liver, and kidneys [50], in which they cause serious damage and cancer [51].

Even with the prohibition of the PCP use in Brazil since 1985 (Ministry of Agriculture in Portuguese: Ministério da Agricultura), many areas remain contaminated. The main reason of the pollution is the indiscriminate use of PCP for several decades [38]. Studies show that PCP residues are still measured at high level in several environmental matrices, such as

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soil, water, sediment, organic matter suspension, atmosphere, and even in many organisms [52-53]. Thus, the use of biological degradation techniques is very important because these methodologies promote the complete mineralization of this compound or conversion to harmless products [54].

1.4. Microbial biodegradation of pesticides

The microorganisms are adaptable to adverse conditions and find ways to grow even in challenging environments [55]. Its potential for biotechnological applications are justified by their tolerance to extreme environmental conditions, rapid growth, low cultivation cost [56], and mainly by their enzymes, which can transform a wide variety of nonnatural chemical compounds [57].

Microorganisms can degrade xenobiotics contained in dyes, cosmetics, detergents, medicines, agricultural chemicals and can mineralize and degrade pesticides to nontoxic compounds [58, 59]. Therefore, microbial biodegradation is an effective method to reduce the harmful effects of pesticides. Biodegradation is considered the main process of pesticides elimination in soil [60] since microorganisms are capable of use these compounds as nutrients source for its enzyme-catalyzed transformations, which lead to changes of structure and toxicological properties and consequently, its polluting potential [61].

Organochlorine compounds are known to undergo dehydrochlorination, oxidation, dechlorination, rearrangement, hydrolysis, and photochemical reactions [65]. Among the pathways observed in microorganisms, the dechlorination under anaerobic condition and dehydrogenation under aerobic condition are the most important [18].

The selection of an appropriate microorganism is an essential step to perform a microbial biotransformation. If a microorganism can proliferate efficiently in environments with high concentrations of certain pollutants, such strain might be more adapted for the remediation of these contaminants [62]. Different bacterial and fungi genera had been used as efficient pesticides metabolizing organisms such as *Rhodococcus*, *Pseudomonas* and *Flavobacterium* [61], *Lentinula edodes*, *Phlebia radiata*, *Phanerochaete chrysosporium* [63], *Trametes hirsutus*, *Phanerochaete sordia*, and *Cyathus bulleri* [64].

In the biodegradation of organochlorine pesticides, some bacterial genera have been proven to be good biocatalysts, i.e., *Klebsiella* [66], *Staphylococcus* [67], and *Pseudomonas* [68]. Some fungi are also effective, i.e., basidiomycetes [69, 70] and white-rot-fungi, such as *Trametes villosa* [71], *Phaneroachaete chrysosporium*, *P. sordida* [72], *Phlebia radiata* [73], which are commonly used to biodegrade organochlorine compounds. But there are also reports of other fungal species involved in biodegradation of these compounds, i.e., *Trichoderma harzianum* [74], *Aspergillus niger* [75], and *Fusarium verticillioides* [76] with excellent results.

1.5. Biodegradation of PCP

The degradation of PCP in the environment can occur through chemical, microbiological, photochemical, electrochemical, and thermal processes [77, 78]. Microbial decomposition is an

important removal mechanism of this compound [78]; however, PCP causes oxidative phosphorylation and membrane cell disruption. Therefore, its toxicity slows biodegradation because of the growth inhibition effects on microorganisms [79].

Despite having these biodegradation unfavorable attributes, some microorganisms have the ability to use PCP and its metabolites as carbon and energy sources [80, 81]. Among the reported species, *Pseudomonas fluorescens* (TE3) [82], *Pseudomonas aeruginosa* (PCP2) [83], *Serratia marcescens* [84], *Pseudomonas stutzeri* (CL7) [81], and *Comamonas testosteroni* (CCM7350) are important examples [85].

Figure 2 shows the biodegradation pathway of PCP by *Sphingobium chlorophenoculium* ATCC 39723 [86]. This strain can degrade PCP to carbon dioxide and water (Figure 2).

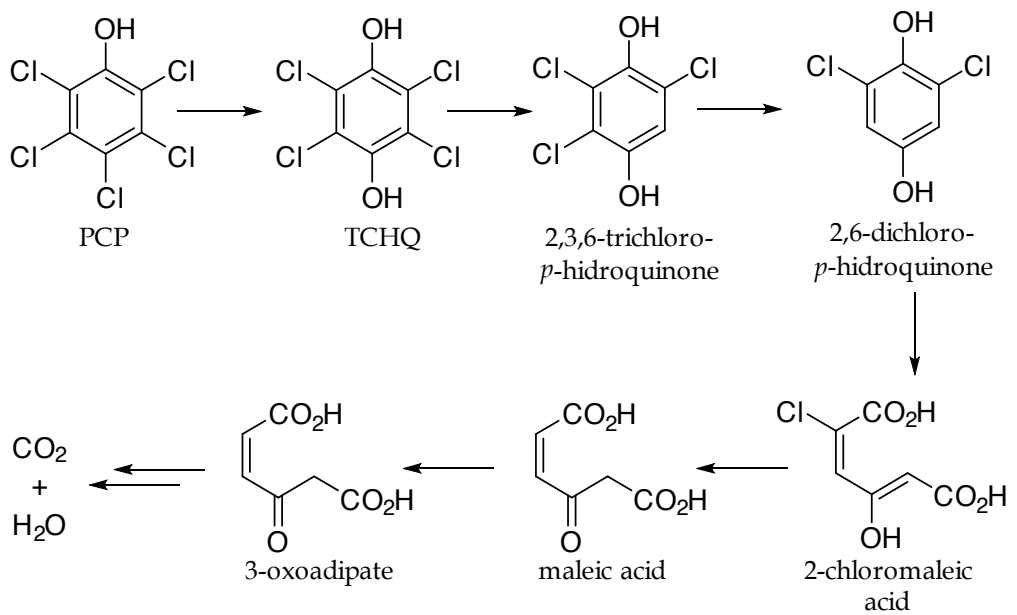

Figure 2. Biodegradation pathway of PCP by the *Sphingobium chlorophenolicum* ATCC 39723 bacteria (adapted from Cai and Xun [86]).

Usually, metabolic transformations in biological systems can be divided into two phases. The reactions of phase I promote changes in xenobiotics such as oxidation, reduction, hydrolysis, and other reactions. After this step, the phase II reactions known as conjugations occurs, in which endogenous groups, which are usually polar and present in abundance *in vivo*, are added to the xenobiotic resulting in more polar products (except in alkylation reactions) and therefore, more easily eliminated compounds. It is noteworthy that conjugated xenobiotics can undergo inverse reactions and regenerate the original compound [87]. Thus, the compound can be degraded (into smaller molecules which can be toxic or not), absorbed, adsorbed, or conjugated during the biodegradation [88].

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There are many reports involving the use of terrestrial fungi in the biodegradation of PCP. Among them, white-rot fungi are highly tolerant to toxic compounds and are widely used in biodegradation techniques [71]. These fungi are effective in the degradation of PCP by having ligninolytic and peroxidase enzymes [89] that act by generating free radicals [90], which can also degrade a variety of recalcitrant pollutants (Figure 3) [91].

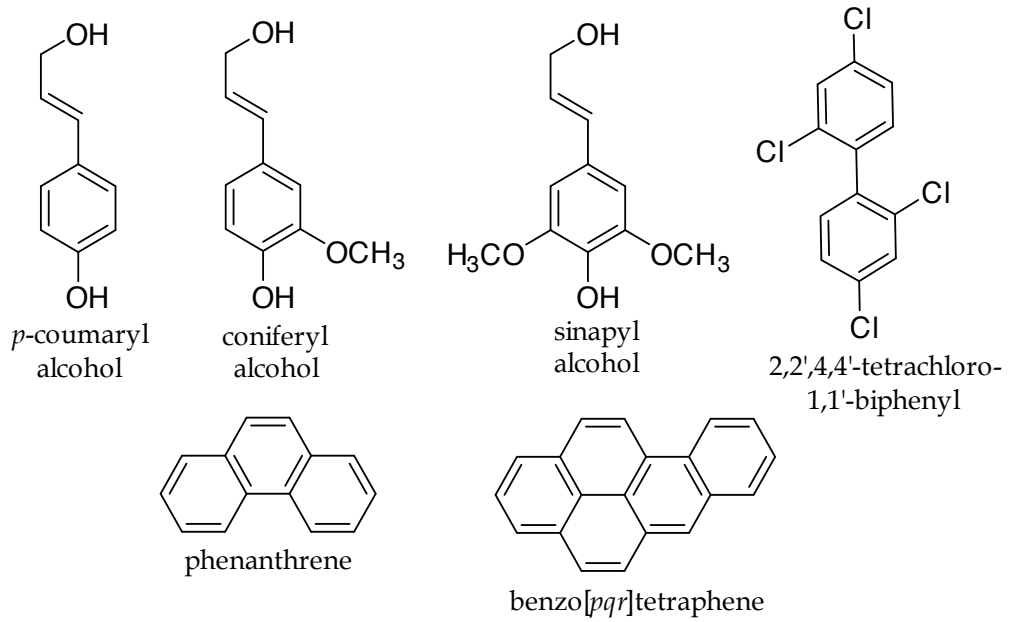

Figure 3. Examples of recalcitrant compounds biodegraded by ligninolytic and peroxidase enzymes: lignin monomers, polycyclic aromatic hydrocarbons (PAHs), and halogenated compounds (Adapted from Pointing [90]).

The ligninolytic extracellular activity of some fungal enzymes is considered a promising method for PCP degradation [92, 94]. The *Phanaerochaete chrysoporium* [95, 96], as well as *Phlebia brevispora* [97], *Phlebia radiata*, *Trametes versicolor* [98], and *Mucor plumbeus* [99] showed great ability to degrade organopollutants (including PCP). Fungal species belonging to the genus *Trichoderma*, such as *T. virgatunil* [100] and *T. harzianum* [74], were efficient in the mineralization of PCP and *Anthracophyllum discolor* mineralized this polutant in reactors containing soil slurry according to Rubilar [101]. Figure 4 shows the PCP biodegradation pathway by *A. discolor*. It is noteworthy that this pathway is different from that by *S. chlorophenolic* ATCC 39723.

The use of filamentous fungi in biodegradation is increasing considerably in recent years, due to the high rates of biodegradation, sortion, and resistance in adverse environmental conditions [102]. According to Sankaran et al. [103], the interest in the use of filamentous fungi in bioremediation is due to high species diversity, high resistence for recalcitrant compounds, and high production of extracelular enzymes.

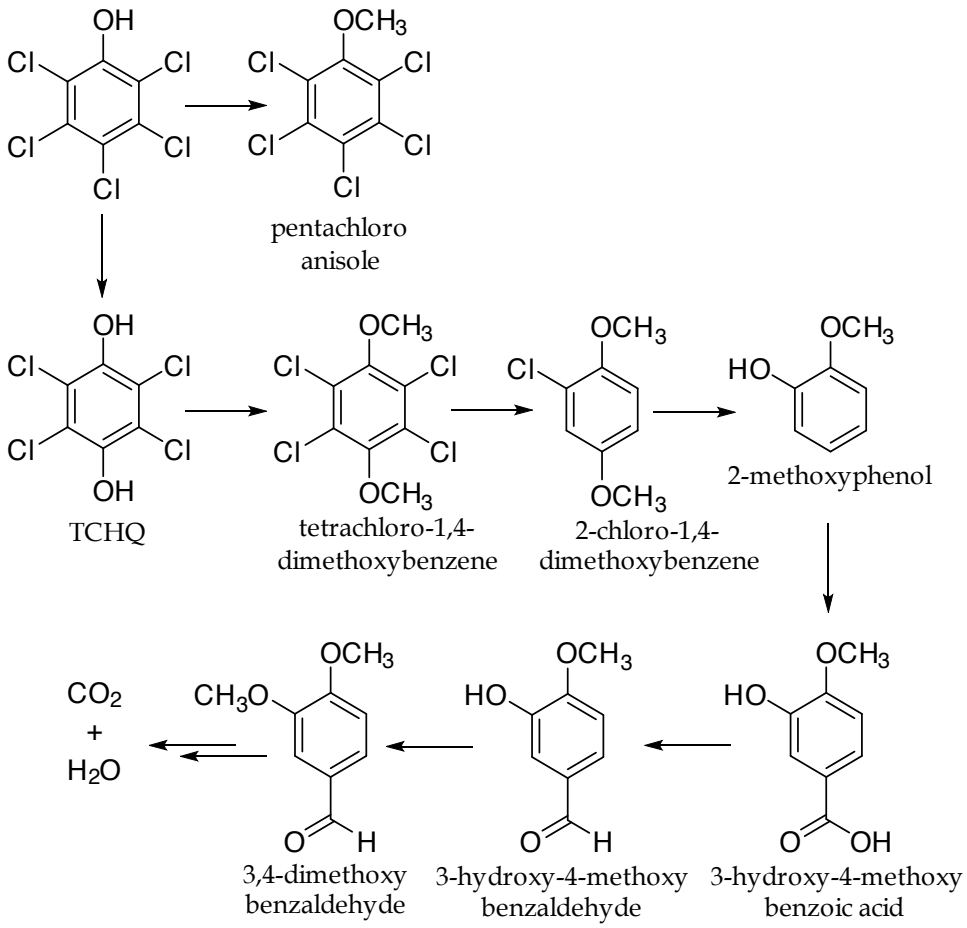

Figure 4. Degradation of PCP by the fungus *Anthracophyllum discolor* (Source: modified from Rubilar et al. [101]).

1.6. Marine fungi

The marine environment covers more than three quarters of the Earth's surface and is a promising source of new enzymes [104]. These enzymes show great potential for use in biocatalytic reactions by possessing unique characteristics related to the marine environment. In recent years, a wide variety of enzymes and microorganisms with specific activities have been isolated from marine environments [105] and have been extensively studied, particularly proteases, carbohydrases, oxidoreductases, peroxidases [106].

The words “marine fungi“ are not derived from a taxonomic class and they are not classified by their physiological characteristics. These fungi considered as an ecological group, and the most suitable definition was proposed by Kohlmeyer and Kohlmeyer [107]: "Mandatory marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat; facultative marine fungi are those from freshwater or terrestrial water environments and are able to grow and even sporulate in the marine environment " [108]. In the marine environment,

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many fungi strains can be found in a wide variety of habitats such as open sea, sediment, mangroves, surface of wood, shells of molluscs, corals, marine vertebrates and invertebrates, on the surface or interior of algae and even in hydrothermal vents. The variety of habitats also influences their metabolic diversity, which contributes to their potential use as source of enzymes and bioactive molecules [109].

Unlike terrestrial fungi, which were initially exploited for drug discovery, marine fungi have attracted the attention of researchers as a source of new natural products and enzymes [110]. Marine fungi are adapted to high salinity and extreme conditions, developing attributes that give them the ability to produce a different enzymatic metabolism from their respective representatives from the terrestrial environment [111]. Researches had been generally focused on biological activities such as antibiotic and by marine fungi [112]. However, recently they have been investigated for dechlorination and detoxification of effluents [113], biodegradation of polycyclic aromatic hydrocarbons [114], lignin [115], pesticides [116, 117], and polyethylene [118], and more recently, studies on biocatalytic reactions for organic synthesis [106].

Filamentous fungi *Aspergillus sydowii*, *Penicillium raistrickii*, *Trichoderma* sp., and *Penicillium miczynskii* isolated from marine environment and cultured in artificial sea water were capable of catalyzing the hydrolysis of benzyl glycidyl ether and allyl glycidyl ether [119, 62]. Bonugli-Santos et al. [120] found interesting results in a study of ligninolytic enzyme production by the marine fungi *Aspergillus sclerotiorum* CBMAI 849, *Cladosporium cladosporioides* CBMAI 857, and *Mucor racemosus* CBMAI 847. The marine fungi *Microsphaeropsis* sp., *Acremonium* sp., and *Westerdykella* sp. promoted the biodegradation of esfenvalerate (pyrethroid pesticide) with formation of several metabolites [121].

The enzymatic reactions catalyzed by marine-derived fungi can be carried out in laboratory using artificial seawater. Studies have shown that marine bacteria and fungi cultured in laboratory have specific requirements of salts, especially the sodium ions, potassium, magnesium, and chloride [122, 119]. According to Rateb and Ebel [123], for biotransformation studies and production of secondary metabolites, marine-derived fungi strains have been isolated mainly from inorganic substrates, plants, marine invertebrates, and vertebrates. In this context, studies on enzyme production by filamentous marine-derived fungi are important for future applications in bioremediation techniques. Thus, this work aimed the exploration of the potential biodegradation of the pesticide PCP by strains of marine-derived fungi isolated from a marine invertebrate, the ascidian *Didemnun ligulum*.

2. Materials and Methods

2.1. Isolation of fungi strains

Marine-derived fungi were isolated from the ascidian *Didemnum ligulum* according to the method described by Kossuga et al. [124]. The ascidian samples were collected in São Sebastião, South Atlantic Ocean, in September 2005 at the northern coast of São Paulo state, Brazil, by Prof. Roberto G.S. Berlinck (IQSC-USP, Brazil). After the isolation and purification of the

strains, the marine-derived fungi were deposited in the microbiology laboratory of the Department of Ecology and Aquatic Microbiology supervised by Mirna H.R. Seleghim (UFSCar, Brazil). They were preserved by two techniques: in distilled water according to Castellani [125] and in inclined tubes containing agar, both stored under refrigeration. The strains were reactivated for the experiments by streaking or aseptic transfer of mycelial discs to solid culture media (3% malt).

In the laboratory, samples collected from the ascidian were subjected to surface sterilization by successive washes with 0.001 g.L^{-1} solution of $HgCl_2$ in 5% ethanol for 1 minute, followed by 3 washes with sterile sea water [126]. Then, portions of about 1 cm^2 were taken from the inside of the ascidian with a sterile scalpel. These fragments were inoculated in Petri dishes containing agar medium with artificial sea water (ASW - Artificial Sea Water) and the broad-spectrum antibiotic rifampicin (0.3%) to inhibit bacterial growth [127]. Plates were incubated for 7 d at 25° C. Eight culture media were prepared (Table 3) in order to expand the possibilities of obtaining different strains that may be associated with the ascidian *D. ligulum*.

Culture media	**Composition**
2% Malt Extract Agar (MA2%)	Malt extract (20 g L^{-1}), agar (15 g L^{-1}) in artificial seawater
3% Malt Extract Agar (MA3%)	Malt extract (30 g L^{-1}), mycological peptone (5 g L^{-1}) and agar (15 g L^{-1}) in artificial seawater
Glucose agar, Peptone, and Yeast extract (GPY)	Glucose (1 g L^{-1}), soy peptone (0.5 g L^{-1}), yeast extract (0.1 g L^{-1}), agar (15 g L^{-1}) in artificial seawater
Potato Carrot Agar (PCA)	Cooked and mashed potatoes (20 g L^{-1}), cooked and mashed carrots (5 g L^{-1}), agar (20 g L^{-1}) in artificial seawater
Corn Meal Agar (CMA)	Maize flour (42 g L^{-1}) stirred in 500 mL of distilled water at 60°C for 12 h, filtered, and then the supernatant was diluted with artificial seawater to 1 L with agar (15 g L^{-1})
Oat Meal Agar (OMA)	Rolled oats (30 g) were boiled in 500 mL of distilled water for 1 h, filtered, and then diluted with artificial seawater to 1 L with agar (20 g L^{-1})
Tubaki Agar (TA)	Glucose (30 g L^{-1}), yeast extract (0.5 g L^{-1}), peptone (1.0 g L^{-1}), dibasic potassium phosphate (1.0 g L^{-1} KH_2PO_4), magnesium sulfate heptahydrate (0, 5 g L^{-1}, $MgSO_4.7H_2O$), 0.01 g of iron(II) sulphate heptahydrate (0.01 g L^{-1} $FeSO_4.7H_2O$), agar (15 g L^{-1}) in artificial seawater
Cellulose Agar (CA)	Cellulose (10 g L^{-1}), yeast extract (1 g L^{-1}), agar (15 g L^{-1}) in artificial seawater

Table 3. Culture media composition for isolation of marine-derived fungi from *Didemnun ligulum* [124].

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2.2. Purification

The Petri dishes with different culture media containing the filamentous fungi strains were examined periodically. The isolated strains were subjected to successive inoculations to obtain pure cultures. Initially, the pure cultures were described by morphological method and coded as DL. The DL code was related to the organism from which the strains were isolated, the ascidian *Didemnum ligulum*, and the abbreviation for the culture medium used in the isolation. Eight different culture media for strain isolation were used; however, fungi growth was not observed in the cellulose agar and Tubaki agar media.

The 15 isolated strains were coded as; DL5A, DL6A, DL11A (oatmeal agar medium), DL2B, DL5B (potato carrot agar medium), DL1F, DL2F, (corn meal agar medium), DL5G, (glucose agar, peptone, and yeast extract culture medium), DL3M2, (2% malt extract agar medium), DL1M3, DL4M3, DL6M3, DL7M3, DL8M3, and DL9M3 (3% malt extract agar medium). The detailed methodology for the isolation and purification were described by Kossuga et al. [124]. The procedures were performed at the Department of Ecology and Evolutionary Biology at UFSCar, São Carlos, Brazil.

2.3. Identification of strains by molecular biology

The 15 fungal strains were characterized and identified by techniques based on the molecular identification of genes rRNA, ITS1 and ITS4. These analyzes were carried out under the supervision of Prof. Dr. Suzan Pantaroto de Vasconcellos at the Federal University of São Paulo (UNIFESP), Campus Diadema.

The isolates were grown on yeast extract sucrose agar (YES) (10 g yeast extract, 75 g sucrose, 10 g agar, and 500 mL distilled water). Then, DNA was extracted with the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The DNA concentration and purity (relative to proteins and salts) were determined by optical density at 260 nm (OD260) and ratios of OD260/280 and OD260/230, respectively. The internal transcribed spacer (ITS) region of rDNA were amplified with primer pairs and ITS1/ITS4 using the protocol described by Gonçalves et al. (2012). The reactions were performed with PCR master mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. After amplification, the fragments were sequenced following the protocol provided with the BigDye reagent kit (Applied Biosystems, Foster City, CA, USA) in an ABI 3130 (Applied Biosystems, Foster City, CA, USA) automatic sequencer. PCR products were sequenced with the same primers used for amplification. Contig assembly and editing were performed with Sequencher DNA sequence assembly software 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Successful assembly of the contigs required a minimum match percentage of 85 and a minimum overlap of 20.

Complete ITS consensus sequences were used to conduct BLAST search analysis for species identification from the NCBI genomic database (http://blast.ncbi.nlm.nih.gov/).

For all regions analyzed by BLAST search, the sequences that were presented with high identity (99%), queries, and E values of $e10^{-5}$ were considered for the final species identification using the sequencing method.

2.4. Growth of fungi strains in solid medium

The strains of marine-derived fungi were cultivated on Petri dishes containing 3% malt solid medium using artificial sea water with the following composition: malt extract (30.0 g L^{-1}), soy peptone (3.0 g L^{-1}), and agar (20.0 g L^{-1}). The pH was adjusted to 8 with KOH solution (0.1 mol L^{-1}), similar to the pH of the marine environment [124]. Artificial seawater composition was: $CaCl_2.2H_2O$ (1.36 g L^{-1}), $MgCl_2.6H_2O$ (9.68 g L^{-1}), KCl (0.61 g L^{-1}), NaCl (30.0 g L^{-1}), Na_2HPO_4 (0.014 mg L^{-1}), Na_2SO_4 (3.47 g L^{-1}), $NaHCO_3$ (0.17 g L^{-1}), KBr (0.10 g L^{-1}), $SrCl_2.6H_2O$ (0.04 g L^{-1}), and H_3BO_3 (0.03 g L^{-1}).

2.5. Selection of fungal strains resistant to PCP in solid culture medium

Fifteen fungal strains were grown in Petri dishes and inoculated in solid medium containing 3% malt extract medium without PCP (control) and with different concentrations of the organochlorine pesticide; 10, 25, 30, 40, and 50 mg L^{-1} per plate (98%, analytical standard commercially obtained from Sigma-Aldrich, Brazil). The experiments were prepared in triplicate. Ethyl acetate was used as solvent to prepare the stock solution of the pesticide in the proportion of 5.0 mg of PCP / 100 μL of ethyl acetate.

The culture media were sterilized in an autoclave at 121 °C for 20 minutes, cooled to about 40-50 °C, and then the pesticide stock solution was added, according to the desired concentration. The mixture was homogenized and then added in Petri dishes. The inoculation of fungi was made by transferring the mycelium of pure cultures precultivated in 3% malt medium after 5 d of growth by a platinum needle insertion point into the plate center. The plates were incubated at 32 °C (B.O.D. 411D, Nova Ética) and the radial growth of the fungus were observed for 21 d. The diameter of the colony formed was measured at 7 d intervals, as performed by Birolli et al. [128]. The strain that showed the highest radial growth was selected for the PCP biodegradation in a liquid medium. The experiments were performed in triplicates.

3. Results and Discussion

The aim of this chapter was the isolation and selection of marine-derived fungi with potential for PCP biodegradation. So the PCP biodegradation details will not be discussed because they already were published. Figure 5 shows the 15 fungi strains isolated from *Didemnum ligulum* cultivated in 3% malt extract medium in absence of pesticide.

The isolated fungi were identified by molecular biology and exhibited a variety of genera and species illustrating the fungi diversity in marine environment (Table 4): *T. harzianum* CBMAI 1677 was deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI - http://webdrm.cpqba.unicamp.br/cbmai/, WDCM 823).

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Strains	Identification	CBMAI deposit
DL1M3	*Stagonosporopsis cucurbitacearum*	
DL4M3	*Penicillium citrinum*	
DL6M3	*Mycosphaerella crystallina*	
DL7M3	*Didymella phacae*	
DL8M3	*Phoma* sp.	
DL9M3	Not identified*	
DL1F	*Pleosporales* sp.	
DL2F	*Cladosporium cladosporioides*	
DL3M2	*Cladosporium cladosporioides*	
DL5B	*Cladosporium cladosporioides*	
DL5G	*Cladosporium cladosporioides*	
DL6A	*Aspergillus versicolor*	
DL5A	*Aspergillus versicolor*	
DL11A	*Fusarium fujikuroi*	
DL2B	*Trichoderma harzianum*	1677

* Not cultured strain

Table 4. The codification and identification of the strains employed in this study.

For the evaluation of the fungi inhibition caused by the presence of the xenobiotic compound, radial growth experiments were performed. The marine-derived fungi were cultivated in various concentrations of PCP (10, 25, 30, 40, and 50 mg.L^{-1} per plate). The inoculation was carried out by a central insertion point using an inoculation needle. After incubation, the colonies' diameters were measured at 7, 14, and 21 d. The results are summarized in Tables 4-6.

All marine-derived fungi showed excellent growth after 7 d of cultivation in solid culture medium (3% mat extract agar) without PCP. The results showed that 3% malt extract medium was suitable for growth of marine-derived fungi as suggested by Kjer et al. [130]. After 21 d of incubation, 60% of the strains have grown throughout the plate surface, reaching 8.0 cm of colony diameter (diameter of the employed Petri dish). The cultivation of the fungus in the absence of PCP was important to assess the development of the pure cultures isolated from the sponge *D. ligulum*.

In the presence of the organochlorine pesticide, the strains coded as DL1M3, DL4M3, DL6M3, DL7M3, DL8M3, and DL9M3 failed to grow in any of the plates containing PCP, showing low

Figure 5. Colonies of marine-derived fungi isolated from the ascidian *Didemnum ligulum* grown in 3% malt extract medium.

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resistance and adaptation to the organochlorine presence, and thus suggested low potential for biodegradation. It is noteworthy that these microorganisms were isolated from environments without PCP contamination; therefore its presence caused growth inhibition because this is a very toxic compound for living organisms and these strains were not adapted to its effects on their metabolism.

Colony diameter (cm)[a]						
Strains	**Petri dishes without PCP**	**Concentration of PCP in Petri dishes with PCP**				
		10 (mg.mL^{-1})	**25 (mg.mL^{-1})**	**30 (mg.mL^{-1})**	**40 (mg.mL^{-1})**	**50 (mg.mL^{-1})**
S. cucurbitacearum DL1M3	4.2	-	-	-	-	-
P. citrinum DL4M3	1.5	-	-	-	-	-
M. crystallina DL6M3	1.9	-	-	-	-	-
D. phacae DL7M3	0.9	-	-	-	-	-
Phoma sp. DL8M3	0.6	-	-	-	-	-
Not identified DL9M3	3.9	-	-	-	-	-
Pleosporales sp. DL1F	1.9	0.2	-	-	-	-
C. cladosporioides DL2F	2.1	0.4	-	-	-	-
C. cladosporioides DL3M2	2.5	0.5	-	-	-	-
C. cladosporioides DL5B	1.6	0.6	-	-	-	-
C. cladosporioides DL5G	1.5	0.9	-	-	-	-
A. versicolor DL6A	5.1	1.8	0.7	-	-	-
A. versicolor DL5A	3.1	1.6	1.0	0.1	-	-
F. fujikuroi DL11A	6.6	2.2	0.8	0.7	-	-
T. harzianum CBMAI 1677	7.8	4.1	3.0	1.8	0.7	0.9

[a]Standard deviation: minimum (0.07 cm) and maximum (0.4 cm).

- not grown.

All experiments in plates were performed in triplicate.

Table 5. Average diameter of fungi colonies isolated from the ascidian *D. ligulum* after 7 d of growth (32° C, 3% malt extract medium) in the presence and absence of PCP.

Strains	Colony diameter (cm)[a]					
	Petri dishes without PCP	Concentration of PCP in Petri dishes with PCP				
		10 (mg.mL^{-1})	25 (mg.mL^{-1})	30 (mg.mL^{-1})	40 (mg.mL^{-1})	50 (mg.mL^{-1})
S. cucurbitacearum DL1M3	6.6	-	-	-	-	-
P. citrinum DL4M3	3.6	-	-	-	-	-
M. crystallina DL6M3	4.2	-	-	-	-	-
D. phacae DL7M3	3.1	-	-	-	-	-
Phoma sp. DL8M3	1.5	-	-	-	-	-
Not identified DL9M3	5.4	-	-	-	-	-
Pleosporales sp. DL1F	2.6	0.4	-	-	-	-
C. cladosporioides DL2F	4.3	1.2	-	-	-	-
C. cladosporioides DL3M2	4.3	1.3	-	-	-	-
C. cladosporioides DL5B	3.1	1.5	-	-	-	-
C. cladosporioides DL5G	4.8	3.4	-	-	-	-
A. versicolor DL6A	6.7	3.6	1.7	0.1	-	-
A. versicolor DL5A	4.8	3.0	1.6	0.3	-	-
F. fujikuroi DL11A	8.0	4.9	1.8	1.6	-	-
T. harzianum CBMAI 1677	8.0	6.6	5.9	2.7	1.4	2.1

[a]Standard deviation: minimum (0.0 cm) and maximum (0.2 cm).

- not grown.

All experiments in plates were performed in triplicate.

Table 6. Average diameter of fungi colonies isolated from the ascidian *D. Ligulum* after 14 d of growth (32° C, 3% malt extract medium) in the presence and absence of PCP.

As shown in Tables 5-7, some strains did not grow in the presence of PCP. In addition, the strains capable of growth in the employed conditions showed that the more concentrated the PCP, the less growth presented in the culture medium. These results indicated that PCP causes a toxic effect on these microorganisms. However, the fact that the majority of the strains subjected to this experiment grew, at least, in one of the tested concentrations indicates that

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Strains	Colony diameter (cm)[a]					
	Petri dishes without PCP	Concentration of PCP in Petri dishes with PCP				
		10 (mg.mL^{-1})	25 (mg.mL^{-1})	30 (mg.mL^{-1})	40 (mg.mL^{-1})	50 (mg.mL^{-1})
S. cucurbitacearum DL1M3	8.0	-	-	-	-	-
P. citrinum DL4M3	6.5	-	-	-	-	-
M. crystallina DL6M3	8.0	-	-	-	-	-
D. phacae DL7M3	6.2	-	-	-	-	-
Phoma sp. DL8M3	2.8	-	-	-	-	-
Not identified DL9M3	7.1	-	-	-	-	-
Pleosporales sp. DL1F	6.1	0.9	-	-	-	-
C. cladosporioides DL2F	8.0	3.0	-	-	-	-
C. cladosporioides DL3M2	8.0	3.2	-	-	-	-
C. cladosporioides DL5B	8.0	3.3	-	-	-	-
C. cladosporioides DL5G	8.0	6.3	-	-	-	-
A. versicolor DL6A	8.0	5.4	2.5	0.2	-	-
A. versicolor DL5A	6.6	4.5	3.2	0.4	-	-
F. fujikuroi DL11A	8.0	7.2	3.1	2.8	-	-
T. harzianum CBMAI 1677	8.0	7.6	7.2	3.8	2.2	3.5

[a]Standard deviation: minimum (0.1 cm) and maximum (0.5 cm).

- not grown.

All experiments in plates were performed in triplicate.

Table 7. Average diameter of fungi colonies isolated from the ascidian *D. Ligulum* after 21 d of growth (32 C, 3% malt extract medium) in the presence and absence of PCP.

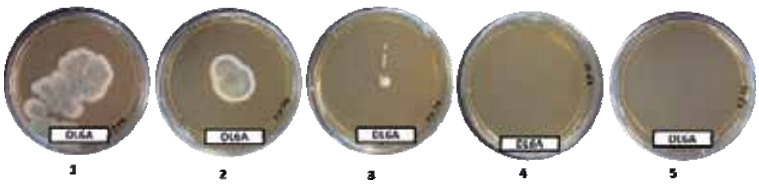
the toxic effect exerted by the compound was not enough to prevent fungal resistance and consequently, biodegradation potential.

According to Bonugli-Santos et al. [129] and Ortega et al. [116], marine-derived microorganisms tend to be resistant when subjected to adverse conditions and can be used in bioremediation techniques because they have enzymes adapted to complex environments such as those with extreme pressure, salinity, and temperature variations. They are able to develop impor-

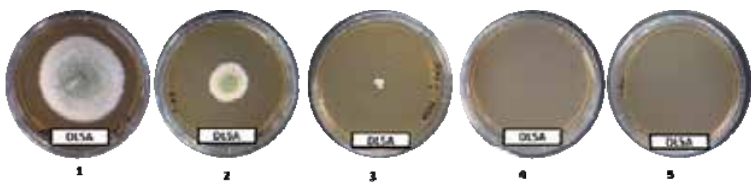
tant metabolic and physiological activities, for example, degradative potential of organochlorine pesticides.

The best adapted strain to the presence of PCP were by DL6A, DL5A, DL11A, and DL2B strains, which were capable to grow at concentrations above 10 mg L^{-1}. The colony diameter of the strains DL6A and DL5A increased in the concentrations of 10, 20, and 30 mg L^{-1}, but the sizes were inferior in comparison with DL11A and DL2B strains in the same concentrations (Figure 6).

Aspergillus versicolor DL6A



Aspergillus versicolor DL5A



Fusarium fujikuroi DL11A

Trichoderma harzianum DL2B

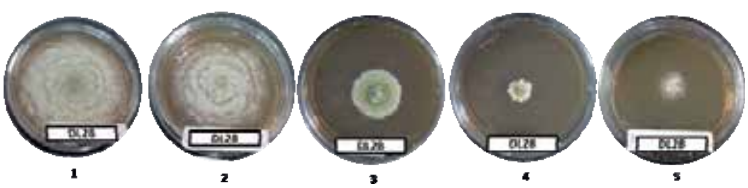


Figure 6. Growth of marine-derived fungi (DL6A, DL5A, DL11A, DL2B) in 3% malt extract agar containing different concentrations of PCP after 21 d at 32° C. The plate numbers of 1, 2, 3, 4, and 5, respectively, correspond to 10, 25, 30, 40, and 50 mg L^{-1} of PCP.

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The most part of microorganisms show increasing growth inhibition in increasing xenobiotics concentrations, especially on those with high toxicity. However, the strain DL2B, which showed the best results in the solid media experiment also grew well at the highest pesticide concentration (50 mg L^{-1}). Thus, this fungus showed resistance to toxicity, adaptive capacity, and biodegradation potential for PCP, even at high concentrations. Creswell and Curl [131] achieved similar results assessing the growth of the fungus *Trichoderma harzianum* in the presence of herbicides such as prometryn, norflurazon, and ciazine. In this work, the fungal growth was significantly increased at the highest dose of the herbicide norflurazon. According to Tomasini et al. [132] fungi need a period of adaptation in high toxicity conditions and, if they were resistant, in the final period of cultivation they tend to grow more. If a group of microorganisms can proliferate efficiently in environments with high concentrations of certain pollutants, it is an indication that these microorganisms have a metabolism adapted to the presence of these contaminants [62]. The increased growth in the presence of the xenobiotic can occur because of its use as nutrient, especially carbon source.

Earlier studies have shown that adaptation experiment with fungi in solid culture medium is a simple and important methodology to screen microorganisms for pesticide biodegradation [133]. After the adaptation experiments with the 15 isolated strains, *Trichoderma harzianum* DL2B (CBMAI 1677) was selected for studies of biotransformation and biodegradation of PCP. In a later study, Vacondio et al. [134] observed that after 7 d of incubation with 20 mg L^{-1} of PCP in liquid medium, it was no longer detected in the presence of PCP in the samples, showing the biodegradation of the pesticide by *Trichoderma harzianum* CBMAI 1677. In addition, the metabolites pentachloroanisole (PCA) and 2,3,4,6-tetrachloroanisole (2,3,4,6-TeCA) were identified. *T. harzianum* was also able to biodegrade PCA and 2,3,4,6-TeCA in liquid medium (Figure 7). These results confirmed the efficiency of marine-derived fungi in the biodegradation of persistent compounds and contributed to the improvement of decontamination techniques. Detailed results were published recently in the literature [134].

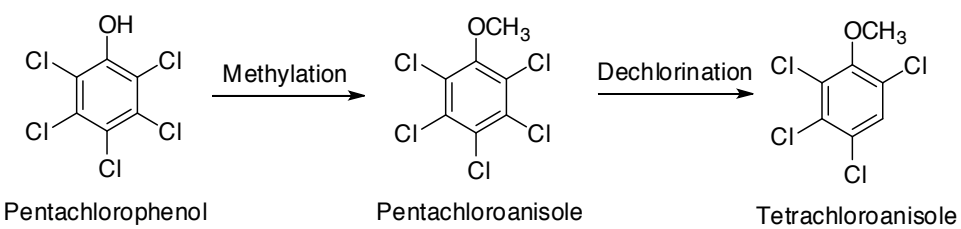

Figure 7. Proposed PCP biodegradation pathway by marine fungus *T. harzianum* DL2B (CBMAI 1677).

4. Conclusions and perspectives

Fifteen marine-derived fungi associated with the ascidian *Didemnun ligulum* were isolated and identified by molecular techniques based on the genes rRNA ITS1 and ITS4. They were tested

for toxicity resistance and biodegradation of PCP, and promising results were obtained. Experiments with these strains using culture medium containing 3% malt extract agar in the presence of PCP enabled the selection of a resistant strain (*Trichoderma harzianum* CBMAI 1677) capable of biodegrading this compound. This fungus grew well in high concentrations of PCP; therefore, showed resistance to its toxicity and potential for the biodegradation of this xenobiotic. This work showed the great potential of microorganisms from marine environment for biotransformation and biodegradation of anthropogenic compounds. The biomethylation and dechlorination of PCP gave the pesticide metabolites PCA and 2, 3, 4, 6-TeCA.

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Chapter 10

The Characteristics of Phytoremediation of Soil and Leachate Polluted by Landfills

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Additional information is available at the end of the chapter

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Abstract

Current landfill regulations provide for the responsible management of solid waste and a safer alternative to the outdated practices of open or illegal dumping. Aside from imparting aesthetic value, natural or planted vegetation on landfill sites has an important role in erosion control and removal of contaminants, and may also be used in leaching treatment. The use of leachate for the irrigation of landfill vegetation reduces its harmful effects, and the reuse of water aids in water conservation. The aim of this study was to search for ways to use leachate water from solid waste landfill sites for irrigation of plant species that normally grow in the wild. The study focuses on the plant species *Alcea rosea* (hollyhock), *Cynodon dactylon* (Bermuda grass) and *Melilotus officinalis* (yellow melilot). Over the 2-year study period, plants were irrigated with tap and leachate water under drought conditions. Wild plant diversity was identified, and the landfill was rehabilitated with various plant species. After the experiment, populations of *Escherichia coli*, total coliforms and fecal coliform bacteria in soil samples were analyzed. We observed that the use of leachate water for cultivation of different kinds of plants affected the density of total and fecal coliforms in the soil.

Keywords: Landfill, Leachate water, Remediation, Coliform bacteria

1. Introduction

With the rapid increase in population and urbanization, solid waste landfills are emerging as a major problematic urban infrastructure. Urban solid waste can be stored both underground and aboveground, but each creates environmental and human health risks.

Those involved in solid waste management continue to search for ways to reduce these risks. Landfills are required to use synthetic and/or soil liners of site-specific thickness and hydraulic conductivity as well as various other safeguards to isolate the waste from nearby groundwater, air and soil [1].

Waste landfill management is an important concern, as negative effects are often caused by discontinuation of traditional management practices. The adverse effects on flora and fauna resulting from changes inland use patterns have been described by landscape ecologists throughout Europe [2], and have been reported by several authors for Mediterranean landscapes such as the montado and agro-silvopastoral systems in Portugal [3], the Tuscany region of Italy [4], the mountainous landscapes in northern Italy [5], and the shrub and woodland areas in Israel and other Mediterranean countries [6]. Aside from imparting aesthetic value, natural or planted vegetation on a landfill has an important role in erosion control and removal of contaminants, and may also be used in leaching treatment. Although phytoremediation of various contaminants has been investigated, the practical application of this technology to the remediation and rehabilitation of municipal solid waste landfill sites has not been sufficiently studied [7].

Areas of bare soil, where vegetation is not present, are open to erosion. The soil of landfills is not generally suitable for growing plants, and protective soil cover is needed. Additionally, planting will have no effect on erosion in the short term without the selection of optimal plants. In our previous study, we examined species best suited for this purpose and found that certain plants could be used for remediation of cover soil [7]. Other studies have found approximately 120 plant species, consisting of trees, shrubs, and grasses, that are appropriate for establishing plant cover in different types of vegetative amelioration [8, 9]. One of the most common plant mixtures used in rehabilitation is grasses and legumes. Grasses are regarded as most appropriate for protection from soil erosion, while legumes grow rapidly, particularly in soils with a low concentration of nitrogen [8, 10, 11].

Leachate is a major issue in landfills and surrounding areas, as it is very harmful to the environment. Leachate interacts with soil and ground and surface waters, and contains high degrees of organic and inorganic pollutants. It is the longest-lasting emission from landfills [12], and therefore the liquid waste causes considerable pollution [13]. One of the most promising methods for mitigating these effects is the use of leachate water for irrigation of vegetation planted on landfills. Research has shown that this technique enables the reuse of polluted water as a result of the remediating effects of plants and microorganisms. The landfill cover soil was irrigated with leachate for maintaining appropriate moisture content for methane oxidation reaction. Municipal solid waste compost was found to be an effective landfill cover material for controlling landfill gas emissions, exhibiting the highest methane oxidation rate [14]. Soil is a habitat for a great number of organisms, but at the same time, it is perhaps the most endangered component of our environment, and can be altered by the different pollutants arising from human activity [15, 16]. Prevention of soil pollution and its harmful effects, however, requires some basic knowledge of the soil characteristics. Soil has a very complex structure and exhibits greatly different properties among various regions [17].

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Trace elements such as metals in contaminated soil also have negative impacts on human health and the environment, and thus their removal is often required. Metals can be stabilized by soil amendments to increase metal adsorption or alter their chemical form [18]. There have been few experiments comparing different in situ remediation treatments under similar environmental conditions or investigating whether all soil components or properties (e.g., microbes, soil fauna, plants, soil retention and colloid stability) are similarly protected. As part of the EU FP7 Greenland project (reference number 266124), we compared the impact of novel soil amendments and their combinations with traditional materials with regard to metal solubility and the response of plants, soil organisms and microbial activity [19]. Soil metal bioavailability is often cited as a limiting factor in phytoextraction (or phytomining). Bacterial metabolites such as organic acids, siderophores, and bio surfactants have been shown to mobilize metals, and the use of microbial inoculants to improve metal extraction has been proposed by several authors [20].

Plants, in combination with their associated micro flora, have a prominent role in remediating soils contaminated with organic pollutants such as petroleum hydrocarbons. Several plant-associated bacteria have the capacity to degrade hydrocarbons, promote plant growth and alleviate plant stress. In many cases, this is due to the fact that inoculant strains may not adequately interact with or colonize plants used for phytoremediation and/or cannot compete with the resident micro flora under certain environmental conditions. However, colonization and the competitive ability of inoculants trains is generally rarely addressed, despite the fact that an understanding of the efficiency of inoculation is essential [21].

In cases when phytoremediation is successfully performed, target pollutants play roles such as enhancing bioavailability by altering the flora or microbial community structure, either through stimulation of existing microbial degraders or through the introduction and establishment of new organisms [22]. For example, surface flow wetlands have proved to be successful in removing selenium (Se) from wastewater. Researchers also reported that constructed wetlands can remove up to 90 % of Se contained in the inflow of oil refinery waste water and up to 80 % from agricultural irrigation drainage [23].

As a result of manmade activities, large areas of soil are contaminated with multiple pollutants, and these high concentrations of pollutants have toxic effects on the environment. Plant microorganism-based technologies can supply a strategy for soil remediation and for the restoration of soil functionality after treatment [24].Soil conditions such as pH, the composition of organic matter and vegetation, and supplements influence soil micronutrient dynamics [25]. Soils may become polluted with high concentrations of heavy metals that are naturally produced by the melting of ore or artificially produced by industrial activities [26, 27].Among pollutants, heavy metals exceeding specific thresholds have been the subject of particular attention because of their long-standing toxicity. Their mobility in the ecosystem and transition through food chains are key issues in environment research [28–33]. Organic amendments may influence soil properties for years after application, as only a fraction of the organic material may be initially degraded or become available to plants and soil microorganisms [34, 35].

Issues with heavy metal contamination at landfill sites have recently been noted. Landfill remediation is generally performed by restoration of the site through the creation of a low

hill planted with plants indigenous to the area. The aim of the current study was to search for ways to use leachate water from solid waste landfill sites for irrigation of plant species that grow wild under normal climate conditions. The study focuses on the plant species *Alcea rosea* (hollyhock), *Cynodon dactylon* (Bermuda grass) and *Melilotus officinalis* (yellow melilot). During the 2 years of the study, plants were irrigated with tap and leachate water under drought conditions. The wild plant species were determined in the hollyhock, Bermuda grass and yellow melilot parcels. After the experiment, populations of *E. coli*, total coliforms and fecal coliform bacteria in the soil samples were analyzed. Results showed that using leachate water to cultivate various types of plants affected total and fecal coliform populations in the soil.

2. Characteristics of the Adana-Sofulu landfill and the plants used for remediation

The Adana-Sofulu landfill is the first major landfill in Turkey at which scientifical planting rehabilitation has been conducted. The population of Adanais 2,026,319, and Adana Province is the political and economic center of the Cukurova region. Field experiments were conducted between 37°03′12″N and 37°03′12.1″N and between35°23′34.3″E and 35°23′35″E [8] (Figure 1).

The city of Adana is in the Mediterranean climate region, and during this study,plants were irrigated with leachate water and tap water during the drought season. Figure 2 shows temperatures and rainfall amounts, including the drought season, for the city of Adana [36]. The irrigation was conducted from May to September.

Until recently, there has been no urgent need for planting of landfill areas. Landfills were generally transformed into small copses within 20 years after the planting of populous species that would grow to a depth of 30 cm from the soil surface. In later years, however, toxic degradation products of waste compounds have created issues that have brought about the need for new planting strategies [37].

Typical plant remediation methods in landfill areas include “grassing” and “grassing and reforestation”. In the grassing method, 20-cm class I and II soil layers are laid on wasteland, and a mixture of *Lolium*, *Dactylis*, *Poa, Agrostis*, *Cynodon*, *Trifolium*, *Medicago* and *Vicia* seeds are planted there. This method of rehabilitation of landfill areas is very common in England. In grassing landfill areas, trees are just an ornamental element. With the grassing and reforestation method, it is important to determine the timing of landfill closures. With old landfill sites, there are no serious problems with the timing of planting. Soil that has been incubated in a fermentation process is laid on the waste site at a thickness of 30 cm, and grassing then begins with the seeding of *Lolium*, *Dactylis*, *Trifolium* and *Poa.* In this process, the grassy vegetation is first laid on the soil layer. After the vegetation has been established, the *Populus* and *Salix* species are planted to begin reforestation [38, 39].

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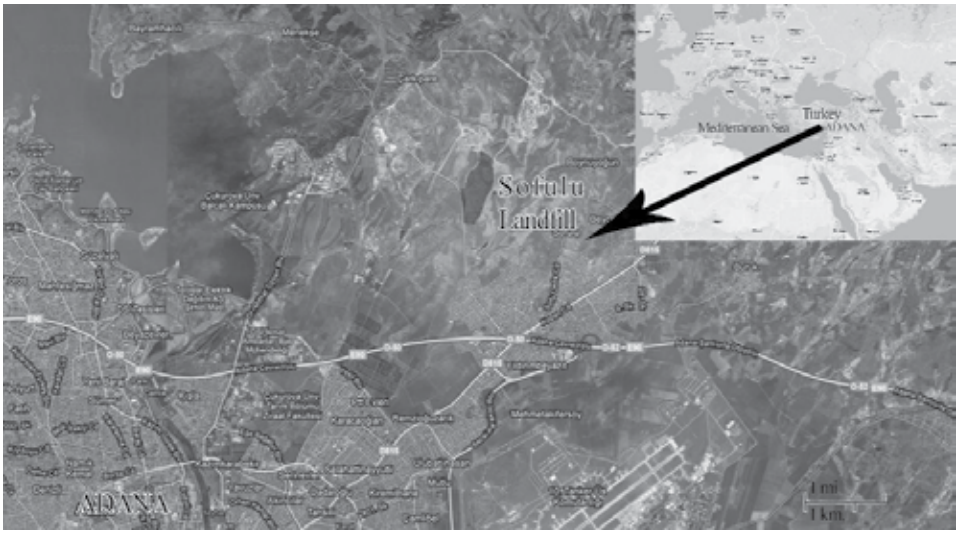

Figure 1. Map showing location of the Sofulu landfill [8]

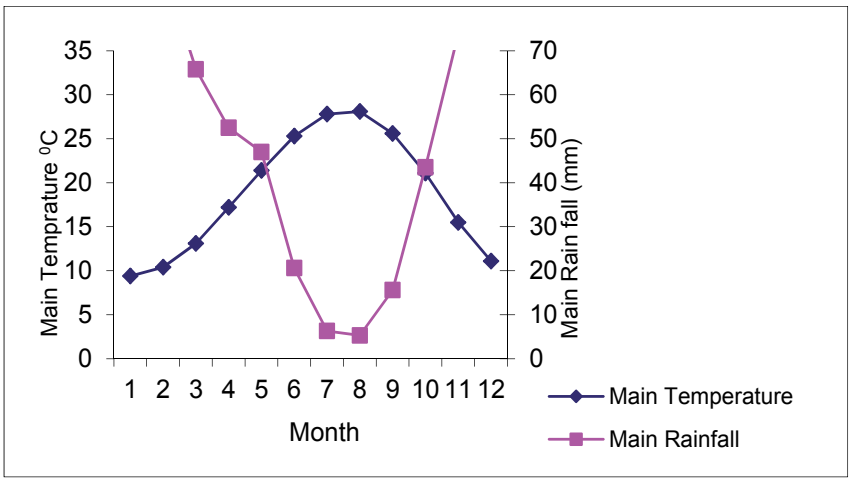

Figure 2. Drought index of study area [36]

In the Sofulu landfill rehabilitation study, three plant species—*Alcea rosea* (hollyhock), *Cynodon dactylon* (Bermuda grass) and *Melilotus officinalis* (yellow melilot)—were selected on the basis of their ecological characteristics including area of spread, the need for soil nutrient sand their tolerance to extreme temperatures. These plants require few nutrients. Each plant species has a different seed weight. To obtain equal numbers of seeds, we followed the recommended weights for plants. Accordingly, 80 g of *A. rosea*, 600 g of *C. dactylon* and 3 g of *M. officinalis* seeds were sown on each parcel. Figure 3 shows photographs of the three plant species [8].

A. rosea flower (hollyhock) (Original)

A.rosea plant group in the plot (Original)

M. officinalis flower (yellow melilot)(Original)

M. officinalis plant group in the plot (Original)

C. dactylon (Bermuda grass) (Original)

C. dactylon covering in the plot (Original)

Figure 3. Photographs of plant species in the plots

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3. Change in vegetation at the planted plots

The experimental design of the study was based on the plot applications, and the study was conducted at three independent plots. Treatment equipment was constructed in the Sofulu waste landfill, which was covered with 30 cm of soil in the B soil horizon. Experimental plots 6 m^2 (2 m × 3 m) in size were prepared, and three types of plants (*A. rose, C. dactylon* and *M. officinalis*) were each planted on 6 plots. The climate in the site is typical of the Mediterranean, and is characterized by a hot, dry period between May and September. The mean annual rainfall in the area is 647 mm, which occurs in the winter and spring seasons, and therefore irrigation is necessary in the summer for any plant growth [8].

The plants in the experiments were irrigated with either tap water or leachate wastewater under drought conditions. Nine plots were not irrigated and were used as controls in order to evaluate the effects of irrigation. The leachate waste water was taken from the basin of the Sofulu landfill site [8]. Figure 4 shows the changes in vegetation at the plots of south waste landfill over 2 years. Few plants were observed in the spring after plant seeds were sown in February. However, growth of *A. rosea* was observed, and the flower bloomed in autumn of that year; *M. officinalis* and *C. dactylon* also grew in the spring of the following year. Moreover, the mixed vegetation of the three plants was found in the next autumn. Subsequently, 41wild plant species were also seen in the experiment parcels. The names of the species are given in Table 1. In this study, gramines (*Bromus arvensis, Lolium temulentum* and *Polypogon monspeliensis)* grew in high numbers in the plot irrigated with tap and leachate wastewater.

Figure 5 shows high numbers of *L. temulentum* and *Silybum marianum* in the plots. In contrast, legumes (*Lathyrus annuus, Psoralea bituminosa, Trifolium campestre* and *Trifolium speciosum*) were not able to propagate insufficient numbers. A study by Arambatsiz et al. [11] was able to achieve significant rehabilitation after mining activities with gramines and legumes, and another study[40] reported that gramines and legumes were grown for the rehabilitation of a degraded study area.

We were able to obtain a sufficient number of wild plants on the landfill and achieve a green landscape when the field was irrigated with leachate water during the drought season. Therefore, the planting of appropriate plants and the use of irrigation by leachate water appears to be an efficient means of rapid landfill remediation as well as removal of pollutants contained in leachate water.

As shown in Table 1, the highest number of wild plant species, 21, was in the *M. officinalis* plots. The *C. dactylon* plots had 17 and *A. rosea* plots had 16 wild plant species with landfill leachate irrigation. *C. dactylon* is so dominant plant species [41, 42], Figure 6 shows that it did not permit to sprawl the wild plants in some plots. *M. officinalis* was also dominant in the spring in some plots.

The first spring in the study area

The second spring in the study area

The first autumn in the study area

The second autumn in the study area

Figure 4. Change in vegetation at the plots of the Sofulu landfill

Lolium temulentum in the plot

Silybum marianum in the plot

Figure 5. High-number species in the plots

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C. dactylon plot

M. officinalis plot

Figure 6. Some of the dominant species in the plots

Wild species	**Waste leachate irrigation**			**Tap water irrigation**		
A.rosea	*C. dactylon*	*M. officinalis*	*A.rosea*	*C. dactylon*	*M. officinalis*	
Ainsworthia trachycarpa		*r*			*r*	
Anagallis arvensis				*r*		
Avena sterilis					*r*	*r*
Alcea rosea		*r*				
Bromus arvensis	*r*					
Capsella bursa-pastoris	*r*			*r*		
Carthamus lanatus		*r*	*r*			
Carthamus dentatus			*r*			
Catapodium rigidum				*r*		
Chenopodium album	***r***			*r*		
Conyza canadensis			*r*			
Carduus pycnocephalus			*r*	*r*	*r*	*r*
Crepis sp.	*r*			*r*		
Cynodon dactylon	*r*		*r*	*r*		*r*
Echinops ritro					*r*	
Helminthothecaechioides		*r*	*r*	*r*		*r*
Hordeum marinum	*r*	*r*	*r*		*r*	*r*
Lathyrus annuus			*r*			

Wild species	Waste leachate irrigation			Tap water irrigation		
Lactuca serriola	*r*	*r*	*r*		*r*	*r*
Lolium temulentum	*r*	*r*	*cd*	*cd*	*cd*	*r*
Melilotus officinalis	*cd*	*r*		*r*	*r*	
Polygonum aviculare		*r*	*r*			*r*
Polygonum equisetiforme						*r*
Polygonum lapathifolium		*r*	*r*			
Polypogon monspeliensis	*r*		*r*	*r*	*r*	*cd*
Psoralea bituminosa					*r*	
Psoralea bituminosa		*r*				
Rumex acetosa		*r*				
Scrophularia canina				*r*		
Senecio vernalis				*r*		
Setaria viridis	*r*	*r*	*r*	*r*	*r*	*r*
Silene colorata	*r*		*r*	*r*		
Silybum marianum		*r*	*cd*	*r*	*r*	
Sinapis arvensis	*r*	*r*	*r*	*r*	*r*	*r*
Sonchus oleraceus		*r*	*r*	*r*	*r*	*r*
Stellaria media					*r*	
Trifolium campestre	*r*		*r*			
Trifolium lappaceum				*r*		
Trifolium speciosum	*r*					
Triticum aestivum L.	*r*					
Verbena sp.				*r*		

cd co-dominant presence (50–80 %), *r* rare presence (1–20 %), *blank columns* no presence

Table 1. Wild plant species grown in plots irrigated with leachate wastewater and tap water [8, 36]

The dominance of plants belonging to four families, viz., Poaceae, Asteraceae, Polygonaceae and Chenopodiaceae, while other species were found to occur only sporadically in the Stockholm, Malmo and Helsingborg landfills of Sweden [43].At the Kodungaiyur and Perungudi dumping grounds in Chennai, India, the dominant plant species recorded were *Acalypha indica*, *Solanum lycopersicum*, *Parthenium hysterophorus*, *C. dactylon* and *Cucurbita maxima* [44].

The wild plant species in the Sofulu landfill experimental plots are shown in Figure 7.

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Figure 7. The wild plants in the Sofulu landfill experimental plots

4. Microbial soil analysis

Over the2-yearstudy period, plants were irrigated with tap water and landfill leachate water during drought conditions (May to September). The landfill leachate water was taken from the collection basin of the Adana Sofulu landfill site. Tables 2 and 3 show the physiochemical, biological and microbial characteristics of landfill leachate water used for the irrigation.

There are three groups of coliform bacteria. Each is an indicator of water and soil quality, and each has a different level of risk. Total coliforms are a large collection of different kinds of bacteria. Fecal coliforms are types of total coliforms that exist in feces, and *Escherichia coli* is a subgroup of fecal coliforms. Total coliform bacteria are common in the environment (soil or vegetation) and are generally harmless [45].

Landfill leachate water showed high concentrations of nitrogen, phosphate and minerals at pH values of 7.9–8.4, and therefore, chemical oxygen demand (COD) and biochemical oxygen demand (BOD) were high, suggesting that landfill irrigation with leachate water may enhance microbial concentration. Microbial density at the plots was thus examined, and these characteristics were analyzed according to standard methods[46].

Parameters	July 1st year	August 1st year	May 2nd year	June 2nd year	July 2nd year	August 2nd year
COD (mg/l)	960	3750	4315	3100	3585	4060
BOD(mg/l)	552	1950	52	47	50	41
pH	8.2	7.9	8.0	8.3	8.1	8.44
NO_2-N (mg/l)	-	-	1.6	2.54	0.40	0.37
PO_4-P (mg/l)	-	-	2.832	7.560	4.880	5.284
Zn (mg/l)	0.2	0.4	0.06	0.04	0.02	0.07
Fe (mg/l)	0.8	1.4	3.5	2.6	0.868	15.2

COD chemical oxygen demand, *BOD* biochemical oxygen demand

Table 2. Landfill leachate water properties [8, 36]

Soil samples were collected from three locations within each treatment parcel and mixed. The soil was used for analysis of fecal total coliforms and fecal coliform bacteria. Each soil sample was mixed with sterile water or physiological saline and divided into three subsamples. A membrane filter technique was used for all bacteriological assays [47]. Microorganisms containing thermo tolerant fecal coliforms transferred onto the membrane were incubated on fecal coliform (M-FC, Difco Laboratories, Inc.) agar medium for 24 h at 44.5 °C, and the number of colonies was counted. In the case of *E.coli*, the cells transferred onto the membrane were incubated in M-FC agar medium containing 4-methylumbelliferyl-β-D-glucuronide for 4 h at

Parameter	July 1st year	August 1st year	May 2nd year	June 2nd year	July 2nd year	August 2nd year	Limit value
Total coliform bacterial count (CFU/100ml)	7×10^3	7.1×10^2	3.2×10^6	2×10^7	-	-	-
Fecal coliform bacterial count (MPN/100ml)	>1100	>1100	>1100	>1100	>1100	>1100	1000
*E. coli*count (MPN/100ml)	>1100	>1100	>1100	>1100	>1100	>1100	1000

CFU colony-forming units, *MPN* most probable number

Table 3. Landfill leachate water microbial properties [8, 36]

35 °C. The colonies generating blue fluorescence by exposure to a longwave UV light (366 nm) were counted as *E. coli* cells. The minimum indication level was approximately 30colony-forming units (CFU)/ml, whereas the maximum cutoff level was 300 CFU/ml.

The mean values were compared between groups using one-way ANOVA. A significance level of $P<0.05$ was used throughout the study. The SPSS Version 10.0 software program [48] was used for these statistical analyses. Duncan's multiple range test was applied to bacterial count data.

5. Effect of landfill and leachate water on microorganisms

Soil is generally a favorable habitat for the proliferation of microorganisms, with micro-colonies developing around soil particles [49]. Bacteria comprise the most abundant group of microorganisms in the soil ($3.0\times 10^6$to 5.0×10^8 per gram of soil), followed by the actinomycetes ($1.0\times 10^6$to 2.0×10^7), fungi ($5.0\times 10^3$to 9.0×10^6), yeast ($1.0\times 10^3$to 1.0×10^6), algae and protozoa ($1.0\times 10^3$to 5.0×10^5), and nematodes (50–200 per gram of soil),with wide differences in the relative proportions of individual bacteria genera found in particular soils[50, 51]. In this study, the total coliform bacteria count varied from $2.1\times10^5$to 7.4×10^5 in landfill soil.

The number of extant bacterial species is thought to range from 3×10^4 to 3×10^6[52], of which only a small fraction have been cultured and identified [53,54]. Mayr et al. reported that due to differences in cultivability among soils, the number of cultivable bacteria per ml inoculums ranged from 0.6×10^3 (forest soil) to 7×10^3 (agricultural soil),with significant variability [55]. *E. coli* and thermo tolerant coliform bacteria are widely used as indicators of soil characteristics. However, many microorganisms, including ente rococci, coliphages, and sulfite-reducing clostridial spores, have been suggested as microbial indicators of fecal pollution [56], and anaerobic digestion processes, if operated properly, have long been known to successfully

reduce the number of pathogens and indicator organisms [57,58]. In a study by Zhang et al. investigating microorganism concentration in raw sewage, the bacterial indicators total and fecal coliforms were enumerated. The average concentrations of total and fecal coliforms were 2.5 $\times 10^7$ CFU/100 ml and 9.6$\times 10^6$ CFU/100 ml, respectively [59].

In the last a few years, researchers have reported higher fecal coliform populations, on a dry solids basis, in centrifugally dewatered bio solids compared to digester effluents [60–67]. Therefore, landfill irrigation by leachate water may represent a key process for landfill remediation and rehabilitation.

Tables 4 and 5 show the microbial density at the plots. As shown in Table 4, the density of fecal and total coliforms increased with the use of leachate water, with almost equal amounts between landfill and clean areas, which suggests that leachate water irrigation is an effective method of landfill remediation. The effects of the plants were also examined, as shown in Table 5. The types of plants affected the amount of fecal coliforms, with the highest concentration in the area planted with *A. rosea* (8.6 $\times 10^3$).

	Fecal coliform bacteria (Count per gram)	**Total coliforms (CFU per gram)**
Factors		
Clean area + tap water	3.2 $\times 10^2$ *a*	2.1$\times 10^5$ *a*
Clean area + leachate water	1.0$\times 10^3$ *b*	7.4$\times 10^5$ *b*
Landfill + leachate water	1.0$\times 10^3$ *b*	5.0$\times 10^5$ *ab*

Data analyzed using Duncan's multiple range test. *b* maximum value, *ab* intermediate value, *a* minimum value. Alpha = 0.05

Table 4. Changes in fecal coliform bacteria and total coliform density in soil for different factors

Plots	**Fecal coliform bacteria (count per gram)**
M. officinalis	6.0$\times 10^2$ *a*
A. rosea	8.6$\times 10^3$ *b*
C. dactylon	1.0$\times 10^3$ *ab*

Data analyzed using Duncan's multiple range test. *b* maximum value; *ab* intermediate value, *a* minimum value. Alpha = 0.05

Table 5. Changes in fecal coliform bacterial density in the soil with different plant species

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6. Conclusions

Solid waste landfill sites pose a significant hazard to natural life, and mitigation of these harmful effects has posed a major challenge. This study proved that the use of leachate wastewater for plant breeding on a landfill in drought weather conditions caused a change in microbial activity in the landfill cover soil. If leachate is used for irrigation, the site should be safely enclosed by fencing, given the negative microbial effect on human health.

Landfill rehabilitation has a positive effect on the landscape. In today's world, waste reduction is critically important, as is the need to ensure that people are able to live in a healthy and beautiful environment. The transformation of brownfield areas into healthy green landscapes using recycled wastewater is an area of research that should be a primary focus of scientists.

This paper examines reasons for considering the use of plant remediation for microbial pollution in landfills. These areas offer the potential for improving biodiversity, and turning these problem areas into opportunities requires the selection of the appropriate plants and the most effective technique. The re-vegetation of landfills can increase biodiversity as well as reduce microbial pollution. This article provides an example of such a strategy for landfills in the Mediterranean climate zone.

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Chapter 11

An assessment of the Causes of Lead Pollution and the Efficiency of Bioremediation by Plants and Microorganisms

Naofumi Shiomi

Additional information is available at the end of the chapter

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Abstract

A rapid increase in mining industries associated with an increase in lead demand has resulted in the problem of lead poisoning. In this study, the initial causes of lead pollution were investigated. The results suggest that soil pollution from lead did not occur in urban and agricultural areas due to the efforts of decreased lead use and an increase in recycling; however, serious pollution locally occurred in the areas where metallurgy and mining industries were present. Therefore, remediation must be carried out in the latter areas. Next, the efficiency of lead remediation by plants and microorganisms in the areas with increased lead pollution was assessed. The plants showing high potential have been developed, and phytoextraction is a promising process. However, a more cost-effective method is necessary to achieve widespread implementation. Thus, a novel remediation method (the landfarming with immobilized microorganisms (LIM) method) to overcome the problem of cost was proposed. The LIM method combines the immobilized technique with landfarming. As the treatment period is short and the lead can readily be recycled from the soil, the LIM method may be a better alternative to phytoextraction for lead remediation.

Keywords: Lead, Phytoextraction, Bioremediation, Landfarming

1. Introduction

Pollution by heavy metals has gradually worsened in Asian, African, and South American countries, partly due to the excessive use of pesticides for growing crops which may in turn lead to the pollution of groundwater and well water. An investigation by the Food and Agriculture Organization (FAO)/United Nations Environment Programme (UNEP)/World Health Organization (WHO) suggests that the number of patients with pesticide poisoning is approximately 1–5 million annually, and several thousand cases are fatal. In some Asian countries, wells reserved for drinking water were dug below the acceptable levels to avoid pesticide pollution, and consequently, many inhabitants developed arsenic poisoning [1, 2]. Rivers and soils are also polluted by industrial wastes in those countries. In particular, wastes from metallurgy and mining industries contain various heavy metal ions, and wastes from leather industries contain cadmium and chromium. These wastes are typically exhausted and discarded in nearby rivers and in the air due to the lack of posttreatment equipment or strictly controlled landfill sites, resulting in detrimental groundwater and soil pollution.

Additionally, environmental pollution by heavy metals has resulted in serious disease. In Japan, four historic cases of metal pollution occurring predominately in the 1950s have been reported, which include "ouch-ouch" disease and Minamata disease. In 1910, a mining company eliminated cadmium waste into a nearby river. The inhabitants who drank directly from the river or ingested food grown along the riverbanks developed cadmium poisoning and exhibited symptoms such as spine and leg pain and fragile bones. In another case of heavy metal poisoning, the improper elimination of methylmercury waste into a nearby river resulted in neuroparalysis (Minamata disease) among the locals. Recent problems due to heavy metal pollution in some countries may be more serious than the previous incidences in Japan. Therefore, it is critical to remediate polluted areas as quickly as possible in order to decrease the risk of disease.

Lead is an important heavy metal because it is widely used to produce electronics, crystal glasses, and batteries. The annual consumption of lead has been increasing at a high rate in China due to the increased demand for cars and power-assisted bicycles according to the rapid economic growth. Recently, a relationship between high blood lead levels and lead pollution in lead mining areas has been proclaimed as a serious problem [3]. Some reports have suggested that many children living in the areas near mining industries developed symptoms of lead poisoning [4]. Therefore, the control of lead emissions and remediation of soil polluted from lead are crucial.

The purpose of this chapter is to assess both the causes of lead pollution and the efficiency of bioremediation by plants and microorganisms. First, the author investigated the causes of lead contamination, and the results suggest that soil pollution from lead occurred particularly in the areas where metallurgy and mining industries were present. Then, the author determined whether phytoextraction is a practical method for lead remediation in these areas. Finally, the author proposed a novel lead remediation process which employs microorganisms. The proposed process, the LIM method, combines immobilization with landfarming. As the

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treatment period in the LIM method is much shorter and lead can be readily collected from the soil, the process may be a viable alternative to phytoextraction.

2. Assessment of the causes of lead pollution

2.1. History of lead use

Lead has been utilized in the production of many products, such as tableware, tubes, and pipes, since the age of Ancient Rome [5] because it can be easily manipulated due to its low melting point and malleability. However, lead poisoning has gradually increased since the 1970s. One major source of lead poisoning has been gasoline. Gasoline containing tetraethyl lead or tetramethyl lead (lead gasoline) was widely utilized to protect car engines. A significant amount of volatile organic lead exhausted by cars triggered air and soil pollution, resulting in the symptoms of lead poisoning. In Japan, the soil near the roads in large cities contained 10–30 g/kg of lead. To remedy this problem, alkylate gasoline was developed and regular and high-octane gasolines were changed to lead-free gasoline in 1975 and 1987, respectively, in Japan. The United States banned lead gasoline sales in 1995 in accordance with the Clean Air Act, and EU banned lead gasoline in 2000. Many other countries (more than 50) have banned or decreased the amount of lead in gasoline [6]. Another major source of lead poisoning was water contamination due to lead pipes. Lead pipes have been used to transport tap water in many countries and were used as service pipes for tap water until the 1970s in Japan. In Uruguay, for example, the inhabitants of old houses had elevated blood lead levels, because most of the old houses used lead pipes for tap water [7]. The dangers of lead poisoning from lead pipes are well recognized, and the use of lead is being reconsidered.

The use of lead in 1996 and 2009 in Japan is depicted in Figure 1A [8]. More than 80 % of lead is utilized for the production of lead-acid batteries for cars and industries. The second most common use is in inorganic chemicals, such as a polyvinyl chloride stabilizer, crystal glass, and paint. A polyvinyl chloride stabilizer containing lead was widely used due to its protective effects in the elimination reaction of vinyl chloride by oxygen. Crystal glass, which contains a high concentration of lead(II) oxide (PbO), is also widely used due to its high degree of transparency and refractive index, similar to crystal. Other common uses for lead include solder for electronic materials, tubes for draining and exhausting, and plates for medical equipment and lagging materials of underground cable. Moreover, lead production is rapidly increasing in some countries due to an increase in the production of lead-acid batteries, especially in China (Fig. 1B) [9].

2.2. Emission control of lead in Japan and other countries

Heavy metal pollution has become an increasing concern in the EU [10]. The amount of waste of electrical and electronic equipment (WEEE) in 2005 was approximately 9 million tons and is steadily increasing at a rate of 5 % annually. The wastes are generally burned or buried without any treatment. If the total amount is calculated based on the assumption that WEEE contains approximately 5 % solder, 22, 500 tons of lead is lost as waste every year. In addition,

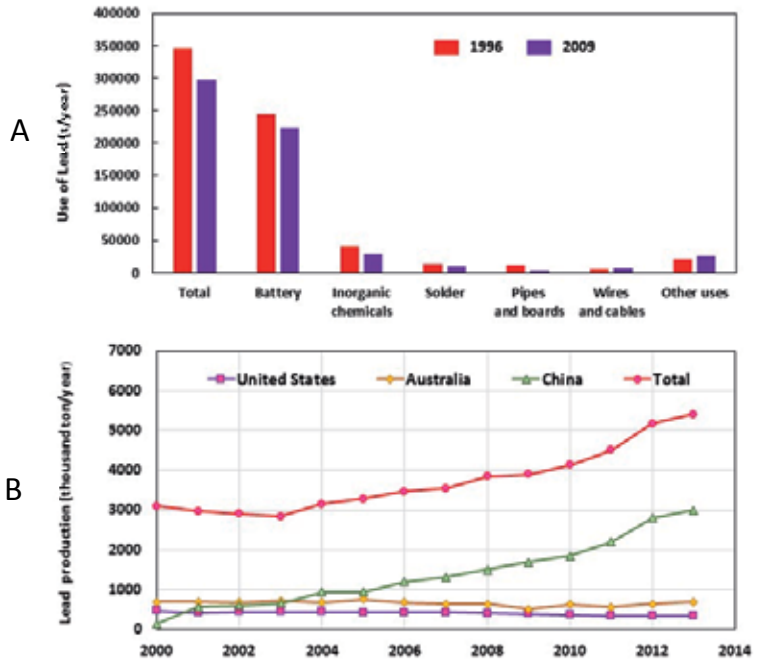

Figure 1. Lead use and lead production. A. The amount of lead used in various products in 1996 and 2009 in Japan. B. Worldwide lead production in leading countries.

it has been reported that lead may leak from WEEE and battery waste by the effect of acid rain. In 2000, the EU developed the "Directive of End-of-Life Vehicle (ELV)" to solve the problem of waste. The directive necessitated the recycling of end-of-life vehicles, and the ratio of lead recycling to collecting lead-acid batteries was improved. A similar recycling system has been constructed in Japan, and lead-acid battery recycling is additionally mandated by law.

The EU also issued a strict directive in 2003 on the restrictive use of certain hazardous substances in electrical and electronic equipment (referred to as the "Restriction of Hazardous Substances (RoHS) Directive"). The directive forbids the use of hazardous heavy metals (e.g., Pb, Hg, Cd, and Cr(VI)) in newly produced products of electrical and electric equipment. The United States also issued a similar law, the "Electric Waste Recycling Act of 2003." In compliance with these directives, lead-free products (solder, glass, and paint) have been developed, and the use of lead-free solder and lead-free paint is now the standard practice in Japan.

Another factor related to lead consumption is polyvinyl chloride consumption. In 2012, global polyvinyl chloride consumption was approximately 36 million tons. Polyvinyl chloride contains approximately 4, 500 ppm of lead stabilizer; thus, 162, 000 tons of lead stabilizer was exhausted as burned ash. The EU issued a strict directive on the management of packaging waste (Directive 94/62/EC), which banned the use of lead stabilizers in vinyl chloride production. To reach the target value of this directive, lead-free stabilizers, such as those containing Ca and Zn, have been developed. Furthermore, old pipes and electrical codes made of polyvinyl chloride are gradually being changed to lead-free ones in homes and industries.

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A schematic illustration of lead recycling is shown in Figure 2. Recycled lead currently occupies 75 % of the total lead produced by metallurgy in the EU, and this ratio is increasing. In Japan, over 90 % of used lead in batteries was collected and reused in 2009. If a 100 % recycling ratio can be obtained and solder, polyvinyl chloride, paint, and glass can be converted to lead-free products as shown in Figure 2, lead emissions should theoretically become negligible.

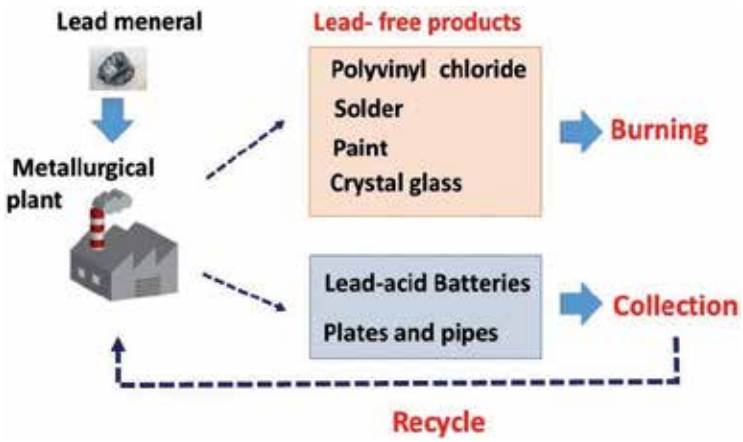

Figure 2. Schematic illustration of lead production and recycling.

2.3. Causes of air and soil pollution from lead

Due to the efforts to decrease lead emissions, air and soil pollution from lead has decreased. According to an investigation from the 1980s to 2000s on lead concentrations in the air and blood by Thomas et al. [11], the lead concentrations decreased after the ban on lead gasoline. Recently, the blood lead levels (BLLs) of inhabitants and the concentrations of lead in the air and soil in urban and agricultural areas have been investigated. The results suggest that the lead concentrations did not exceed nonpoisonous levels, even in the countries in which lead is produced [12, 13].

In the industrial areas where mining and metallurgy occurred, however, a significant amount of unusable lead was discarded in the soil, and effluent from the factories had been directly exhausted to the rivers without any posttreatment removal of heavy metals. In Uruguay, for example, drinking water and the soil are critically polluted by metallurgy industrial wastes because most of the hazardous wastes are dumped in the rivers [7]. Moreover, lead gasoline is still utilized in Uruguay, and many old cubes of tap water are made of lead. The BLLs in many Uruguayans are much higher than in other countries [14]. In mining areas (e.g., Paraná state) in Brazil, 177, 000 tons of waste from metallurgy and mining has remained in the soil for more than 60 years [15]. When lead concentrations in 171 soil portions were analyzed, extremely high concentrations (10, 000–52, 000 mg/kg) of lead were found in the soil near a metallurgy factory. Moreover, the inhabitants near a mining company in the Czech Republic had an average BLL of 37.2 μg/dL, and 40 % of the lead workers in the southwest of Nigeria

had an average BLL of 60 μg/dL [16, 17]. Additionally, the soil near a car battery processing workshop in Kerman City, Iran, was found to contain 5, 780 mg/kg of lead [18]. The BLL of Indians near a residential area was 20–25 μg/dL, and the lead concentration of PM10 (or PM2.5) in the residential area was very high (10–14 mg/m^3) [11, 19].

Soil pollution from lead was not observed in the major cities in China, such as Beijing and Hong Kong; therefore, the BLLs of inhabitants in these cities were normal (4–5 μg/dL) [20-22]. The number of mining and metallurgy factories is rapidly increasing in China due to the increased consumption of lead-acid batteries. However, no formal reports on the lead concentration and BLL have been reported in areas predominantly inhabited by mining and metallurgy factories, such as Zhejiang and Guangdong provinces. According to the WEB report [4], it is suspected that 100, 000 children are suffering due to lead toxicity. Therefore, in China, the areas predominantly inhabited by mining and/or metallurgy industries are thought to contain extremely high lead concentrations which are exhausted into the air, river, and soil.

Another potential cause of soil pollution from lead is a firing range. One of the worst cases of soil pollution from lead at a firing range demonstrated more than 10 kg/kg of lead due to remnant lead alloy bullets. Therefore, lead pollution in firing ranges may be as harmful as in mining areas.

2.4. Effects of lead pollution on the health of inhabitants living near metallurgy and mining areas

The BLL is an indicator of pollution from lead. Lead decreases the IQ value when the BLL is greater than 20 μg/dL [23]. A test to measure the ability of recognition in monkeys suggested that dysgnosia was observed in monkeys with BLLs of 10–13 μg/dL. Moreover, lead toxicity was observed when the BLL exceeded 40 μg/dL. According to recent studies, the BLL should be maintained below 10 μg/dL [24, 25].

The main route of exposure for an elevated BLL is ingestion. The amount of lead that adult subjects ingest from food is generally 20–25 μg/kg and 5–10 % is absorbed. Approximately 100 mg of lead is present in the body. The sensitivity of lead in a child is much higher than in an adult because 40 % of the ingested lead is absorbed. A previous report demonstrated that when more than 5 μg/kg/day of lead was ingested in infants, 32 % of the lead was absorbed, although no accumulation was observed in infants who ingested less than 4 μg/kg/day. The WHO also suggested that the BLL was not increased in those who ingested less than 4 μg/kg/day [26].

The other most common route of exposure is polluted air. Approximately 40–50 % of lead taken in from the nose is absorbed by the lung. The relationship between the concentration of lead in the air and the BLL is shown in Figure 3 (based on the data from the study by Thomas et al. on pollution from lead gasoline [11]). The BLL was found to be strongly correlated with air pollution.

Safety standards are defined to keep the environment safe. In Japan, the lead concentrations in the air and wastewater are below 1 ng/m^3 and 0.01 mg/L, respectively. Moreover, the normal BLL observed in Japanese is 1–3 μg/dL, and the normal concentration of lead in the soil is 15–30 mg/g. The lead concentrations in the areas near mining and metallurgy industries listed in

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Section 2.3 are greater than 1, 000 mg/kg, which are unusually high and dangerous. Therefore, to maintain a safe environment for those living near these areas, an effort to decrease lead emissions and remediation in these areas must be rapidly implemented.

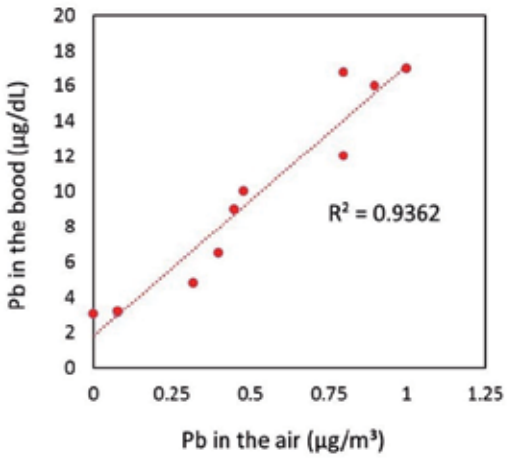

Figure 3. Effect of lead concentration in the air on the BLL. The figure was prepared based on the data from the study by Thomas et al. [11].

3. Assessment of phytoextraction

3.1. Advantages of phytoextraction

The remediation of polluted soil is extremely costly. In the United States, ten billion dollars were invested to remediate soil pollution in the 1990s. According to an approximate calculation of the cost [27], more than 100 billion dollars would be necessary for remediation of polluted soil in the 2000s; thus, an inexpensive process was sought to decrease the investment. Phytoextraction is a remarkable process where heavy metals can be absorbed from the soil and accumulated into plants at high concentrations without the use of expensive equipment. The advantages of phytoextraction also include increased safety and inexpensive running cost compared to physical and chemical methods, such as washing and solidification. Phytoremediation is an expanding market; in the United States and Japan, it is expected to result in 170 million dollars and 800 million yen, respectively.

The plants which contain the highest abilities of absorption are called "hyperaccumulators" and are the most suitable for the phytoextraction of heavy metals. With regard to lead, a hyperaccumulator is defined as a plant that is capable of accumulating greater than 1, 000 mg/kg dry biomass (or 100 times more than other plants) and generally shows a high tolerance to heavy metals. Some prominent hyperaccumulators have been screened and identified [28]. *Stanleya pinnata*, for example, was found to accumulate 3, 000 ppm of selenium in its leaves

when planted on soil containing 6 ppm of selenium [29]. *Rinorea niccolifera* was recently found in Western Luzon, Philippines, and could accumulate an unusually high amount (18, 000 ppm) of nickel by detoxifying with vacuoles [30]. *R. niccolifera* was the most prominent hyperaccumulator, because it was capable of absorbing heavy metals at a value several hundred times higher than other plants.

Additionally, an interesting tree was found in the Sabah Parks in Malaysia. The green sap of the tree contained high concentrations of nickel [31, 32] and could be continuously collected. When the tree was planted in soil containing wastes from nearby mines, a green nickel-rich sap containing 20 % nickel was collected from the trees. The ash of the burned sap additionally contained high concentrations of nickel (10–25 %), which corresponded to 200 kg/ha (2, 000 dollars/ton). Those studies suggest that phytoextraction may be applicable to soil pollution from nickel.

3.2. Phytoextraction of lead by a hyperaccumulator

Hyperaccumulators absorbing lead have been screened as well as hyperaccumulators for other heavy metals. The chief hyperaccumulators introduced in this section are shown in Table 1. *Thlaspi caerulescens* [33], kenaf [34], sunflower [35], *Cannabis sativa* [36, 37], *Tagetes minuta* L. [38], cabbage [39], *Brassica juncea* [40], *Acacia victoriae* [41], and buckwheat are superior hyperaccumulators compared to other weeds and crops, and the following plants are especially remarkable. *A. victoriae* was found to be capable of accumulating 3, 580 mg/kg of lead from a 1, 000 mg/L solution of lead nitrate. *B. juncea* additionally showed a high lead tolerance and could accumulate a significantly high concentration of lead (34, 500 mg/kg). Furthermore, Shinshu buckwheat, an improved breed developed by Shinshu University, was capable of growing in soil containing more than 3, 000 mg/kg of lead and could accumulate 6, 000–10, 000 mg/kg of lead. Buckwheat may be the most suitable plant because it is obtained at a high yield (700 g/ha/year) [42].

Additionally, two types of pteridophytes, *Athyrium yokoscense* and *Pteris vittata*, showed high tolerance to lead. *A. yokoscense* is typically found around mine areas containing high concentrations of heavy metals. It is known as an indicating plant to explore gold veins in Japan, as it lives in a cluster around areas of gold rubbish. One gametophyte of *A. yokoscense* was capable of accumulating high concentrations of lead (10, 000 mg/kg) and showed tolerance to extremely high concentrations of lead [43]. *P. vittata* has been shown to accumulate arsenic. A breed belonging to *P. vittata* could accumulate 16, 257.5 mg/kg of lead and grow in soil containing 92, 900 mg/kg of lead. Additionally, it accumulated 4, 829 mg/kg of lead when it was grown in mine soil for six months [44].

The two types of mosses *Scopelophila cataractae* and *Funaria hygrometrica* were also identified as hyperaccumulators. *S. cataractae*, which is known as "copper moss" in Japan, was found in soil containing high concentrations of copper and could accumulate copper selectively at the cell wall. Moreover, a breed belonging to *S. cataractae* could accumulate lead as well as copper. *F. hygrometrica* additionally showed a very high ability of absorbing lead [45, 46]. The reports by Riken (Japan) suggested that *F. hygrometrica* adsorbed 70 % of lead per dry biomass when an effluent containing lead was supplied to the column containing the moss [47]. An advantage

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of using moss is that it can grow at a fast rate without water. Thus, mosses may be suitable for areas in which there is little water.

Ornamental plants adapted for phytoextraction were also screened. However, one such plant, *Chlorophytum comosum*, could only accumulate 516 mg/kg when 1, 250 mg/kg of lead was supplied in the soil [48]. The advantage of using ornamental plants is that the flowers may be reused after remediation [48, 49]. Moreover, ornamental plants do not show a high tolerance to lead. Therefore, they may be readily adapted for soil pollution in urban places because the lead concentrations in these areas are relatively low and the plants can be used in landscaping.

Trees have interesting characteristics for phytoextraction. The advantage of using a tree is that the root is much deeper, and therefore, it can be applied to the remediation of soil at a depth of 3–5 m. Conversely, the disadvantages are a slow growth rate and low tolerance to lead, although the biomass per land area is high. To overcome these disadvantages, the best trees were screened [50-52] and a combination of aspen and rowan trees [53], a combination of *Ixora coccinea* and *Ficus benjamina* [54], and the use of ornamental trees and timber trees [55] were examined to enhance the remediation efficiency. Additionally, a field trial experiment was performed [56, 57] and the tree showing the highest ability for phytoextraction was *Acacia mangium*, which is widely used in an artificial forest in Malaysia because it is capable of growing well even in nutrient-poor soil and shows a high ability to adapt to its environment. It is known that the forest is the first stage in maintaining air and soil safety by capturing lead in the leaves [58]. The best candidate tree will be one that can effectively transfer lead to its leaves or sap, because leaves and sap can be easily and continuously collected.

Plants	**Species**	**Pb (mg/kg)**
Weeds	*Brassica juncea*	34, 500
Shinshu buckwheat		22, 363
Cabbage		5, 010
Pteridophytes	*Athyrium yokoscense*	16257.5
	Pteris vittata	16257.5
Moss	*Funaria hygrometrica*	1, 000–26, 000
Flower	*Chlorophytum comosum*	516
Tree	*Acacia mangium*	600–800

Table 1. Amounts of lead absorbed by important hyperaccumulators

3.3. Enhancement of the efficiency of phytoextraction using ethylenediaminetetraacetic acid (EDTA)

Lead readily converts to lead(II) oxide (PbO) through contact with oxygen in the air. The resultant PbO slowly converts to inorganic salts of lead, such as $Pb(NO_3)_2$, $PbSO_4$, and $PbCO_3$,

by acid rain containing nitrate and sulfate ions or water saturating carbon dioxide (containing carbonate ions). Table 2 shows the solubility of inorganic salts of lead [59]. The solubility of $PbSO_4$ and $PbCO_3$ is very low. Inorganic salts and free lead ions are adsorbed on particles in the soil by forming a complex with organic compounds contained in the particles [60, 61] and gradually changed to more insoluble compounds by reacting with phosphate. Therefore, the concentration of free lead ions in the soil is extremely low. The rapid absorption by plants is disturbed due to the low concentration of free lead ions or its inorganic salts. The amount of free lead ions must be increased by removing lead salts adsorbed in the soil particles for rapid absorption.

Many methods to increase the efficiency of phytoextraction have been reported [62], and the best method was to supply ethylenediaminetetraacetic acid (EDTA) to the soil. The complex formation constants (pK) of EDTA to Pb(II), Cd(II), Zn(II), and Fe(III) are 18.3, 16.6, 16.7, and 24.2, respectively. Therefore, the lead ions of inorganic salts (or PbO) in the soil positively conform the complex followed by the addition of EDTA (pH < 7) [63]. In a pilot experiment, when the soil containing 1, 935 mg/kg of lead was washed with water containing EDTA, 97 % of lead was extracted from the soil [64, 65].

Compound	Solubility to water g/100g H_2O (20 °C)
Pb	3.1×10^{-5}
PbO	5.04×10^{-3} (α form)
$Pb_3(PO_4)_2$	1.4×10^{-5}
$PbHPO_4$	2.187×10^{-2}
$Pb(NO_3)_2$	54.3
$PbCO_3$	7.269×10^{-5}
PbS	6.77×10^{-13}
$PbSO_4$	3.836×10^{-3}
$Pb(OH)_2$	1.615×10^{-3}
$Pb(CH_3COO)_2$	44.3

Table 2. Solubility of lead and its inorganic salts in water. This table is based on the WEB data [59]

Moreover, the processing time and accumulation in phytoextraction can be drastically shortened and enhanced by EDTA. For example, *Scirpus Maritimus* L. could adsorb 80 % of lead in the root within 60 days when 5 mmol/kg EDTA was supplied [66]. Absorbed amounts of lead in *Zea mays* L. and *Pisum sativum* L. were enhanced to 120 times [67], and Indian mustard *Brassica juncea* had increased absorption when EDTA was supplied [68]. Kos et al. [38] investigated the effect of 5 mmol/kg EDTA or 10 mmol/kg ethylenediamine-N, N'-disuccinic acid (EDDS) on lead accumulation in various plants, and the results suggest that *Cannabis sativa* contained the best phytoremediation potential (26.3 kg/ha) and accumulated 1, 053 mg/

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kg of lead. Furthermore, for cabbage (a high-biomass crop), the accumulation and treatment period was enhanced (5, 010 mg/kg) and shortened, respectively, when 3.0 mmol/kg EDTA was supplied for seven days [40]. When buckwheat was cultivated in soil containing 13, 032 mg/kg of lead and EDTA and citric acid were supplied for two months, 22, 363 mg/kg of lead was accumulated in the shoots and leaves [69]. These results suggest that EDTA is effective for increasing the absorption of lead in plants.

However, there are several disadvantages for the use of EDTA. One is the low degrading characteristic of EDTA [70]. EDTA supplied in the soil exists without degradation for a long period of time and slowly degrades to diketopiperazines, which are toxic compounds. To solve this problem, EDDS, which has a higher biodegradability than EDTA, has been used [71-73], although the complex formation constants (pK) of EDDS are lower than that of EDTA and an unfavorable exchange between lead and the others often occurred [67, 68]. Another disadvantage is the elution of other metal ions following the use of EDTA. The addition of EDTA in the soil causes the leakage of important minerals (e.g., Mg and Ca) necessary for the plant growth, and the effluent containing toxic ions (e.g., Pb, Hg, and As) pollutes the groundwater [74]. Therefore, EDTA or EDDS must be applied to the soil at the lowest effective concentrations.

3.4. Improved procedure for the phytoextraction of lead

Two devises have been proposed to enhance the concentration of free lead ions or its inorganic salts. One is an electro-phytoextraction process. Electro-phytoextraction is performed under an electric field and can enhance the performance. For example, the absorption in ryegrass was enhanced when 1.0 V/cm of DC electrical field was given to the soil in a vertical direction [75], and the absorption in *Brassica juncea* was enhanced when an electric field was given at four times over 30 V [76]. Additionally, when 1.0 V/cm of AC electrical field, as well as DC, was given to rapeseed (*Brassica napus*) and tobacco (*Nicotiana tabacum*), the absorption of Cd and Pb in the shoots was enhanced [77].

Another device used to improve phytoextraction is the use of acid [78, 79]. The concentration of free lead is increased when the pH of the soil is more acidic (near pH 5). The solubility of lead phosphate, which is an insoluble compound, was enhanced by a 0.15 M citric acid solution [80], and oxalic acid was the best at enhancing the solubility of pyromorphite ($Pb_5(PO_4)_3Cl$), an insoluble phosphate [81, 82]. Because microorganisms can secrete various acids, such as acetic, citric, and lactic acids, the soil pH may be decreased by supplements of these microorganisms. The effects of urea [83] and other chelate compounds [84, 85], as well as the effect of acids, were examined. The cells of *Rhizobacteria* could enhance the concentration of free lead by secreting siderophores [86] and aided in plant growth by secreting indoleacetic acid (IAA), a plant growth factor. Such microorganisms are referred to as "plant-growth-promoting rhizobacteria" (PERG) [87, 88].

3.5. Transgenic approach to improve the phytoextraction of lead

The improvement of a hyperaccumulator via gene manipulation is the most effective way to enhance its ability. Transgenic plants have been energetically developed since the 1990s

[89-92]. Transgenic plants which could increase the volatility of heavy metals or decrease the toxicity of heavy metals may be the best candidates because the remediation process can be continuously carried out without removing the plants. Transgenic *B. juncea*, for example, expressing the cystathionine gamma-synthase gene of *Arabidopsis thaliana* L. could convert selenium to volatile dimethylselenium [93, 94], and a plant expressing the methylmercury lyase gene decreased the toxicity by reducing methylmercury to mercury [95]. However, vaporization is not acceptable for lead because methylated lead diffuses into the air and exhibits a high toxicity as previously described.

Thus, the following two mechanisms have been proposed. One method is to enhance the number of compounds capable of combining heavy metals, such as metallothionein, glutathione, and phytochelatin. For example, the absorption efficiency of transgenic *B. juncea* expressing adenosine triphosphate sulfurylase, glutamyl-cysteine synthetase, and glutathione synthetase genes was 4.3 times higher than that in the wild plant [96]. Moreover, the accumulation in *Nicotiana glauca* expressing phytochelatin synthase was also enhanced [97]. The other mechanism is to obtain a high lead tolerance by enhancing the transport into the cell and vascular membranes. Higher tolerance and accumulation of Zn, Mn, and Cd were realized by the plants transformed with a zinc transporter (*ZAT* or *AtMTP1*), *ShMTP*, *CAX2*, *AtMHX* [89-91] or the *AtNramp, AtPDR8*, and *AtATM3* genes of ABC transporters [98, 99]. For lead accumulation, the following transgenic plants were studied: tobacco plants expressing the calmodulin-binding protein gene of *Nicotiana tabacum* (*NtCBP4*) [100] and *Arabidopsis* plants expressing the *ZntA* [101], which codes for the zinc transporter in *E. coli*, and an enhanced accumulation of lead, as well as other heavy metals, was observed. The yeast *YCF1* gene codes for a transporter of vacuolar storage of Cd/Pb. *A. thaliana* expressing the *YCF1* gene showed a high resistance to Cd and Pb and accumulated those heavy metals [102]. Transgenic poplar trees expressing the *YCF1* gene also developed a high resistance to Cd and Pb [103]. Moreover, a study conducted by Mizuno et al. showed that transgenic *A. thaliana* had longer roots (2.5 times longer) and a higher (3–14 times higher) accumulation of lead when the *FeMRP3* gene of buckwheat was expressed in *A. thaliana*.

3.6. Assessment of efficiency of phytoextraction

The author assessed the efficiency of phytoextraction in contaminated soil by lead. The most advantageous point of phytoremediation is its profitability. By the author's rough estimate, the income generated by the phytoremediation process is approximately 340, 000 dollars/ha for cases where it is assumed that (1) pollution is present at 1 m in depth and 10 g/kg of lead is contained in the soil (density: 1.7), (2) 100 % of the lead is extracted from the soil, and (3) the price of lead is 2, 000 dollars/ton. However, the approximation of the necessary expenses is much higher according to some reports and are estimated to be as high as 300, 000–5, 000, 000 dollars/ha (lowest estimation: 2, 500–15, 000) [104]. The difference in the costs suggests that further efforts are necessary to decrease the expenses in order to improve the application of phytoextraction.

The high necessary expenses of phytoextraction are due to the low yield per treatment period and the time-consuming posttreatment heavy metal recycling from the biomass. Even in

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buckwheat, which is one of the best hyperaccumulators for lead, the amount of lead absorption is only 20 kg/year (dual cropping), when the yield and adsorption ability are assumed to be 1 t/ha and 10 g/kg. Moreover, the plant absorbing lead must be dried, burned, and extracted to recycle the lead. The cost for those operations accounts for half of the total cost. Consequently, a hyperaccumulator with a fast growth time and a fast absorption rate, as well as a high accumulation ability, is required to overcome the problem of high necessary expenses.

Furthermore, the effect on the environment should be considered. Indigenous species do not necessarily have a superior ability for phytoremediation, although the use of indigenous species is acceptable [105]. The planting of a nonnative hyperaccumulator often changes the natural flora or may destroy the indigenous species because hyperaccumulators have an increased ability to adapt to the environment. This is particularly true for transgenic plants. Therefore, special consideration for the environment and a general consensus in the society are necessary.

In conclusion, further efforts to decrease the necessary expenses must be undertaken for the widespread use of phytoextraction, although phytoextraction is a remarkable procedure for recycling lead in the soil. For example, some weeds are capable of easily and rapidly reproducing even when most of the shoots and leaves are removed. The advantages of using a weed for phytoextraction include the following: (1) lead can be continuously obtained from the leaves and shoots, (2) the growth and absorption can be completed in a short period of time, and (3) the posttreatment is inexpensive. Therefore, the author suggests that the ideal phytoextraction process includes the use of such a weed.

4. Assessment of the remediation of lead by microorganisms

4.1. Microorganisms adapted for lead absorption

Some microorganisms contain a high ability to adsorb and absorb lead [106-108], and the mechanism through which microorganisms achieve this can be classified into four mechanisms (Table 3). The first mechanism is the absorption of lead by secreting extracellular polymers. The typical extracellular polymer is polysaccharide, which rapidly combines lead at a high affinity. *Halomonus* sp. [109], *Staurastrum* sp. [110], *Bacillus firmus* [111], *Paenibacillus jamilae* [112], and *Pseudomonas* sp. are known as microorganisms that secrete polysaccharides. The polysaccharide secreted by *B. firmus* cells, for example, was capable of adsorbing 98.3 % of Pb at an optimum pH, and 2 g/L of polysaccharide produced by *P. jamilae*, an endospore-forming bacillus, specifically adsorbed 230 mg/g of lead.

The second mechanism is adsorption at the cell wall. *Bacillus* sp. [113], *Pseudomonas aeruginosa* [114], *Synechococcus* sp. [115], *Saccharomyces cerevisiae* [116], and fungi (such as *Aspergillus flavus* [117] and *Corollospora lacera* [118]) were highly efficient in the adsorption of lead; the amounts of lead absorbed by *P. aeruginosa*, *S. cerevisiae*, and *C. lacera* were 123, 250, and 270.3 mg/g dry biomass, respectively.

The third mechanism is the binding of lead inside the cell through phytochelatins, metallothioneins, and siderophores. Phytochelatins are produced by some microorganisms, such as *Schizosaccharomyces* sp. Metallothioneins produced by *Bacillus* [119], *Streptomyce*s sp. [120], and *P. aeruginosa* [121] are capable of combining with Pb(II), although metallothioneins typically combine with copper or zinc ions. Moreover, the yellow-green fluorescent pyoverdine and pyochelin produced by *Pseudomonas putida* KNP9 [122] and *P. aeruginosa* PAO1 [123] were capable of combining with Pb(II).

The fourth mechanism is the precipitation of lead inside the cell. For example, *Staphylococcus aureus* [124], *Vibrio harveyi* [125], and *Enterobacter cloacae* [126] are capable of producing $Pb_3(PO_4)_2$, $Pb_6(PO_4)_6$, and $Pb(PO_4)_3Cl$, respectively, by binding with phosphate, and sulfur-reducing bacteria produce PbS [127].

Furthermore, using genetic techniques, these abilities could be enhanced. For example, the amount of Cd accumulation was seven times higher when the phytochelatin synthesis gene of *Schizosaccharomyces pombe* was expressed in *P. putida* KT2440. The genes associated with metallothioneins, siderophores, and phytochelatins were precisely examined [128-131], and recombinants expressing these genes at a high level may be useful for enhancing the accumulation of lead.

4.2. Novel bioremediation process of heavy metals using microorganisms

As introduced in Section 4.1, some microorganisms showed a high ability for lead accumulation (> 300 mg/g), which was higher than the plant hyperaccumulators. However, few microorganisms have been utilized for the bioremediation of soil polluted by lead. The reason is that the collection of such microorganisms from soil followed by adsorption is extremely difficult. If the microorganisms can be readily collected from the soil, then bioremediation with microorganisms becomes an effective process. Thus, the author developed a novel bioremediation method which combines the immobilized technique with landfarming, referred to as the landfarming with immobilized microorganisms (LIM) method.

The LIM method consists of four steps, shown in Figure 4. In the first step, the beads (approximately 0.35–0.4 cm in diameter) immobilized with microbial cells which demonstrate a high ability of absorption to lead are prepared and mixed with contaminated soil while plowing the field by the landfarming process. The soil is oxygenated by the operation, and the immobilized microbial cells contained in the beads are activated by the increased oxygen supply. In the second step, the plowed soil containing the cell beads is incubated for a defined period. The lead is absorbed (or adsorbed) by the microbial cells during this period. In the third step, the soil containing the beads is collected, and the beads are separated from the soil with sieves of adequate mesh sizes (0.25 and 0.50 cm). Thus, the beads can be easily collected. In the fourth step, lead absorbed (or adsorbed) in the cells is extracted with a small amount of nitric acid. The separated soil by the sieves is recycled by returning it to its point of origin, and the beads flowed by extraction are reused in the next remediation.

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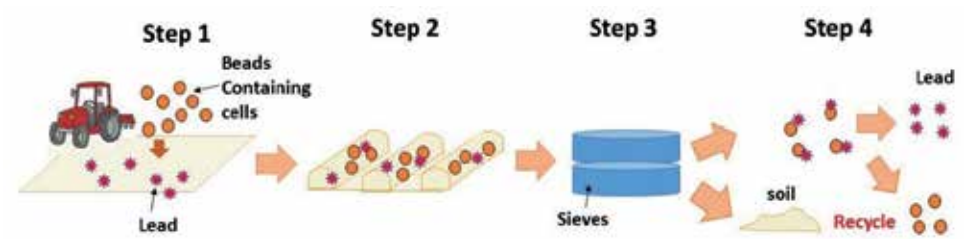
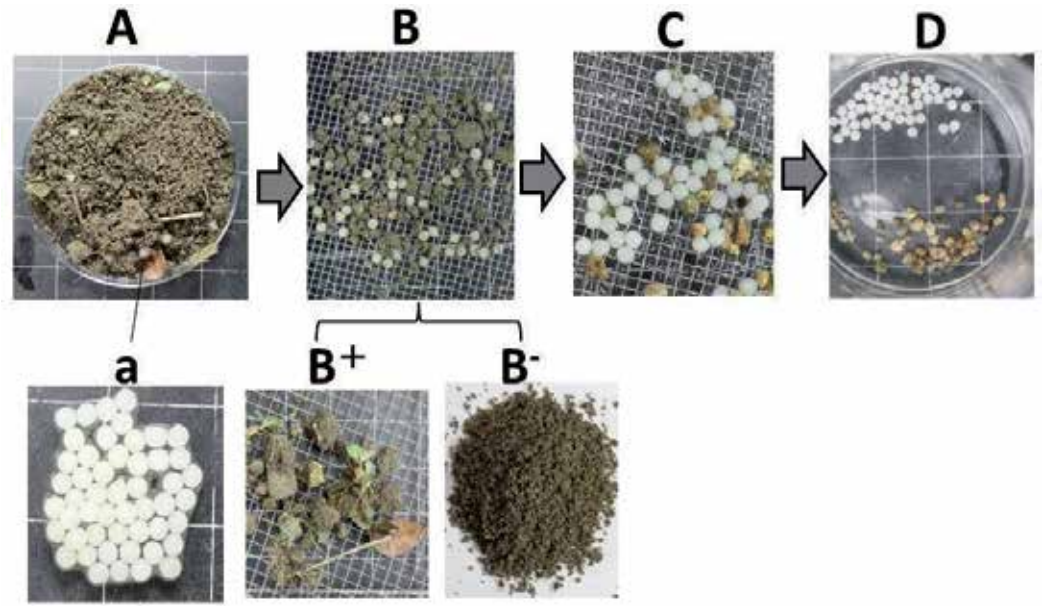
Mechanism	Compound for absorption	Typical species
Absorption by extracellular polymer	Polysaccharides	*Halomonus* sp.
		Staurastrum sp.
		Bacillus firmus
		Paenibacillus jamilae
Absorption by cell wall	Peptidoglycan	*Pseudomonas aeruginosa*
		Bacillus sp.
		Synechococcus sp.
		Saccharomyces cerevisiae
		Aspergillus flavus
		Corollospora lacera
Binding within cells	Phytochelatin	*Schizosaccharomyces* sp.
	Metallothionein	*Bacillus* sp.
		Streptomyces sp
		Pseudomonas aeruginosa
	Pyoverdine	*Pseudomonas putida*
	Pyochelin	*Pseudomonas aeruginosa*
Precipitation within cells	$Pb_3(PO_4)_2$	*Staphylococcus aureus*
	$Pb_6(PO_4)_6$	*Vibrio harveyi*
	$Pb(PO_4)_3Cl$	*Enterobactor cloacae*
	PbS	Sulfur-reducing bacteria

Table 3. Various mechanisms to adsorb or absorb lead by microorganisms



Step 1: The field is plowed and the soil is mixed with beads of the immobilized cells. Step 2: The plowed field is incubated for a defined period to absorb lead. Step 3: The beads are separated using sieves. Step 4: Lead is extracted from the beads, and the resultant beads and sifted soil are recycled.

Figure 4. The LIM method.



A. Soil containing the beads. Fifty beads were mixed with 50 g of soil (Hyoko, Japan). a. Beads made of alginate gel (0.38 mm in diameter). B. The soil and beads were separated by sieves with mesh sizes between 2.5 mm and 5 mm. B+ and B-. The impassable soil through the 5 mm mesh sieve and the passed soil through the 2.5 mm mesh sieve are shown. C. The washed soil and beads in water. D. Extraction of lead from the beads by nitric acid.

Figure 5. Schematic illustration of the LIM method using alginate gel beads.

The beads made of alginate gel are most suitable for the LIM method because (1) they can be easily and inexpensively produced at a uniform size, (2) they can immobilize microbial cells at a high density (approximately 100–1, 000 mg dry cells/cm^3), and (3) they have an appropriate hardness. If it is assumed that the immobilized cell can accumulate lead at 300 mg/g, one bead should be able to absorb 3–30 mg of lead. Additionally, if cells secreting polysaccharides are utilized, each bead may be applied several times for remediation because polysaccharides are not leaked from the beads. Therefore, alginate gel beads can be utilized as a superior absorbent of lead.

Figure 5 shows the separation experiment of the beads and soil from the soil and beads mixture (Fig. 4, Step 3). The experiment was performed to examine the separation efficiency of the beads; the absorption by the immobilized cells was not conducted. Fifty beads (0.38 mm in diameter) were mixed with 50 g of soil (Fig. 5A) and separated with 2.5 mm and 5 mm mesh sieves. All beads were collected between 2.5 mm and 5 mm mesh sieves (Fig. 5B) and the soil was eliminated from the 2.5 mm mesh sieve by rinsing with water (Fig. 5C). Heavy metals were extracted by a small amount of nitric acid (Fig. 5D). Following extraction, the beads may be reused in the next remediation because the beads are not broken by the operation and can be easily separated with small stones (Fig. 5D).

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The advantage of the LIM method is that the processing time is short and the beads may be readily collected and reused for the extraction operation. Therefore, the LIM method has a high potential for remediating the soil contaminated by lead. This method may become an important process for remediation of soil in the future, although the proper procedure and efficiency of the LIM method must be further investigated.

5. Conclusion

A review of the estimated causes of pollution from lead and the following results were discussed. The principal use of lead is due to the production of lead-acid batteries; other uses include inorganic chemicals, solder, tubes, and boards. Following the RoHS Directive, the recycling percentage of lead-acid batteries and crystal glass has gradually increased, and solder, paint, and vinyl chloride containing lead have been converted to lead-free products. Over 80 % of lead is currently recycled in the developed countries. Therefore, serious pollution from lead is low in urban and agricultural areas. However, life-threatening levels of pollution from lead exist in areas containing metallurgy and mining industries in Asia, Africa, and South America due to the dumping of wastes in the rivers and in the air without any posttreatments. The soil in these areas should be promptly remediated.

Next, the author estimated whether phytoextraction is a practical method for remediation. Many native or transgenic plants showing a high accumulation ability to lead were screened or developed, and the ability could be enhanced using EDTA and microorganisms. Therefore, phytoextraction is a promising method for remediation. However, further improvement of the method is necessary due to the long processing time and low capacity (biomass/planted area).

Finally, the author proposed a novel process for remediation using microorganisms. Few microorganisms have been used for the bioremediation of polluted soil by heavy metals because it is often exceedingly difficult to collect the microorganisms from the soil after absorption. The LIM method, which is proposed by the author, is the improved landfarming process which employs beads with immobilized cells. In the LIM method, the processing time is short and the beads may be easily collected from soil with sieves after absorption. Therefore, the LIM method has high potential and may become the ideal process for the remediation of soil contaminated by lead.

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The pollution of soil and groundwater by heavy metals and other chemicals is becoming a serious issue in many countries. However, the current bioremediation processes do not often achieve sufficient remediation, and more effective processes are desired. This book deals with advances in the bioremediation of polluted soil and groundwater. In the former chapters of this book, respected researchers in this field describe how the optimization of microorganisms, enzymes, absorbents, additives and injection procedures can help to realize excellent bioremediation. In the latter chapters, other researchers introduce bioremediation processes that have been performed in the field and novel bioremediation processes. Thus, the readers will be able to obtain new ideas about effective bioremediation as well as important information about recent advances in bioremediation.

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