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The use of microorganisms in environmental remediation*

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Abstract - Due to their high metabolic diversity and high adaptability, microorganisms are able to live in the most varied of "natural" and "artificial" habitats created by environmental contamination. Different microbes can use a great variety of refractory pollutants, thus permitting their use in *ex* and *in situ* bioremediation. The implementation of biotreatment processes requires the use of evaluation methods of pollution and the success of bioremediation. The microorganisms can also be used as biosensors. Laboratory microcosm experiments would allow isolation of new microorganisms, assessment of the methods for evaluating pollution and the risk of implementation of non- and recombinant microorganisms. Bioremediation technologies have an open future linked to multidisciplinary scientific work.

Key words: pollution control, microcosm studies, microbial evolution, bioremediation technologies.

INTRODUCTION

Due to their high metabolic diversity, microorganisms are able to live in the most widely varied "natural" habitats on Earth, thus indicating that they take advantage of any ecological niches found in the environment. To live in an intensely competitive environment, microorganisms must exploit any advantage available, to colonize the ecological niche; they must metabolize common nutrients more rapidly, or use nutrients that competing microorganisms can not metabolize. In non-competitive environments with extreme conditions of temperature, salinity, acidity, etc., microbes need special physiological characteristics, which permit life, as is the case of the extremophiles. Some microorganisms can use other strategies, such as the production of acids, antibiotics, etc., to inhibit the growth

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of competing microbes. However, in all cases survival in ecological conditions is a matter of adaptation.

The actual microbial population from the most primitive kinds of cells, called progenotes (Woese, 1987), has been the result of billions of years of evolution directed by the environment (Wright, 2000). This microbial population is the consequence of the adaptation of different species to changes that occur naturally on Earth. Human activities have always interfered with the environment, but for many years their effects have been minimal and well tolerated by nature. We may say that since the Industrial Revolution this situation has changed; the residues of human activities are generating new environmental conditions, which can be termed "artificial habitats", with a high impact on nature.

Since the 1990s society has been aware of the problem, as well as the need to find effective measures of remediation in order to reverse the negative environmental health conditions that severely threaten plant, animal, and even human health (Bonaventura and Johnson, 1997; Cihacek *et al.*, 1996). Microbiota has already proved an important tool to control environmental waste pollutants and it increasingly plays bioremediation roles as a result of the application of the new technologies and processes. The purpose of this review is to outline current possibilities and future perspectives for the use of microorganisms in bioremediation, although in no case are these supposed to be exhaustive.

ECOSYSTEM SELECTION

Traditionally, natural ecosystems have been considered on a scale too large for understanding microbial interactions, such as a forest, seawater or even the entire globe (Lenton, 1998). Microorganism associations form "microecosystems" for individual situations within large ecosystems, such as ecosymbiotic associations (Margulis, 1996). The increases in environmental contamination lead to a progressive formation of "artificial" microecosystems, in which non-refractory components (e.g. sewage) are together with refractory compounds released from industrial activities. Since evolution usually occurs in response to stress (Jablonski *et al.*, 1983), and there are biochemical mechanisms for non-random mutations and evolution (Wright, 2000), the different kinds of refractory components or situations might facilitate and accelerate the adaptation of microorganisms to the new conditions. The adapted species capable of using non-natural compounds, or adapted to living in stress conditions created artificially, facilitate the growth of other microorganisms surrounding the "leader" species, thus creating a new microecosystem.

The "artificial" ecosystem-selection permits the reproduction of conditions for practical purposes, giving rise to laboratory microcosm experiments to characterize which important microorganisms can be used in bioremediation of these or other similar contaminants (Swenson *et al.*, 2000). This approach has been used, for example, to analyze microbial communities in aromatic hydrocarbon contaminated aquifers from a military installation (Shi *et al.*, 1999) or soils (Boonchan *et al.*, 2000). Although the behavior of microorganisms could be addressed by direct analysis of the contaminated areas, the microcosm experimental approach can answer questions about the mechanism controlling the activities, how the contaminants are metabolized etc.; these are questions that can not be resolved by the analysis of field samples alone. Once information about the microorganisms and the process of biotreatment has been obtained, studies must extend through pilot scales before full-scale implementation. Scientific work would increase our knowledge regarding the possibilities of existing microorganisms, the isolation and utilization of new ones, and the possibility to design novel microorganisms using genetic engineering.

CONTAMINATION AND MICROORGANISMS

Various human activities, such as agriculture, fishing and industry, produce abnormal accumulation of different materials which, due to concentration and/or characteristics, produce degradation of the environment, and occasionally ecological catastrophes such as the Exxon Valdez incident, Chernobyl, the Gulf-war, and recently in Spain, the problem of Aznarcollar. Although most of the contaminants are biodegradable, containing as they do natural matter (agriculture or animal residues), the quantities of production render it impossible to eliminate them in short time-periods. Other natural organic matter, such as oils or petroleum hydrocarbons, could be degraded very slowly, given that microbes preferentially use nutrients in aqueous solution, and petroleum is also deficient in essential elements, for instance nitrogen and phosphorous.

Industrial activities produce many chemicals that are not present in nature, as plastics, pesticides, etc., that enter the ecosystems in large amounts and are refractory to biodegradation. Hence, in this case, their characterization as non-biodegradable could be incorrect or, at least, too drastic, since the majority of synthetic chemicals have bonds or subunits that exist in nature and are susceptible to attack by microbial enzymes. Further problems present contaminants that are toxic for the microorganisms, such as heavy metals or chemicals used to control plant diseases, that in short-term reduce the microbial populations retarding bioremediation of organic pollutants (Konopka *et al.*, 1999).

The aim of this work is not to give an exhaustive description of the different microorganisms capable of using different contaminant materials, as examples, which, by their health or socio-economic importance, are of interest due to their characteristics or difficulty in their elimination. In this sense, fungal species able to eliminate from the soil heavy metals that are inaccessible to plants and radioactive metals after the Chernobyl disaster have been described (Gray, 1998; Smith *et al.*, 1993). The contamination of soils and sediments by petroleum is a matter of international concern, and a new strain called CKB, capable of degrading aromatic hydrocarbons in absence of oxygen, has been isolated (Coates *et al.*, 1998). *Agrobacterium radiobacter* J14a can utilize atrazine (Struthers *et al.*, 1998), one of the herbicides most currently used, and Kohler *et al.* (2001) have reported bacterial degradation of organoarsenic warfare compounds. Even microbial populations capable of degrading plastics in soil waste disposal landfill sites have also been described (Ishigaki *et al.*, 2000). The above examples indicate that practically no natural or artificial substance exists that can not be used, at least in part, by a microorganism. Therefore scientific work must be directed towards obtaining efficient systems that permit the identification of these microorganisms; in this context, the use of microcosm experiments could be a good approach.

BIOREMEDIATION

Increases in environmental contamination lead to a progressive deterioration of ecosystem qualities, that can affect animals and plants and, as a consequence, directly or indirectly, human health (Bonaventura and Johnson, 1997). This condition challenges society to find effective measures of remediation. Conventional treatments of pollutants, such as incineration, volatilization or immobilization of pollutants, simply transfer the pollution, creating new waste, and fail to eliminate the problem. Bioremediation technology, which leads to detoxification and mineralization of pollutants, is an attractive alternative that in some cases could produce economic benefits. As indicated above, successful bioremediation depends on the availability of appropriate microorganisms. Although it is generally accepted that more than 80% of the total microorganisms are unknown, reactions mediated by both the known and the unknown microorganisms are already employed in biotreatment and in bioremediation (Hamer, 1993). This consideration, together with the potential use of engineered microorganisms, offers an expanded time scale technology (Pieper and Reineke, 2000).

There are several means of implementing bioremediation *in situ*. The environment could be inoculated with non-indigenous microorganisms selected in other sites (bioaugmentation) or periodically supplemented with nutrients (biostimulation) assuming that the indigenous microorganisms are able to metabolize the contaminants due to adaptation processes. Combinations of these basic technologies, that represent a continuous supply of nutrients, oxygen and microorganisms, depending on each case, as a bioreactor-based method would provide a range of bioremediation technologies. Bioaugmentation and/or biostimulation also can be employed in *ex situ* methods to stimulate the biodegradation of pollutants. Bioreactors represent a highly controlled method of treating contaminants, because temperature, pH, nutrient amounts and agitation can all be controlled in batch- or continuously-fed reactors. In the compost- or slurry-based reactors, microbial activity, and thus contaminant degradation, can be maximally optimized. Land-based treatment, or solid-phase remediation, are normally used an as *ex situ* remediation methods and can be treated in piles or in constructed treatment cells. Land-farming techniques have also been utilized by many oil companies to treat contaminated soils (Caplan, 1993).

EVALUATION OF POLLUTION AND BIOREMEDIATION

As in the treatment of diseases in medical practice, to establish an effective treatment, it is first necessary to diagnose correctly, and the parameters for monitoring health could also be used to evaluate the degree of recovery of patients; as ecological illnesses, the polluted ecosystems must be detected and evaluated. Depending upon the kind of contamination, the “diagnosis” is occasionally easy, and a simple observation suffices (i.e. garbage, fuel, etc.), whereas others require the use of quantitative strategies (i.e. pesticides, metals, etc.). In all cases however, monitoring the efficacy of bioremediation requires systematic interdisciplinary technologies; microorganisms can also be used for these purposes.

It is clear that before taking any decision to initiate any bioremediation process, a detailed site characterization has to be made, so as to provide information on the chemical nature of the pollutants, the geochemical properties of the site, and its microbiological characteristics (Heitzer and Sayler, 1993). Before determining the degree of contamination it is first necessary to know which are the concentrations of the pollutants in non-polluted conditions, but this information is not available. Due to the success of implementation of bioremediation technologies, and their increase in the near future, it would be important to open research lines to define the above characteristics of the non-polluted environments.

Different types of methods using microorganisms or specific microbial activities can be used for evaluating pollution and the success of bioremediation (Heitzer, 1998; White *et al.*, 1998). If the microbial process is well characterized, progression of bioremediation can be evaluated by monitoring the metabolites derived from the degradative activity or the changes corresponding to the microbial ecology of the treated system, as the increase of specific microbial populations or relevant genetic elements (Shannon and Unterman, 1993). Rapid and sensitive methods to measure ecotoxicity have been developed using bacterial luciferase of *Vibrio* spp (Engebrecht *et al.*, 1985; Steinberg 1995). Using the bacterial luciferase as a reporter gene, whole-cell biosensors that can detect alkanes (Sticher *et al.*, 1997), naphthalene and salicylate (Heitzer *et al.*, 1994), toluene (Willardson *et al.*, 1998), various heavy metals (Selifonova *et al.*, 1993; Karube and Nakanishi, 1994), and petroleum hydrocarbons (Dorn and Salanitro, 2000), etc., have been described. As many different microbial species can live in the presence of different pollutants by developing the capacity to use such compounds and, often, the transcription of the genes encoding the degrading-enzymes is regulated by the presence of the pollutant, the discovery of transcriptional activators and their corresponding promoter sequences would facilitate the development of bacterial sensors for many pollutants. A biosensor can be engineered by placing a reporter gene, such as *lac* or *luc*, under the control of a transcriptional activator. Given appropriate conditions, a direct correlation between contaminant concentration and enzyme activity would be established.

The use of monitoring instruments, based on a combination of biological sensing elements and an electronic signal-traducing element, provides an attractive approach which can open the way to monitoring different biological parameters in order to obtain real-time information about global bioremediation processes (Wood and Gruber, 1996; Karube *et al.*, 1998; Margesin *et al.*, 2000). In the context of this revision we must not forget that the idea of the entire process is to treat contamination, and the natural or engineered microorganisms obtained for biosensor purposes can also be used for bioremediation.

RELEASE OF GENETIC ENGINEERED MICROORGANISMS

As in practically all microbial applications, the use of genetic engineering to improve microbial capacities opens unknown possibilities to obtain new species that are able to use or to degrade different contaminants with high efficiency. In the case of bioremediation there is much scientific work suggesting that engineered microorganisms have greater potential for environmental clean-up than natural ones. However, due to the versatility and adaptation capacity of the naturally occurring microorganisms, they offer many possibilities for bioremediation, and thus scientific effort must be directed in both approaches, so as to isolate new non-recombinant species and to obtain new recombinant ones. Because very few engineered microorganisms have yet been released into the environment (Sayler and Ripp, 2000), the results of their application are preliminary. Dispersal of non-recombinant strains provides further valuable information required for the assessment of the risk associated to the future release of recombinant derivatives of those strains. On the other hand, genetic manipulation of species able to live in contaminated environments, that already have certain conditions to use pollutants, must be more appropriate to obtain successful results in bioremediation than the use of laboratory strains. Moreover, for *in vitro* construction of degradative pathways through genetic engineering, the encoding genes must first be identified and isolated from natural microorganisms. The release of non-recombinant microorganisms into the environment has long been used for different purposes, such as biological control of plant diseases and insects, nitrogen fixation, etc., and this release of microbes into nature has long been an integral part of the research activities of several biological disciplines, including phytopathology and entomology. Most of the studies show that dispersal of microorganisms has apparently not resulted in significant perturbations of the habitats into which the non-indigenous microorganisms have been introduced (Wilson and Lindow, 1993).

Although the number of engineered microorganisms introduced into the open environment remains small in contrast to the larger introduction of non-recombinant ones (Wilson and Lindow, 1993; Sayler and Ripp, 2000), it is difficult to understand the legislation and public opposition to their use (Hamer, 1993). Because indigenous microorganisms often are genetically manipulated by the environment (Goodnight, 2000; Schloter *et al.*, 2000), the engineered microorganisms will frequently be introduced into habitats, wherein similar organisms already exist, producing minimal alterations of the ecosystem. There is a vast literature that discusses the problems and risk of release of recombinant microbes, due to the diversity of the microorganisms themselves, the large number of traits that potentially might be modified, and the diverse habitats into which they will be introduced. Assessment of risk should be based on reasonable interpretations of logical studies, and microcosm studies would be the most appropriate ones. Comparative laboratory microcosm experiments should be undertaken to determine the effects of the new traits introduced into the engineered microorganism on the ecosystem, before large-scale implementation. In this context, the extension of the use of *ex situ* treatments, such as composting or bioreactors, with recombinant microorganisms under correct scientific evaluation, will contribute to a better understanding of the biological effects on the nature of the genetically engineered microorganisms.

CONCLUDING REMARKS

This review was not intended to address the voluminous literature on bioremediation, but rather to demonstrate that the application of biotreatment is growing rapidly due to the fact that it is a safe, natural, and cost-effective process. The excessive localized pollution of the natural environment by industries is a sign of industries in decline (Hamer, 1993); waste-minimization technologies would open ways for a new kind of commercially attractive industrial activities with a promising future.

The application of diverse bioremediation technologies must be based on scientific information obtained in both fundamental as well as research environmental laboratories. The “artificial “ ecosystems created in laboratory microcosm experiments allow the reproduction of conditions in contaminated sites, for practical purposes, such as isolating new microorganisms, assessing methods for evaluating pollution and bioremediation, as well as the risks of implementing non- and recombinant microorganisms. For the development of bioremedial processes to succeed commercially, it is essential to link different disciplines as microbial ecology, biochemistry and microbial physiology, together with biochemical and bioprocess engineering. Attention needs to be directed towards the integration of individual chemical transformation in the metabolism of microbial cells and consortia, and to define the rate-limiting steps in bioremediation. The challenge is to continue developing the scientific and engineering work that provides the real bases for both the technology and its evaluation. Because of political and public pressure, the scientific remediation work ought also to provide legitimate reasons that permit scientists to explain and justify the use of these technologies to the concerned public.

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