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Biodegradation and Bioremediation

With 40 Figures and 12 Tables



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Preface

The diverse metabolic capabilities of microorganisms and their interactions with complex organic and inorganic substrates have long been recognized and are now being exploited for the treatment of hazardous soil contaminants. Microbial reactions range from single-step transformations of pollutants into more or less toxic forms (biodegradation) to complete mineralization, yielding water and either carbon dioxide or methane. Microbial processes are environmentally compatible, operate under mild conditions and can be integrated with non-biological processes with treatment goals including detoxification, destruction and immobilization of the contaminants. The application of biological methods, through bioremediation, has been used successfully for remediation of soils with a variety of pollutants.

Biodegradation and Bioremediation, Volume 2 of the series *Soil Biology*, presents a selection of contributions related to microbiological and biochemical processes with an emphasis on their use in bioremediation. Topics include bioavailability and biodegradation, anaerobic biodegradation of environmental pollutants, microbial community dynamics during bioremediation of hydrocarbons, biodegradation of halogenated compounds, polycyclic aromatic hydrocarbons and nitrogen-containing xenobiotics. The roles of aromatic hydrocarbon dioxygenases and bacterial reductive dehalogenases and engineering of improved biocatalysts in bioremediation are discussed. Innovative methods for monitoring bioremediation processes and approaches for combined biological and abiological degradation of xenobiotic compounds are also included. Volume 1 of the series *Soil Biology, Applied Bioremediation and Phytoremediation*, included topics related to the applied aspects of bioremediation and phytoremediation technologies.

This book contains contributions from experts in the area of environmental microbiology, biotechnology and bioremediation. The authors are from diverse institutions, universities, government laboratories and industry, with basic, applied and industrial research background. This book should prove to be useful to under- and post-graduate students of biotechnology, microbiology, biochemistry,

and soil and environmental sciences and engineering. We hope that teachers, scientists and engineers, whether in academia, industry or government, will find the contents, including its basic and practical aspects, helpful.

We gratefully acknowledge the continuous support of all the contributing authors, help and encouragement provided by our colleagues and valuable guidance provided by Dr. Ajit Varma, Editor-in-Chief of the series *Soil Biology*, and Dr. Jutta Lindenborn, Springer-Verlag, during the preparation of this volume and at various stages of this editorial work.

Guelph, Waterloo, Ontario, April 2004
Ajay Singh and Owen Ward

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1 Biotechnology and Bioremediation – An Overview

Ajay Singh¹ and Owen P. Ward²

1 Introduction

The large-scale manufacturing, processing and handling of chemicals have led to serious surface and subsurface soil contamination with a wide variety of hazardous and toxic hydrocarbons. Many of the chemicals, which have been synthesized in great volume, including polychlorinated biphenyls (PCBs), trichloroethylene (TCE) and others, differ substantially in chemical structure from natural organic compounds and are designated as xenobiotics because of their relative recalcitrance to biodegradation. Other compounds, for example the polycyclic aromatic hydrocarbons (PAHs), are also toxic and the high molecular weight PAHs (having four or more fused rings) are typically recalcitrant to biodegradation. The latter compounds, the products of incomplete combustion of natural organic materials and hydrocarbons, occur in the soil environment as a result of naturally ignited forest fires. However, intensification of energy-related and other industrial processes with associated production of wastes and by-products, rich in PAHs, has led to serious soil contamination of many industrial sites. The resultant accumulations of the various organic chemicals in the environment, particularly in soil, are of significant concern because of their toxicity, including their carcinogenicity, and also because of their potential to bioaccumulate in living systems. A wide variety of nitrogen-containing industrial chemicals are produced for use in petroleum products, dyes, polymers, pesticides, explosives and pharmaceuticals. Major chemical groups involved include different nitroaromatics, nitrate esters and nitrogen-containing heterocycles. Many of these chemicals are toxic and threaten human health and are classified as hazardous by the United States Environmental Protection Agency.

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Interest in bioremediation of polluted soil and water has increased in the last two decades primarily because it was recognized that microbes were able to degrade toxic xenobiotic compounds which were earlier believed to be resistant to the natural biological processes occurring in the soil. Although microbial activity in soil accounts for most of the degradation of organic contaminants, chemical and physical mechanisms can also provide significant transformation pathways for these compounds (Rogers 1998). Bioremediation is generally considered a safe and less expensive method for the removal of hazardous contaminants and production of non-toxic by-products (Providenti et al. 1993; Ward et al. 2003).

There have been many experimental successes with the more difficult to degrade contaminants, but there have also been many notable failures. However, it has been suggested that, although microorganisms have the primary catalytic role in bioremediation, our knowledge of the alterations occurring in the microbial communities remains limited and the microbial community is still treated as a “black box” (Iwamoto and Nasu 2001; Dua et al. 2002). Put in a more positive light, bioremediation remains a developing field, largely because it has traditionally been carried out in a natural environment where many of the organisms are uncharacterized and because no two environmental projects are identical (Watanabe 2001; Verstraete 2002).

Biotechnology has the potential to play an immense role in the development of treatment processes for contaminated soil. As with any microbial process, optimizing the environmental conditions in bioremediation processes is a central goal in order that the microbial, physiological and biochemical activities are directed towards biodegradation of the target contaminants. Environmental factors influencing microbial growth and bioactivity will include moisture content, temperature, pH, soil type, contaminant concentrations and oxygen for aerobic degradation and redox potential for anaerobic degradation. Deviations of these parameters away from optimal conditions will reduce rates of microbial growth and transformation of target substrates and perhaps cause premature cessation and failure of the bioremediation process. Biodegradation potential may also be limited by the toxicity of the pollutants to the degrading microbes. Some species have developed cellular defenses enabling them to tolerate high concentrations of toxic contaminants.

Understanding the biochemical and physiological aspects of bioremediation processes will provide us with the requisite knowledge and tools to optimize these processes, to control key parameters and to make the processes more reliable. Since the majority of bioremediation processes rely on the activities of complex microbial communities, we

have much to learn about the interactive and interdependent roles played by individual species in these communities. We need to develop strategies for improving the bioavailability of the many hydrophobic contaminants which have an extremely low water solubility and tend to be adsorbed by soil particles and persist there. We need to continue to elucidate the complex aerobic and anaerobic metabolic pathways which microbes have evolved to degrade organic contaminants and to understand the nature of rate-limiting steps, bottlenecks and underlying genetic and biochemical regulatory mechanisms. We need to continue to characterize many of the key enzymatic reactions that participate in contaminant transformation and to relate contrasting reaction rates, substrate specificities and enzyme mechanisms to differences in protein structures. Such new knowledge can provide us with the requisite information to test, design and engineer biocatalysts with improved substrate specificities, reaction rates or other desired catabolic properties and ultimately to engineer improved catabolic pathways for bioremediation. We must recognize that some chemical species are inherently intractable to enzyme transformation and we should be open to the possibility of combining chemical or physical strategies with biological systems to achieve overall effective remediation. We must also continue to devise better methods for monitoring and assessing the progress and effectiveness of microbial biodegradation processes at both the research and process implementation level. Clearly, the availability of advanced molecular techniques provides a new impetus and enhances our abilities to address many of these issues.

These topics, introduced below, represent the main focus of this book.

2

Microbial Communities and Bioremediation

Since microbial communities play a significant role in biogeochemical cycles, it is important to analyze the community structure and its changes during bioremediation processes. The challenge of characterizing the roles of a range of hydrocarbon-metabolizing organisms in degrading the myriad of petroleum substrates present in hydrocarbon-contaminated soils is clearly substantial. However, such studies can provide major insights into important biochemical and physiological aspects of bioremediation and microbial catabolism. Culture-dependent and -independent methods are being applied to microbial community characterization. The temporal and spatial changes in bacterial populations and the diversity of the microbial community during bioremediation can be determined using sophisticated

molecular methods (Stapleton et al. 1998; van Elsas et al. 1998; Widada et al. 2002). Recent advances in molecular techniques, combined with genomic information, are greatly assisting microbiologists in unraveling some of the mysteries related to the diverse roles of microbes in these communities. One of the exciting outcomes of these studies is an advance in our understanding of the degree and importance of lateral gene transfer in complex microbial communities. Catabolic genes have the ability to spread through a microbial community at high frequencies (Top and Springael 2003; Van der Meer and Senchilo 2003). An assessment of microbial community dynamics during hydrocarbon bioremediation is presented in Chapter 2.

3

Contaminant Bioavailability

For efficient microbial degradation of chemical contaminants to occur, the contaminants must be bioavailable to the degrading cells. The biodegradation rate of a contaminant depends on the rate of contaminant uptake and mass transfer. Bioavailability of a contaminant in soil is influenced by a number of factors such as desorption, diffusion and dissolution. The decrease in bioavailability due to long-term contamination of soil, often referred to as aging or weathering, is a result of chemical oxidation reactions and slow chemical diffusion of the contaminant into small pores incorporating contaminants into the organic matter. Use of chemical or bio-surfactants during the biodegradation process helps overcome bioavailability problems (Van Hamme et al. 2003). The molecular structure of the contaminant and hydrophobicity may also affect the pollutant uptake by the microorganisms. Indeed, the cells may also have active or selective systems for transporting the contaminants into the cell. Given that many of these contaminants have low solubility in aqueous media, understanding mechanisms of their uptake by the degrading microbes and developing strategies to promote or accelerate their accession represent important aspects of effective bioremediation processes. Chapter 3 provides a critical analysis of the bioavailability of organic pollutants. The hydrophobicity/low water solubility properties of PAHs cause them to associate with hydrophobic components in soil, thereby limiting their accession to microorganisms. This topic is discussed in detail in Chapter 5.

4

Microbial Catabolism of Organic Pollutants

Biodegradation involves the breakdown of organic compounds either through biotransformation into less complex metabolites or through mineralization into inorganic minerals, H₂O, CO₂ (aerobic) or CH₄ (anaerobic). Both bacteria and fungi have been extensively studied for their ability to degrade a range of environmental pollutants including recalcitrant polycyclic aromatic hydrocarbons, halogenated hydrocarbons and nitroaromatic compounds. The biochemical pathways/enzymes required for the initial transformation stages are often specific for particular target environmental contaminants, converting them to metabolites which can be assimilated into more ubiquitous central bacterial pathways. An overview of some of the biodegradation systems used by microorganisms in the catabolism of key organic contaminants in soil is presented in Table 1. The extent and rate of biodegradation depend on many factors including pH, temperature, oxygen, microbial population, degree of acclimation, accessibility of nutrients, chemical structure of the compound, cellular transport properties, and chemical partitioning in growth medium. Figure 1 provides a schematic of aspects of the biodegradation system involved in bioremediation.

Some recalcitrant chemicals contain novel structural elements that seldom occur in nature and which may be incompletely transformed as microbes lack the degradative pathway for complete degradation of these xenobiotics. While microbes may not have the metabolic pathways for mineralization of certain newly introduced synthetic chemicals, there is evidence that microorganisms have the capacity to evolve such catabolic systems over time. In bioremediation processes, it is generally an objective to exploit microbial technology to accelerate the rate of pollutant removal.

Many contaminants in soil exist in anaerobic environments. A couple of decades ago, by observing the anaerobic dechlorination of PCBs over time in Hudson River sediments, it became clear that microbes could transform contaminants under anaerobic conditions. By the late 1980s, there was conclusive evidence that hydrocarbons could be degraded in the absence of oxygen. These anaerobic degradation systems required terminal electron acceptors such as iron (III), manganese oxide or nitrate to replace that function of oxygen in aerobic systems. We have now entered a period of intensive research and discovery focused on the catalytic mechanisms which facilitate the anaerobic catabolism of pollutants. Anaerobic aspects of hydrocarbon bioremediation are discussed in detail in Chapter 4. As is mentioned above, anaerobic processes are

Table 1. Overview of some of the biodegradation systems used by microorganisms in catabolism of key organic contaminants in soil

Contaminant	Catabolism	References
Petroleum hydrocarbons	<p><i>Alkanes (aerobic)</i></p> <ul style="list-style-type: none"> - Monooxygenase/hydroxylase-mediated conversion of alkanes to alcohols with subsequent aldehyde and acid formation prior to b-oxidation (example: OCT plasmid of <i>Pseudomonas putida</i> Gpo1) - Also a dioxygenase-mediated conversion of alkanes to aldehydes (e.g. <i>Acinetobacter</i> sp. M1) - Other mechanisms initiated by desaturation reactions <p><i>Monoaromatics (aerobic)</i></p> <p>Oxygenase-mediated attack of the aromatic ring typically producing a dihydrodiol, with subsequent conversion to catechol, oxygenase-mediated catechol ring cleavage with production of muconic acid-type metabolites which can be degraded via the TCA cycle</p> <p><i>Polycyclic aromatic hydrocarbons (PAHs) (aerobic)</i></p> <ul style="list-style-type: none"> - Bacteria: complex initial PAH ring cleavage reactions involving monooxygenases or combinations of mono- and dioxygenases - Fungi: ligninolytic and non-ligninolytic PAH co-oxidation <p><i>Anaerobic Degradation</i></p> <ul style="list-style-type: none"> - Conversions of alkanes, monoaromatics, some PAHs and hydrocarbon mixtures under Fe(III), Mn(IV) or sulfate-reducing or -denitrifying conditions or using other terminal electron acceptors including CO₂ - Initial activation mechanisms involving carboxylation, methylation, hydroxylation, dehydrogenation and addition reactions (especially fumarate addition) 	<p>van Beilen et al. (1994); Atlas and Cerniglia (1995); Ward et al. (2003)</p> <p>Prince (1998); see also Parales and Resnick (Chap. 8)</p> <p>Cerniglia (1992, 1997); Paszczynski and Crawford (1995); see also Aitken and Long (Chap. 5)</p> <p>Lovely (2000); Widdel and Rabus, (2001); see also Coates (Chap. 4)</p>
Chlorinated hydrocarbons	<p><i>Chlorinated aliphatics</i></p> <ul style="list-style-type: none"> - Aerobic transformations by oxidations, typically by hydroxylation or substitution of a hydroxyl group on the molecule or, in the case of unsaturated chlorinated aliphatics, by epoxidation - Anaerobic transformations are typically reductions such as hydrogenolysis, substitution of a chlorine atom by a hydrogen atom or elimination of chlorine atoms from adjacent carbons with formation of a C=C bond 	<p>Janssen et al. (1994); Peyton et al. (1995); see also Habash et al. (Chap. 9) and Mohn (Chap. 6)</p>

<p><i>Mono- and poly-chlorinated phenols (chlorinated aromatics) Pentachlorophenol (PCP)</i></p> <ul style="list-style-type: none"> - Aerobic production of tetrachloroquinone mediated by NADPH-dependent oxygenolytic dechlorination or hydrolytic mechanisms, followed by its further catabolism and mineralization - Non-specific degradation by fungal ligninases - Anaerobic conversion of chlorinated phenols via one or more reductive dehalogenation reactions, leading to phenol which may be mineralized to methane and CO₂ 	<p>Kennes et al. (1996); McAllister et al. (1996); see also Habash et al. (Chap. 9) and Mohn (Chap. 6)</p>
<p><i>Polychlorinated biphenyls (chlorinated aromatics)</i></p> <ul style="list-style-type: none"> - Aerobic bacterial dioxygenase-mediated attacks, most commonly by 2,3 dioxygenase attack at an <i>ortho</i> chlorinated carbon with the resulting dihydroxy metabolites transformed via <i>meta</i>-cleavage products to chlorobenzoate. Attacks at other unsubstituted positions, by different dioxygenases, for example 3, 4, have also been characterized - Non-specific degradation by fungal ligninases - Extensive stepwise removal of <i>meta</i> and <i>para</i> chlorines by reductive dechlorination under anaerobic conditions typically produces mono- to trichlorinated biphenyls that are amenable to aerobic bacterial degradation 	<p>Abramowicz (1990); Paszczynski and Crawford (1995); see also Mohn (Chap. 6)</p>
<p><i>2,4-D (2,4-dichlorophenoxy)acetate and related compounds</i></p> <ul style="list-style-type: none"> - Aerobic degradation involves removal of acetate producing 2,4-dichlorophenol, which is converted to a dichlorocatechol followed by ring cleavage and mineralization - Fungal ligninolytic mineralization - Anaerobic degradation is initiated by reductive dechlorination <p><i>DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane] and related compounds</i></p> <ul style="list-style-type: none"> - Aerobic degradation with initial ring hydroxylation - Anaerobic dechlorination 	<p>Singh et al. (1999); see also Mohn (Chap. 6)</p> <p>Foght et al. (2001); see also Mohn (Chap. 6)</p>
<p>Nitroaromatics</p> <p><i>Aerobic degradation</i></p> <ul style="list-style-type: none"> - Aromatic ring dioxygenation with release of nitrite and production of dihydroxy intermediates - Monoxygenation to epoxides - Partial reduction of nitro group forms NH₃ and hydroxylaminobenzene, which rearranges to a catechol with release of another NH₃ molecule 	<p>Nishino et al. (2000); Ye et al. (2003)</p>

Table 1. Continued

Contaminant	Catabolism	References
	<p><i>Nitrobenzene</i></p> <ul style="list-style-type: none"> - Aerobically metabolized by a partial reduction route, being converted to hydroxylaminobenzene, 2-aminophenol with dioxygenase-mediated ring cleavage or by an initial dioxygenation, producing catechol - An anaerobic reduction route produces aniline <p><i>Tritrotoluene (TNT)</i></p> <p>Initial reductive attack mechanisms appear to predominate (the nitro group makes TNT vulnerable to reductive attack and resistant to oxygen attack from aerobes), facilitating subsequent mono- and dioxygenase attack leading to ring fission</p>	<p>Hawari et al. (2000); Zhao et al. (2001)</p> <p>Esteve-Nunez et al. (2001); Popesku et al. (2003)</p>
Organophosphate derivatives	<p>Hydrolytic conversion of parathion and methyl parathion to dialkyl phosphates and 4-nitrophenol</p>	<p>Ye et al. (2003)</p>
Nitrate esters	<ul style="list-style-type: none"> - Sequential esterase-initiated hydrolytic denitration conversions with nitrate release - Nitroreductase transformations with production of nitrite or - A glutathione transferase-like conversion with production of nitric oxide and nitrite 	<p>Christodoulatos et al. (1997)</p>
Nitrogen heterocycles	<p><i>s-Triazine herbicides</i></p> <p>Biodegradation by oxidative N-dealkylation of the side chain, dechlorination and ring cleavage</p> <p><i>Hexahydro-1,3,5-trinitro-triazine</i></p> <ul style="list-style-type: none"> - Aerobic or anaerobic denitration followed by hydrolytic ring cleavage and further metabolism or autodecomposition - Anaerobic reductive nitroso route followed by hydrolytic ring cleavage - Direct anaerobic hydrolytic ring cleavage 	<p>Ralebitso (2002); see also Ye et al. (Chap. 7)</p> <p>Halasz et al. (2002)</p>
Plastics	<ul style="list-style-type: none"> - <i>Polyester</i>: many bacterial esterases degrade these polymers in nature - <i>Polyhydroxyalkanoates (PHA)</i>: degraded by bacterial PHA depolymerases - <i>Poly(lactic acid)(PLA)</i>: biodegradation with protease/esterase activity - <i>Polyurethane(PUR)</i>: incomplete biodegradation widely reported, PUR does not support growth - <i>Polyvinyl alcohol(PVA)</i>: completely degraded by some <i>Pseudomonas</i> strains mediated by either dehydrogenase or oxidase-catalyzed reactions attacking the main C-C chain links - <i>High molecular weight Nylon 66</i>: degraded by white-rot fungi using Mn-peroxidase 	<p>Prijambada et al. (1995); Deguchi et al. (1997); Li (1999); Shimao et al. (2000); Shimao (2001)</p>

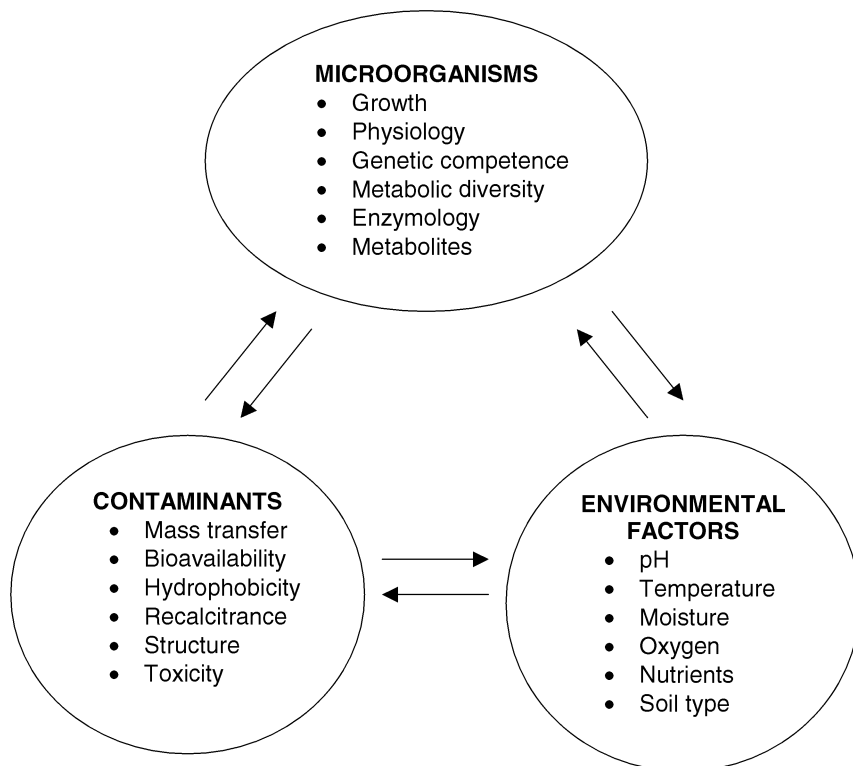


Fig. 1. The biodegradation system in bioremediation

very effective in removing chlorine atoms from PCBs and, indeed, in degrading other halogenated contaminants. These metabolic processes are described in Chapter 6 and one of the enzyme groups involved (reductive dehalogenase) is discussed in Chapter 9. Some anaerobic processes for degradation of nitrogen-containing contaminants are discussed in Chapter 7.

The inability of microbes to mineralize particular contaminants, but to partially transform them, typically means that these organisms require other substrates to support their growth. In such situations, the contaminants are transformed, i.e. 'cometabolism'. The diverse range of structures of PAH molecules requires the PAH-degrading microbes to have a range of enzymes capable of accepting the different PAHs as substrates or, alternatively, having a lesser number of enzymes having broad substrate specificity to PAHs. Consequently, in a particular bioremediation system, some of the more complex PAHs may not get transformed at all or may be partially catabolized into dead-end products. This topic