Chapter 1
Stepwise Strategies for the Bioremediation of Contaminated Soils: From the Microbial Isolation to the Final Application

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R. Prasad, E. Aranda (eds.), *Approaches in Bioremediation*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-02369-0_1

1.1 Introduction

As a matter of fact, increasingly widespread soil pollution has caused vast areas of land to become unproductive for agriculture and hazardous for both wildlife and human populations (Garbisu and Alkorta 2003). This pollution can be caused by a wide range of contaminants as heavy metals, pesticides, xenobiotics, hydrocarbons, wastes from mining industry, agrochemicals, poor disposal of hazardous waste, and poor management of urban solid wastes, among others.

Soil is a nonrenewable resource because the rates of formation and self-restoration exceed the human life span. Despite the inability to completely recover a soil to its previous state to a contamination event, a partial recovery can be carried out by microorganisms or microorganisms' products, such as polymers and enzymes.

Bioremediation processes consist in using microorganisms and/or manipulating the metabolic activities or compounds produced by them, to eliminate, decrease, or, at least, turn pollutants into less aggressive chemical species, minimizing environmental commitment by facilitating the biodegradation processes (EPA 2004).

1.2 Niche: Selection of the Right Place for Microbial Isolation

1.2.1 Bioremediation Strategies and Microorganisms

When microorganisms are subjected to an environment that contains toxic concentrations of a recalcitrant element or compound, special mechanisms to allow them tolerate those conditions are developed. In that context, the concept of resistance can be defined as the microorganism's ability to survive stress, using response mechanisms that are activated by the presence of the stressor (Gadd 2010).

As not all microorganisms are able to grow, survive, or tolerate every polluted environment, to ameliorate contaminated soils, each kind of pollutant requires specific microorganisms (Fuentes et al. 2010). So, to ensure this, the most common

strategy is to use autochthonous microorganisms, which means to isolate microorganisms from the same polluted environment to be recovered (Fuentes et al. 2011). These species will have already been able to adapt to such stressful conditions in that environment by developing different intra- and/or extracellular mechanisms (Prasad 2017, 2018) (Tables 1.1 and 1.2).

Examples of the strategies developed by microorganisms are excreting the metals through transport systems and sequestering compounds through cytosol agents which may bind to the metal and detoxicate the interior of cells (Majzlik et al. 2011). Bioaccumulation, performed by bacteria and fungi (Suarez 2002; Elangovan et al. 2006; Chojnacka 2010), is a mixture of intra- and extracellular mechanisms, because it requires metabolic activity of living cells involving intracellular sequestration, extracellular precipitation, metal accumulation, and complex formation. It comprises two stages: metabolism-independent passive biosorption (e.g., physical and chemical adsorption, metal ion exchange, chelation, coordination, surface complexation, and microprecipitation) and metabolism-dependent active bioaccumulation (e.g., transport of metal ions into microbial cells including complex permeation, carrier-mediated ion pumps, and endocytosis) (Ma et al. 2015, 2016).

Other adaptive mechanisms proposed are the organic acid excretion to make metals soluble (Gadd 1999) and developing cytoplasmatic protection mechanisms through inclusion bodies which retain a great number of metal cations (González and Jensen 1998; Gentili et al. 2006), biomineralization (Lowenstam 1981; Pérez-González et al. 2011), and EPS formation (Ryder et al. 2007).

Table 1.1 Intracellular mechanisms involved in detoxification aim to reduce metal burden in the cytosol

Mechanism	Description	Organism	Reference
Organellar location/ chelation	Metal sequestration in intracellular compartments (mainly cell vacuole) or binding to nonprotein thiols and transport into intracellular compartments	Ectomycorrhizal Fungi and bacteria	Bellion et al. (2006) and Ma et al. (2016)
Efflux or enzymatic detoxification	Exclusion of metal chelates in to the extracellular space	Bacteria and fungi	Ma et al. (2016)
Biologically controlled mineralization (BCM)	Formation of minerals on or inside organic matrices or vesicles within the cell	Bacteria	Frankel and Bazylinski (2003) and Moraga et al. (2017)
Bioleaching	Extraction of heavy metals from sludge, sediments, and soils, therefore alleviating metal phytotoxicity directly or indirectly through various metabolic activities such as oxidation, reduction, and complexation	Bacteria and fungi	Mulligan and Galvez-Cloutier (2003), Kletzin (2006), Navarro et al. (2013), and Ma et al. (2016)
Bioexclusion	Active transport or efflux of toxic metals from the cytoplasm	Bacteria	Ma et al. (2016)

Table 1.2 Extracellular mechanisms involved in detoxification mainly in avoidance of metal entry

Mechanism	Description	Organism	Reference
Complexation	Secretion of extracellular polymeric substances (EPSs) protects against harmful effect of metal	Bacteria	Guibaud et al. (2005), Zhang et al. (2006), Vodnik et al. (2008), Slaveykova et al. (2010), and Hou et al. (2013)
	Production of insoluble metal-sorbing glycoprotein (glomalin) reduces metal mobility or sequesters metals		
Precipitation	Formation of insoluble precipitates by the rapid reaction of inorganic acid with certain dissolved metals (such as Cu, Fe, Zn, and Pb)	Bacteria	Park et al. (2011) and Zhou et al. (2013)
Chelation	Binding of organic molecules (particularly di- and tricarboxylic acids), metallothionein, and siderophores within fungi walls and outer layers	Ectomycorrhizal fungi and bacteria	Bellion et al. (2006) and Dimkpa et al. (2008)
Redox transformations	Enzymatic redox reaction to convert a metal ion into a nontoxic or less toxic state	Ectomycorrhizal fungi and bacteria	Ma et al. (2016), Chatterjee et al. (2009), Olegario et al. (2010), Majumder et al. (2013), and Oves et al. (2013)
Biologically induced mineralization (BIM)	Mineral formation as a consequence of changes in the supersaturated system due to the intake or excretion of different metabolites and to the contribution of substances that can act as nuclei of crystallization, such as cell surfaces (cell wall, membranes, excreted organic compounds, cell debris, etc.)	Bacteria	Lowenstam (1981) and Moraga et al. 2017
Release of metabolites	Mobilization, which helps the removal from contaminated soils, of metals facilitated by metabolites such as metallophores, biosurfactants (BSs), sophorolipids, and rhamnolipids	Bacteria	Mulligan et al. (2001), Juwarkar et al. (2007), Venkatesh and Vedaraman (2012), and Deicke et al. (2013)

As different species may have different adaptive mechanisms, the use of autochthonous consortiums or mixed cultures is a widely applied strategy to enhance remediation processes (Fuentes et al. 2011, 2013a, b). In particular, extreme environments are a major source of microbial diversity for potential uses in biotechnological processes and products; thus, to study in different bioremediation processes their microbial diversity would enhance the challenges in understanding

their involvement in (Nesme et al. 2016). Direct access to the genomic DNA of coexisting microbial species can give a better understanding of evolution, lifestyle, and diversity of the microorganisms and expose more of the hidden world of microbes (Krause et al. 2008). In order to find out "who is there" and with which frequency, metagenomics rises nowadays as a very powerful tool.

Metagenomic analysis of nucleic acids provides direct access to the genomes of the "uncultivated majority." Using amplicon surveys or metagenomic approaches for comparing soil microbial communities and correlating indicator species with specific environmental perturbations or specific land usage tend to produce statistically valid trends whether the selection of the different methods minimizes the bias of subsequent results or not (Nesme et al. 2016). Thus, as suggested by Lynch and Neufeld (2015), the objectives should include minimally biased methods (or combinations of methods) for soil characterization, differentiating between active soil microorganisms and dormant cells (and extracellular DNA). They should also cover seasonal variability, quantification of the full extent, and scale of functional diversity and microbial soil taxonomy, together with the diversity of "biosphere rare" microorganisms that typically dominate assessments of soil microbial diversity.

Step-by-step, from sampling a contaminated soil to identifying potential bioremediation, strategies are listed below (Fig. 1.1):

1. Sampling
2. Isolation of microorganisms and metagenomic analysis
3. Physicochemical soil analysis
4. Identification of toxic tolerance levels by isolated bacteria
5. Analysis of tolerance mechanisms
6. Identification of compounds of interest involved in tolerance
7. Identification of genes or proteins differentially expressed in the presence of toxic

In the next sections, each one will be developed.

1.3 Microbial Isolation

The standard sampling design for the characterization of a soil differs from that carried out for microbial isolation. In the first, it is of utmost relevance to collect sufficient samples to have a representative overview of the situation of the soil, i.e., as close to reality as possible. However, in the case of soil sampling for microbial isolation, with the final aim of bioremediation or biotechnological purposes, the priority is to target the site and sampling point, minding the final application. Such is the case, for example, of studies that focus on the search of enzymatic activities in extremophiles to optimize existing industrial processes. One of the best-known examples is the application in biotechnology of the thermostable DNA polymerases Taq, Pfu, and Vent, all of bacterial origin (from the thermophile bacteria *Thermus aquaticus*, *Pyrococcus furiosus*, and *Thermococcus litoralis*, respectively).

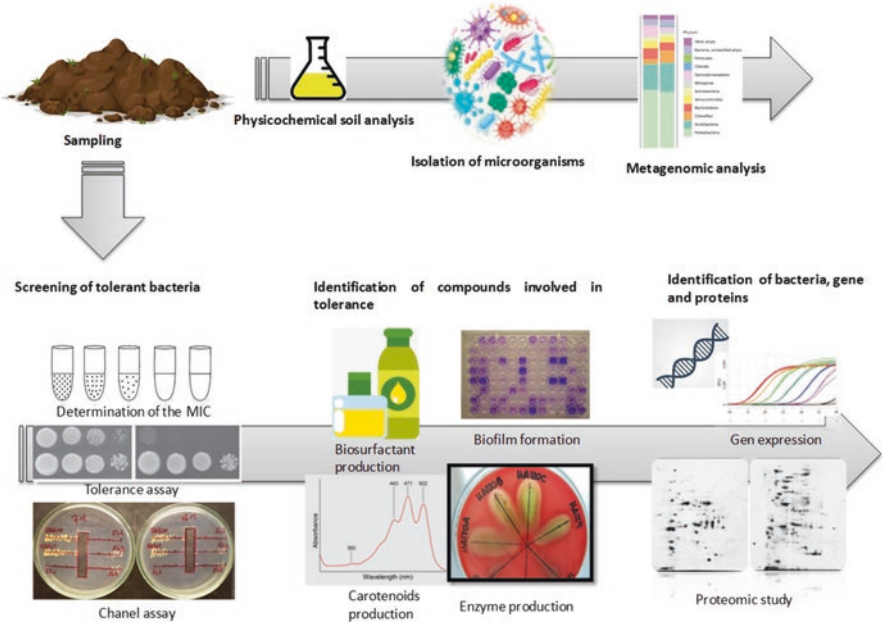

Fig. 1.1 Scheme of the steps to follow from sampling a contaminated soil to identifying potential bioremediation strategies

1.3.1 Soil Sampling Design

The soil matrix is a complex heterogeneous living system, influenced by many biotic and abiotic factors. For example, on a hillside, there will be differences between the soil in the lower, middle, and upper part of the slope. However, on the surface of different points on flatland, it is possible to find differences as well. The soil is heterogeneous in space and time, mostly as the product of variations in topography, original material, environmental forming processes, and human intervention. Thus, this heterogeneity must be considered in the planning of the sampling.

From a horizontal point of view (i.e., in the extension of the surface), the soil has a patchy nature. Slight variations of terrain elevation, soil texture, or surface vegetation can produce essential differences in the environment for the soil microbial community. Also, from a vertical point of view (i.e., in depth), the different horizons that comprise the soil will present specific properties regarding air and water movement and overall transport of specific compounds through the soil profile.

Therefore, always minding the final goal of the isolates, a specific point in the selected location can be targeted for sampling. For such selection, the premise to consider is that the bacterial community in a specific point is the fittest for the prevailing conditions there. This strategy was followed in several bioremediation studies that aimed to degrade hydrocarbon compounds (Zheng et al. 2018) and pesticides (Campos et al. 2015) or sequester toxic compounds such as those with boron or

heavy metals (Moraga et al. 2014b; Gillan et al. 2017). Also, when the aim is to positively enhance plant-microorganism interactions, this approach is followed by isolating bacteria from the plant's surfaces like the rhizosphere and phyllosphere and also from the interior of the plant in the search for endophytic bacteria (Karnwal 2017).

For rigorous systematization of the sampling procedure, normalization guidelines for soil quality assessment can be obtained from the International Standardization Organization (ISO). They provide guidance on the collection, handling, and storage of soil samples on its norms (both part of the former ISO 10381-6:2009) ISO 18400-105:2017 and ISO/FDIS 18400-206 (under development).

1.3.1.1 Sample Collection and Transportation

Once the specific point and depth of sampling are determined, the collection and transport of samples must be planned considering two essential requisites: (1) avoid cross-contamination and (2) ensure the integrity of the bacterial community until its arrival to the laboratory for processing. The minimal set of elements, for the collection of soil aiming to the isolation of aerobic mesophilic microorganisms, include black plastic bags, transparent plastic bags, tags, a cord, a plastic knife, a shovel, gloves, a nice box, and a cooling agent (ice or ice packs). These elements can be complemented with a GPS (to mark the exact location of the sample collection point) and portable devices to measure environmental or soil variables (temperature, humidity, and pH in a soil suspension in water), depending on the objective of the study and the availability of time and resources. Also, it is useful to keep a log of the information about the site: details on topography; weather conditions at the moment of sampling, e.g., if it rained the day before; and any other data relevant to the study, e.g., surface cover (crops, vegetation, roadside, brine, frost, burning signs, oil) and land use history (application of pesticides, oil spill, mining tailing), must be taken account, among others.

Briefly, before collecting the sample, clean the ground surface by removing those elements that are not part of the soil (stones, salt crust, not decomposed organic matter such as branches, leaves, and any other strange material), and dig a hole with the shovel. The hole should be at least 10 cm deeper than the previously set sampling depth and of a diameter such that allows comfortable hand and arm movement inside. Then, inside the hole, scrape a section of the wall with a plastic knife to remove the soil surface that was in contact with the shovel. Scraping is required to eliminate any metal contamination or toxicity from the shovel, and it also provides visibility for qualitative identification of the horizons in the sampled soil profile, as well as the texture, structure, humidity, the presence of macrofauna, and others. To collect the sample, first clean the hole as thoroughly as possible removing the loose soil, and place a black plastic bag inside. Make 5-cm-deep cuts in the sides and bottom (at the previously determined sampling depth) of the scraped section in the hole's wall, and place one hand against it, cutting the back carefully with the knife to obtain a slice of soil. Place the soil slice in the bag with a tag identifying the

sample, and close it with a cord to ensure gas diffusion. Place the black bag inside a transparent bag with a second tag, and close again with a piece of cord. Finally, place the bagged sample inside the cold box with a refrigerating agent to transport it readily to the laboratory for processing.

The operation described can be more or less complicated depending on the texture of the soil (heavy soils are too hard, and light texture soils are too lose), the water content, the structure, and the density. Therefore, other sampling tools like a manual or electrical augers and soil core samplers can be more appropriate to drill the soil and collect samples. There is a large variety of augers in the market depending on the specific characteristics of sampling site, for example, augers designed for sand, clay, and mud and of one piece, open-faced, screw type, or gauged; cutters for hard soil; and others. These tools, commercialized in different sizes, many times can be customized according to the requirements of the study.

The amount of sample to collect will be given by the accessibility to the sampling site, the transportation and storage capability, fundamentally the minimum required amount to carry out the previously planned analysis, and the availability of the sample. The two latter must be well identified, since sometimes a sample can only be taken at a specific moment or opportunity, after which the surrounding conditions vary, changing as well the intrinsic properties of the sample itself.

Although during the microbial isolation the culture conditions will mimic those of the sampling site, it is useful to maintain them during the sampling and transportation. Thus, the surrounding conditions should resemble those in the site of origin regarding temperature and oxygen availability. Thence, specific elements and conditions for manipulation and transportation are to be considered for particular cases. For the isolation of anaerobic microorganisms, airtight containers and gas (argon or a mix of CO_2 and N_2) are required to remove the air in them (Wiegel et al. 1979; Song et al. 2000; Kim et al. 2013). Instead for the isolation of psychrophiles, a portable cooler with temperature control is necessary (Vishnivetskaya et al. 2000; Panikov and Sizova 2007).

1.3.1.2 Samples Processing and Storage

The samples can be either processed in situ or taken to the laboratory for storage. For immediate processing after the collection, the soil is placed in containers with specific culture media that will allow the survival of the microbial community targeted (Kim et al. 2013). Thus, the samples can be stored in vials with culture media for further microbial extraction and isolation from the soil. The most common procedures to process the samples in the laboratory, after collecting and transporting them, involve freezing or drying for storage of the soil in cold temperatures ranging from −70 to 4 °C.

The processing of the samples for frozen storage only involves sieving (<4 mm) and fractioning of the soil. Then the homogenized soil is placed in thin layers inside zipper bags that can be readily stored by freezing at −20 °C or ultra-freezing at −70 °C (Wallenius et al. 2010). For the storage of dried soil, the sample is let to dry

at room temperature for 24 h. To avoid contamination, a large volume of soil can be spread as a layer inside a laminar flow hood or similar or by fractioning the soil and placing it inside Petri dishes loosely covered. Then, after removing any remaining organic debris and stones, the dry soil is sieved through a 2 mm mesh to be stored in black plastic bags at 4 °C until use (Romano-Armada et al. 2017). In the case of aggregates larger than 2 mm, the soil is ground before sieving. The bags, filled with 1 kg of dry homogenized soil (maximum), are closed with a cord and must not be stacked one over another. These conditions allow gas diffusion inside the bag, which will preserve the aerobic microbial community in the soil sample.

There are some advantages on freezing the soil over drying it regarding the preservation of the soil's enzymatic and microbial community activity (Wallenius et al. 2010). However, in the case of microbial isolation, it is advisable to process the samples and extract the microorganisms of interest as soon as possible, regardless the storage method. If the target of isolation does not comprise the aerobic mesophilic microbial community, specific care, concerning temperature and oxygen availability, must be considered for the manipulation of the sample in the different steps (Vishnivetskaya et al. 2000; Panikov and Sizova 2007).

1.3.2 Isolation of Microorganisms and Metagenomic Analysis

1.3.2.1 Metagenomic Analysis

A total soil metagenomic DNA extraction must be performed and then an amplification of the hypervariable regions V1-3 and V4-7 of the 16S rRNA belonging to the bacterial microbial community using PRBA63f and UN518r primers. Then a hypervariable region search in databases of other report community members must be done (Tekere et al. 2011).

1.4 Bacterial Population Extraction from the Soil Matrix

Overall, the extraction of microorganisms from the soil consists of the suspension of the latter in a liquid. However, all the procedures aim to accomplish the dispersion of the soil and their microbial aggregates with minimal reduction in the microbial viability. According to the literature, there is little consensus in the ratios of soil-to-liquid for the suspensions (1 g in 10 ml; 5 g in 15 ml, 20 ml, 50 ml, and 250 ml; 10 g in 90 ml and 100 ml; 30 g in 90 ml) and also in the used liquids (0.9% sodium chloride (NaCl), 1% sodium hexametaphosphate ($(NaPO_3)_6$), 0.1% peptone water, specific culture media) (Wiegel et al. 1979; Vishnivetskaya et al. 2000; Panikov and Sizova 2007; Kim et al. 2013; Moraga et al. 2014a; Gillan et al. 2017; Romano-Armada et al. 2017; Zheng et al. 2018). The many combinations for the extraction can encompass steps of suspension, agitation, centrifugation, sonication, and the addition of chemical dispersing agents.

The most straightforward procedure is the extraction for direct plating and isolation from the first extract. Briefly, to break down the soil aggregates without the addition of a chemical dispersing agent, the soil suspension can be agitated at 250 rpm for 30 min with a subsequent 10 min sonication to disperse the microbial aggregates (Romano-Armada et al. 2017). Then the soil particles can be let to settle for 30 min (Moraga et al. 2014b), or a centrifugation step can be carried out to eliminate the soil and collect the microbial cells from the supernatant (Gillan et al. 2017; Zheng et al. 2018).

Also, in the processing of the sample previous to the isolation step, culture media enrichment can be carried out to apply selective pressure to favor the growth of the microorganisms with the targeted metabolic activities. The soil samples and its extract are suspended in culture media added with the compounds to be degraded as sole carbon or nitrogen sources (Song et al. 2000; Zheng et al. 2018) or with heavy metals (Moraga et al. 2014b; Gillan et al. 2017).

Some procedures or reagents can be avoided by taking into account the targeted bacterial community to isolate. While the $(NaPO_3)_6$ method is time and cost effective for dispersing soil aggregates for fast texture analysis, it may be too aggressive for the fraction of microorganisms highly dependent on the soil organic carbon. Also, the saline solution (0.9% NaCl) can interfere with the desired microbial isolation. Both solutions can be replaced by 0.1% peptone water or by sterile distilled water (as long as the salts in the soil sample allow maintaining the osmotic balance in the solution).

1.4.1 Bioremediation-Oriented Microbial Isolation

Following the microbial extraction from the soil samples, the supernatants are plated in culture media for isolation. Aliquots (0.1–1 ml) of the serial dilutions of the supernatants are spread plated in specific media, commonly enriched with the compound to be remediated, culturing under the environmental conditions of interest. Most soil isolates are cultured in the dark at close to neutral pH and between 25 and 30 °C (Song et al. 2000; Moraga et al. 2014b; Gillan et al. 2017; Zheng et al. 2018). Once the microbial colonies were identified, they must be isolated by streak plating following the aseptic technique. The single colonies must be re-streaked until the purity of the culture can be confirmed by microscopy or observation of the colonies morphology, after which they can be stored for further studies.

Note that the isolation procedures are conducted in solid media, which allows differentiation of single colonies. Therefore, particular attention must be paid to the used gelling agents in the case of extremophiles isolation regarding temperature and pH. While for the isolation of thermophiles that grow above 60 °C (Wiegel et al. 1979) agar-agar can be used since its fusion temperature is 85 °C, for the isolation of acidophiles, the use of agarose is necessary (Nancucheo et al. 2016). However, in the case of cryophiles that grow under the freezing point in supercooled media, the

addition of up to 20% glycerol is required as an antifreezing agent (Panikov and Sizova 2007).

Also, in the case of anaerobic microorganisms, the oxygen must be removed from the sealed vials for proper culture (Wiegel et al. 1979; Song et al. 2000; Panikov and Sizova 2007; Kim et al. 2013). Moreover, the culture manipulations should be held inside the anaerobic chambers (Kim et al. 2013).

1.4.2 Selection and Characterization of Microorganisms for Soil Bioremediation

The selection and subsequent characterization of adequate microorganisms are the main steps that will determine the success of the entire bioremediation procedure and the recovery of the affected soil.

Once the selection is done, a good characterization and evaluation in different conditions are particularly important. This is due to the fact that selected microorganisms have, as final destination field application, a much larger scale than laboratory, and therefore all their characteristics and the expected behavior in the conditions they will face in field must be perfectly known. In this sense, while some authors consider that the first part of the selection should be to identify strains which can degrade the target contaminant, others suggest that it is essential to base the selection on a priori knowledge of the population dynamics and their distribution in sampled habitats in the first place, to ensure the success of the bioremediation technique. After that, the second phase of the selection procedure would be to identify strains which can degrade the target contaminant, having better persistence and colonization rates (Thompson et al. 2005).

Beyond the taken position, once the pure cultures are obtained, it is necessary to select the microorganisms of interest according to the contaminant to be remediated. After this, it should proceed to the characterization of the selected microorganisms, in search mainly of those that present greater tolerance and degradative capacity or well looking for specific molecules that promote and favor bioremediation, such as enzymes, biosurfactants, etc., depending on the contaminant of interest. Regarding the characterization, it can be mentioned in the first place basic techniques such as morphological characterization and, later, metabolic characterization, among others.

1.4.2.1 Selection of Microorganisms for Bioremediation

Selective Enrichment This involves the selective culture of some strains over others from polluted samples, with a metabolic advantage, for example, using the target contaminant as the sole carbon or nitrogen source. The technique results in the selection of strains that express the required degradation or metabolic capacity

(Thompson et al. 2005). Its importance lies in the fact that it is a very selective technique where nutritional conditions are manipulated, using the contaminant of interest as energy source as example.

Selection by Channels in Plates This technique involves cutting small channels in the solid medium of the plate. Then, in the channel, a solution with the contaminant of interest in the desired concentration is discharged, and isolated strains are sown around it. The inoculation pattern could vary making the channel in the middle of the plate or at both or one side. The solution diffuses through the channels, and the strains grow more or less near the channels according to their tolerance to the pollutant (Moraga et al. 2014b; Martinez et al. 2018).

Selection by Agar Diffusion Test This technique allows the identification of tolerant strains to the target contaminant. It consists in using paper filter discs embedded in the contaminant of interest in different concentrations. In the first place, the plate has to be filled with the 50% of the final volume and is left until the medium partially solidified. The remaining 50% is subsequently inoculated with the microorganisms of interest and poured, forming a second layer, over the first semi-gelled layer on the plate. After that sterile filter paper discs, impregnated in a solution of the contaminant of interest, are placed on the agar. The appearance of an inhibition growth halo around the filter paper disc is expected in the case of susceptible bacteria (Rosas Hernández 2009).

1.4.2.2 Characterization of Selected Microorganisms

Morphological Characterization

Bacterial colonies that grew on agar plates can be initially characterized according to their morphology in each particular media. Macroscopically, size, shape, pigmentation, elevation, surface, appearance, edges, etc. can be determined in solid medium, and if it is an evaluation in liquid medium, the formation of pellets, exocellular polymeric substances (EPS), and precipitates can also be visually evaluated. In this step, microbial movement can also be evaluated in semisolid medium.

Regarding microscopic evaluation, it allows differentiating structural characteristics of bacteria, for which different stains are commonly used:

Gram staining: This technique is used to classify bacteria into two large groups, Gram-positive and Gram-negative, on the basis of differential staining with a crystal violet-iodine complex and a safranin counterstain. This is due to differences in the cell wall structure: while Gram-positives retain the complex after the treatment with alcohol and appear purple, Gram-negative organisms decolorize and after the treatment with safranin become pink. This staining also facilitates the determination of cell morphology (Madigan et al. 2009). As known, the age of the reagents for the stain as well as that of the microorganisms is crucial for the proper classification.

Endospore staining: Some bacterial genres, like *Clostridium*, produce resistance structures called endospores. The position of endospores in the cell (in the center or in the pole) may be indicative of some species. Malachite green dye is used for this staining (Rosas Hernández 2009).

*Staining with methylene blue***:** This is useful for the identification of morphological characteristics as bacterial motility; for such staining the smear is stained with blue methylene for 1 or 2 min and then washed with tap water and observed at the microscope (Rosas Hernández 2009). On the other hand, it can be used as a quick method to know the viability of yeasts.

Metabolic Characterization

After the general morphological description is done, it is important to proceed to the metabolic characterization in the search of the specific molecules, and if possible the mechanisms involved, that allow microorganisms to degrade or modify the target contaminant or help them in the decontamination process.

Enzymatic Characterization

The process of bioremediation depends on microorganisms that enzymatically attack the pollutants and convert them to innocuous products (Karigar and Rao 2011). However, some enzymatic products can be toxic, in some cases indeed more toxic than the initial pollutant. In this sense, the search can be oriented toward enzymes that play a key role in the degradation of specific pollutants.

Hydrolytic Enzymes These enzymes disrupt major chemical bonds in the toxic molecules resulting in the reduction of their toxicity (Karigar and Rao 2011). Due to their intrinsic low substrate specificity, hydrolases play a pivotal role in the bioremediation of several pollutants including insoluble wastes, oil spill and organophosphate and carbamate insecticides, cellulose materials, chitin, keratin, kraft pulp, sewage sludge, starch materials, polyacrylate, and polyurethane, among others (Gianfreda and Rao 2004). This group of enzymes includes proteases, carbohydratases (ascellulases, amylases, xylanases, etc.), esterases, phosphatases and phytases, lipases, and proteases, among others (Karigar and Rao 2011).

Dehydrogenase and Oxidoreductase These enzymes take part in the metabolism (catabolic and also anabolic reactions) in microbial cells. During catabolism they oxidize organic compounds by the transfer of electron pairs from a substrate to nicotinamide adenine dinucleotide (NAD+) or nicotinamide adenine dinucleotide phosphate (NADP), and then they also act during anabolism to regenerate the electron transporters. Different investigations revealed that oxidoreductase enzymes were involved in removing toxic heavy metals like chromium or cooper. There are microorganisms resistant to chromium and showed Cr (VI) reduction to be undetectable (Irazusta et al. 2018). Experiments with cell-free extracts, mitochondrial

and extracellular yeast extract of *Cyberlindnera jadinii* M9 and *Wickerhamomyces anomalus* M10, indicated that a soluble intracellular type of enzymes was responsible for Cr(VI) reduction. These enzymes usually show a NADH/flavin oxidoreductase activity and have the ability to reduce chromate as a secondary function (Irazusta et al. 2018).

Laccases They are a type of ligninolytic enzymes, polyphenol oxidases, that catalyze the oxidation of various phenolic and non-phenolic compounds, particularly those with electron-donating groups such as phenols (−OH) and anilines (−NH2), by using molecular oxygen as an electron acceptor (Karigar and Rao 2011). They participate in the cross-linking of monomers, involved in the degradation of a wide range of industrial pollutants. Their low substrate specificity makes these enzymes interesting for bioremediation not only for industrial pollutants and effluents but also for wastes of paper, pulp, textile, and distillery industries. Also, and particularly in soils, these enzymes, in conjunction with peroxidases, enhance the natural degradation of xenobiotic and organochlorides, conversion and mineralization of polycyclic aromatic hydrocarbons and pentachlorophenols, oxidation of aromatic hydrocarbon and pentachlorophenol, and degradation of herbicides as bentazon and diuron, among others (Duran and Esposito 2000). They are encoded by different genes and expressed in different organelles and can be readily detected by gel electrophoresis (Chandra and Chowdhary 2015).

Peroxidases These enzymes are oxidoreductases which catalyze reactions in the presence of hydrogen peroxide and act in the oxidation of a variety of organic and inorganic compounds (Duran and Esposito 2000). They are useful in decomposition of pollullants: textil dye degradation, ligning degradation, sewage treatment, and also as biosensor (Bansal and Kanwar 2013). Considering their bioremediation importance, three main enzymes have been studied the most, lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and versatile peroxidase (VP), most of them due to their high potential to degrade toxic substances in nature (Karigar and Rao 2011).

Biosurfactant Production

Biosurfactants are amphiphilic compounds produced by microorganisms with pronounced surface and emulsifying activities (Singh et al. 2007). They consist of a hydrophilic and a hydrophobic group, and due to this structure, they are able to increase the surface area of hydrophobic water-insoluble substances (Pacwa-Płociniczak et al. 2011). They act diminishing the surface tension (σ) between two liquids with different polarity, making the emulsification of one liquid in the other easier (Iustman et al. 2013).

Their importance on bioremediation lays on the fact that biodegradation rate of a contaminant depends on its bioavailability to the metabolizing organisms, and they increase the water bioavailability of such substances and change the properties of

the bacterial cell surface (Pacwa-Płociniczak et al. 2011). In this sense, biosurfactants have two main functions: in one hand, they increase substrate availability, and in the other hand, they enhance biodegradation by mobilization, solubilization, and emulsification (Ahmad Khan et al. 2015).

These compounds are specially used for decomposition of heavy metals and hydrocarbons including polynuclear aromatic hydrocarbons (PAHs) (Rangarajan and Narayanan 2018). They are also used in the oil industry to clean up oil spills and to enhance oil recovery (Jones 1997).

Siderophore Synthesis

These molecules are specially used for detoxification of heavy metals as a consequence of chelation, since they form stable metal-ligand complexes and influence the metal mobility in the environment (Naik and Dubey 2013).

1.5 Molecular Omics Technologies in Microorganisms' Selection and Characterization

Traditionally, studies on bioremediation of target pollutants were made using conventional isolation strategies based on culturing microorganisms. However, these techniques exclude all microorganisms that cannot be cultured, which represent a huge proportion. Culture-independent methods as molecular techniques in general and omics technologies in particular are extremely important to discover a wide range of unidentified pollutant-degrading microorganisms that could have crucial roles in bioremediation (Watanabe 2001). In this sense, "omics"-based approaches can help investigate the genome, transcriptome, proteome, and metabolome of single organisms and even mixed communities, opening new opportunities to decipher molecular mechanisms in pollutant biodegradation (El Amrani et al. 2015).

1.5.1 Metagenomics

It involves the study of entirely genetic information contained within an environmental sample, making possible to identify the functional potential and the taxonomic identity of all organisms in the sample, but without information about the actual active members of the community (El Amrani et al. 2015). Metagenomic approaches often take two forms: targeted metagenomics or shotgun metagenomics. In targeted ones, environmental DNA is extracted, and the gene of interest is PCR amplified using primers designed to amplify the greatest diversity of sequences for that gene. This approach is regularly used to investigate the diversity of small subunit rRNA sequences (16S/18S/ITSrRNA) in a sample. However, it is employed not

only to investigate both the phylogenetic diversity and relative abundance of a particular gene in a sample but also as a tool to investigate the impact of environmental contaminants in altering microbial community structure (Techtmann and Hazen 2016).

In shotgun metagenomics, the total genomic complement of an environmental community is probed through genomic sequencing (Techtmann and Hazen 2016). A typical shotgun study comprises five steps: (i) the collection, processing, and sequencing of the samples; (ii) preprocessing of the sequencing reads; (iii) sequence analysis to profile taxonomic, functional, and genomic features of the microbiome; (iv) statistical and biological postprocessing analysis; and (v) validation (Quince et al. 2017). Its main limitation is the depth of sequencing because gaining a complete inventory of the genes in an environmental sample often requires extremely deep sequencing. Secondly, oftentimes shotgun metagenomics samples the dominant microbes in a community and only sparsely covers the genomic content of the low-abundance members of that community (Techtmann and Hazen 2016).

1.5.2 Metatranscriptomics

It consists in a community RNA analysis that provides an inventory of the actively expressed genes in a sample. RNA is extracted from an environmental sample, converted into cDNA, and sequenced in a similar way than metagenomics (Techtmann and Hazen 2016). This approach facilitates insight into the potential expression of genes at the time of sampling. Its importance relies in the fact that while posttranscriptional and posttranslational gene expression can regulate protein synthesis, transcriptional-level control of gene expression enables organisms such as bacteria to rapidly adapt to changing environmental conditions (Moran 2009). Therefore, immediate regulatory responses to environmental changes may be better reflected by the metatranscriptome (Carvalhais et al. 2012).

1.5.3 Metaproteomics

Metaproteomics consists in the community protein analysis. This approach provides insights into the complement of proteins found in an environmental sample including posttranslational modifications in proteins that may impact their activity (Techtmann and Hazen 2016). It is useful mainly in determining changes in the composition and abundance of proteins, as well as in the identification of key proteins involved in the physiological response of microorganisms when exposed to pollutants (Desai et al. 2010). The first step in any proteomics-based approach is protein extraction, for which, there are numerous mechanisms and the available

instruments (Singh 2006). For protein separation and identification, two strategies have been established: one is the gel-based method. After protein extraction, highly efficient methods of separation based on two-dimensional polyacrylamide gel electrophoresis (2-DE) and modern tools of bioinformatics in conjunction with mass spectrometry (MS) are used. Matrix-associated laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) is the most commonly used MS approach to identify proteins of interest excised from 2-DE gels, by generation of peptide-mass fingerprinting (Singh and Nagaraj 2006). The other strategy is the liquid chromatography (LC)-based method, where the whole proteome is digested into a more complex peptide mixture using proteases without prior protein separation in gel. Then the resulting peptides are separated using strong cation exchange chromatography or microcapillary reverse-phase. In general, the separated peptides are analyzed using liquid chromatography coupled with MS/MS (LC-MS/MS). The produced MS data are interpreted for protein identification and then bioinformatic analysis. The second approach circumvents the limitations of the gel-based approach and greatly increases the proteome coverage compared with the gel-based method, allowing high-throughput identification of thousands of proteins within a short time and especially making detection of insoluble membrane proteins possible. Thus, the LC-based approach has become the main stream of microbial community proteomic studies, although it still suffers from problems of reproducibility, dynamic range, and database availability (Wang et al. 2016).

1.5.4 Metabolomics

Metabolomic addresses the whole complement of metabolites in an environmental sample (El Amrani et al. 2015). Metabolome analysis covers the identification and quantification of all intracellular and extracellular metabolites with molecular mass lower than 1000 Da, using different analytical techniques (Villas-Bôas et al. 2005). This analysis encompasses sampling, sample preparation, metabolite separation and detection, data analysis, and interpretation. Some particular advantages of metabolomics is that it allows monitoring changes in an organism as it is exposed to environmental pollutants, to follow the degradative pathways and to track their intermediates and responses during mineralization (Singh 2006). However, one of its main limitations is the complexity. The convoluted nature of cell metabolism, in which the same metabolite can participate in many different pathways, complicates the interpretation of metabolite data (Villas-Bôas et al. 2005), so it is difficult if not impossible to establish a direct link between genes and metabolites. Likewise, the metabolome consists of extremely diverse chemical compounds with a large variance in chemical structures and properties (Villas-Bôas and Bruheim 2007).

1.6 Microbial Features Involved in Resistance Against Pollutants

Microorganisms able to survive in extreme conditions harbor a wide variety of properties. The processes developed by these organisms often include several characteristics that are coded in genes, which are activated or silenced when needed. Some of the features that characterize extreme microorganisms and that allow them to survive in such hostile environments also provide them other features that are very promising from a biotechnological perspective. As an example, some bacteria able to grow in highly salted environments have been proved to use polycyclic hydrocarbons as a source of carbon and energy (Isaac et al. 2015). They are able to grow in highly salted environments by producing EPS that also functions solubilizing hydrophobic compounds. The solubilization of these compounds increases the interaction of the bacteria with the surrounding organic components, thus favoring its degradation (Isaac et al. 2015). This example shows that a single bacterium owns many interesting features with applicability in biotechnological processes. This is the main goal for the study of extreme organisms, other than their characterization, the search for novel molecules that could be used as an alternative to conventional technologies.

This section aims to highlight some of the mechanisms used by microorganisms when they interact with different contaminants. It is widely known that some organisms have the ability to interact with different contaminants affecting the concentration of the contaminant in the surrounding environment. When microbial interaction produces a decrease in the concentration of the pollutant under study, the biological system can be used to remediate contaminated sites. Some of the examples that have been mentioned involve different metals. In the case of uranium, many researchers have studied the way microorganisms adapt toward its presence. They have encountered biosorption, biomineralization, accumulation inside the cell, and direct reaction with the ions, having them changed their oxidation state, as the main mechanisms (Sánchez-Castro et al. 2017).

In general, microorganisms possess a huge amount of information condensed and coded in their genome. Depending on the environmental conditions, only some of this information (the essential which will allow survival) is expressed. All of the mechanisms involved in DNA replication, expression, and translation are regulated. When environmental and nutritional conditions change, microorganisms adapt to this variation by modifying gene expression and, as a consequence, protein synthesis. The newly synthesized proteins may be involved directly in the interaction with the contaminant or indirectly with participation in synthesis of compounds that interact with the contaminant. Other typical mechanism is the over synthesis of proteins involved in the stress cell protection (Irazusta et al. 2013, 2016, 2018). One example of the latter is the production of EPS. Some extreme bacteria which inhabit environments exposed to UV radiation produce carotenoids, to avoid mutations as a consequence of the highly environmental radiation.

Depending on the genetic properties of microorganisms inhabiting certain niches, different actions can lead to interaction with a contaminant. The contami-

nant can be tolerated through intra- and extracellular mechanisms, extracted from the cell through active transport systems, and the contaminant can also interact with the membrane preventing the influx.

Differential protein synthesis may lead to the activation of pathways involved in the interaction of the microorganism with the contaminant. These pathways can lead to the direct interaction of the microorganism with the contaminant or can be used to produce some substances as a response against the presence of the contaminant in the surroundings of the cell.

The mechanisms that microorganisms use may lead to:

1.6.1 Removal of the Pollutant by Biomineralization

Biomineralization is a general term for the processes by which living organisms form minerals resulting in the remotion of the pollutant and providing a means of detoxification as well as biorecovery depending on the type of pollutant (Gadd and Pan 2016). There are two main mechanisms: biologically controlled mineralization (BCM) and biologically induced mineralization (BIM) (Pérez-González et al. 2011). In controlled ones, the mechanisms are closely regulated, and organisms precipitate minerals that serve physiological and structural roles. This process can include the development of intracellular or epicellular organic matrices into which specific ions are actively introduced and their concentrations regulated such that appropriate mineral saturation states are achieved. Accordingly, minerals can be formed within the organism even when conditions in the bulk solution are thermodynamically unfavorable (Konhauser and Riding 2012). In induced ones, minerals are formed without any apparent regulatory control, as a consequence of changes in the supersaturated system due to the intake or excretion of different metabolites (active mechanism) (Lowenstam 1981) and to the contribution of substances that can act as crystallization nuclei, such as cell surfaces which favor precipitation (passive mechanisms).

The interaction of microorganisms with different pollutants can occur in the surface of the cell. Depending on the nature of the pollutant, the interaction can lead to the modification of the oxidative state of the element. If it is an ion, the variation of the oxidation number may result in its mineralization, and the process is known as biomineralization, as it is a consequence of living organisms. This phenomenon includes the chemical reaction of the ions with membrane transporters, thus inducing the variation of their state to a mineral. The final effect in the surrounding environment is a decrease in the concentration of the soluble ions. This process then can be used as a mechanism to decontaminate polluted sites. This mechanism, though studied in several organisms, is still very interesting in respect of the benefits it provides to the microorganisms involved. Microorganisms able to perform biomineralization may stay trapped between its own biomineral, thus producing its death.

Numerous bacterial genera produce biominerals that have been used in the remediation of soil contaminants, especially in bioremediation of heavy metals such as

chromium (Crean et al. 2012), nickel (Haferburg et al. 2008), cobalt (Handley-Sidhu et al. 2016), and arsenic (Achal et al. 2012), among others.

It is noticeable that mineralization mediated by microorganisms can be a consequence of a fully regulated process or microorganisms can also act as inductor of the biomineralization. For each case, minerals with different characteristics are formed. If microorganisms control the biomineralization, its shape is regular, and it turns into a heterogeneous structure when the cells induce the biomineralization. It is also important to highlight that when this process is highly regulated, the biomineral can be formed extra-, inter-, or intracellularly in respect of its location.

The most common biominerals precipitated by microbes include oxides, phosphates, sulfides, and oxalates, and these can have special chemical properties such as high metal sorption capacities and redox catalysis (Gadd and Pan 2016).

Some marine bacteria are capable of bioaccumulating pollutants from the surrounding environment, like in the case of the bacterial consortium tested for mercury removal by Canstein et al. (2002). They have also been reported to degrade hydrocarbons, chelate heavy metals, and remove pollutants from the environment by production of EPS. Also, *Streptomyces* have been studied for its ability to accumulate heavy metals (Majzlik et al. 2011).

1.6.2 Synthesis of Carotenoids

Microorganisms able to produce carotenoids are widely distributed in nature. It is known that this feature confers some abilities to survive in environments with high levels of radiation (Sandmann 2001). Some researchers have found for microorganisms able to withstand high concentrations of contaminants that the ability to synthetize carotenoids is strongly related to the concentration of the pollutant in the surrounding environment (Ortega-Cabello et al. 2017). An example occurs with the effect of ferrous ions toward the response of *Rhodococcus* and *Gordonia* strains (Ortega-Cabello et al. 2017).

The green algae *Dunaliella salina* and *Dunaliella bardawil* are known for their properties in synthetizing β-carotene in adverse conditions. Some of the carotenoids produced by these algae have great importance for its wide applications and because of the characteristics of the compounds produced, such as liposolubility and antioxidant capacity (Gómez et al. 2003). In the research developed by Gómez et al. (2003), *Dunaliella salina* showed a clear tendency in accumulating high amounts of carotenoids per volume when salinity increased.

Some microorganisms have also been described as able to produce carotenoids when grown under other stresses such as high radiation and high temperature or in the presence of certain compounds like heavy metals (Irazusta et al. 2013). Irazusta et al. (2013) have studied the behavior of the pigmented yeast *Rhodotorula mucilaginosa* RCL-11 in the presence of copper salts and determined that the synthesis of carotenoids increases in the presence of the heavy metal. However, they found an inverse relation between the accumulation of cooper and the carotenoid biosynthesis, indicating that these processes may be related.

1.6.3 Production of Exocellular Polymeric Substances

The components of the extracellular matrixes vary among microorganisms, and this composition is closely related to the function they develop. In general, EPSs are composed of carbohydrates (responsible of the texture of the EPS), proteins (related to the function of the EPS, since they can be enzymes which participate in extracellular degradation processes), extracellular DNA (of main importance in biofilm processes, since the community forming the biofilm may exchange genetic information through this path), and lipids and surfactants (responsible of the EPS hydrophobicity) (More et al. 2014). They play a major role in the architecture of the biofilm matrix being responsible for adhesion to surfaces and for cohesion in the biofilm. They immobilize biofilm cells and keep them in close proximity promoting interactions, including cell-cell communication and the formation of synergistic microconsortia, among other functions (Flemming and Wingender 2010). Considering its bioremediation concern, cells in a biofilm have a better chance of adaptation and survival (especially during periods of stress) as they are protected within the matrix (Singh et al. 2006), so they are especially useful in the immobilization and degradation of pollutants at relatively high rates because of the multiple interactions and high contact surface in the matrix with the target contaminant. EPS produced by algae, bacteria, fungi, and yeasts can be used for bioremoval of toxic compounds from the environment, and they have the characteristics of being a low-cost, nonhazardous, and effective method (Amoozegar et al. 2012).

Many halophilic organisms have been studied in order to describe the composition of the matrices they synthetize in the outside of the cell, since they may be involved in several biotechnological applications. They are also used in decontamination of chlorophenols (such as 2-chlorophenol,2,4-dichlorophenol, pyrene, phenanthrene, o-cresol, naphthalene, phenol, 1,2,3-trimethylbenzene), toluene, azo dyes, and herbicides, among others (Singh et al. 2006).

Amoozegar et al. (2012) have described that a moderately halophilic bacterial strain isolated from saline environments in Iran is capable of removing lead and cadmium from the surrounding environment. They have studied the ability of this microorganism and of the EPS it produces to interact with these heavy metals. The strain, characterized phylogenetically as belonging to the genus *Halomonas*, was able to produce a decrease of 90% and 50% of lead and cadmium, respectively. Since this strain is also known for its EPS formation capacity, the uptake of the heavy metals as a consequence of the interaction with this biological product was also evaluated.

Other than the function they are produced for in the natural environment, EPSs have many other applications. They are being studied to replace chemical polymers and have many advantages in comparison to them. The main advantages include biodegradability, high efficiency, and reduced or absence of toxicity. These features have made them attractive for many industries, including food, pharmaceutical, and medical.

1.7 Taking Advantage of Special Features Observed in Microorganisms That Tolerate Pollutants and Extreme Conditions

All of the microbial processes are highly regulated in microorganisms' cells. These regulations generally occur at gene expression level, modifying protein synthesis. In general, microorganisms with special features may be used for biotechnological applications. In some cases, it is even better to clone a specific gene of interest in a different biological system to optimize the production of the component of interest. For example, in the case of halophilic microorganisms, *Archaea* are known to produce proteins with high functionality at high levels of salt. Enzymes such as hydrolases (proteases, lipases, esterases), biopolymers, and surfactants are some of the components that can be obtained from halophilic *Archaea* (Litchfield 2011). If the element of interest for research or production is a protein, it is possible to clone the corresponding gene in other bacteria easier to grow at the laboratory and obtain the protein of interest in augmented amounts and with improved properties. For example, acute lymphoblastic leukemia (ALL) is a disease that has been treated with L-asparaginase for more than 30 years. Several studies have been developed to obtain an enzyme with better properties. Ghasemi et al. (2017) have found that a recombinant L-asparaginase from the halophilic microorganism *Halomonas elongata* is very promising regarding cancer therapy, because it has more desirable properties than those currently has been used. Proteins from microorganisms isolated from extreme environments are the target of study for any research groups around the world. Enzymes like hydrolases (esterases, glucosidases, and azoreductases, among others) are being the target for research as a consequence of increased enzymatic activities and for being a better alternative compared to the traditional enzymes currently in use (Castilla et al. 2017; Eslami et al. 2016). These enzymes can be used, as previously mentioned, in medical industry, for remediation of azo dyes and in processes for obtaining degraded compounds from complex mixtures (like obtaining sugar residues from cellulose).

1.8 Final Comments

Microorganisms have the ability to colonize a wide variety of environments. Those which are able to survive in extreme conditions often possess several properties which allow not only the colonization of extreme environments but also turn them into targets for biotechnological applications.

The features developed by microorganisms to tolerate adverse conditions can be exploited at an industrial level and turn them into possible candidates for bioremediation.

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