Oil Spill Eater II is mentioned by name on page 4, however the translation converted oil to Petroleum, so Oil Spill Eater II became Petroleum Spill Eater II. The description from the US EPA NCP List, makes it clear its Oil Spill Eater II since OSE II is the only EA type enzymatic additive.

Review Article

Petroleum-Degrading Enzymes: Bioremediation and New Prospects

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Anthropogenic forces, such as petroleum spills and the incomplete combustion of fossil fuels, have caused an accumulation of petroleum hydrocarbons in the environment. The accumulation of petroleum and its derivatives now constitutes an important environmental problem. Biocatalysis introduces new ways to improve the development of bioremediation strategies. The recent application of molecular tools to biocatalysis may improve bioprospecting research, enzyme yield recovery, and enzyme specificity, thus increasing cost-benefit ratios. Enzymatic remediation is a valuable alternative as it can be easier to work with than whole organisms, especially in extreme environments. Furthermore, the use of free enzymes avoids the release of exotic or genetically modified organisms (GMO) in the environment.

1. Introduction

Our planet hosts many different environments. From the Arctic to the Antarctic, there are deserts, rainforests, abyssal regions, and many other places where different forms of life can be found. Not all organisms can adapt and/or survive in diverse environments, but, instead, they inhabit specific environments according to their biotic and abiotic characteristics. However, microorganisms are everywhere; they have colonised diverse environments for thousands of years, including those that, for most organisms, are considered "extreme." In addition to colonising the environment, microorganisms colonise other organisms and are essential to life on our planet as we know it. Only a small proportion of bacteria are harmful. In fact, microorganisms are key components of food webs and biogeochemical cycles and in the maintenance and survival of plants, animals, and other organisms through symbiotic relationships.

Several microorganisms may be involved in the reactions of biogeochemical cycles, and in some cases they are the only biological agents capable of regenerating forms of elements needed for other organisms [1]. Collectively, microorganisms have a great metabolic diversity, which allows their ubiquity. Because of their ubiquitous nature, the biotechnological potential of microorganisms is virtually endless, with many possible applications. One of these applications is the utilisation of microorganisms or their enzymes in petroleum bioremediation approaches [1]. Biocatalysis can provide alternative ways to improve petroleum bioremediation approaches [2]; the screening for enzymes for this purpose is necessary. This paper presents some enzymatic applications for the degradation of petroleum toxic compounds and a discussion about improvements that could be used in petroleum enzymatic bioremediation.

2. Petroleum-Polluted Sites

Petroleum is a heterogeneous mixture of hydrocarbons, including aliphatic (n-alkanes), alicyclic, and aromatic hydrocarbons (i.e., polycyclic aromatic hydrocarbons), which varies in compositional and physical properties accor-ding to the reservoir's origin [3]. These hydrocarbons are organic compounds containing carbon and hydrogen, which are highly insoluble in water. Microorganisms can either degrade or produce hydrocarbons [4], depending on the presence of certain metabolic pathways, specific to each function in the environmental conditions.

Recently, anthropogenic practices such as industrial activities, petroleum and petroleum derivatives (such as gasoline, diesel, and kerosene spills), and incomplete combustion of fossil fuels have caused an accumulation of petroleum hydrocarbons in the environment [5]. In fact, petroleum and

derivatives have a major ecological impact on contaminated marine and terrestrial ecosystems [6]. All along this paper, we will consider the word "petroleum" encompassing also the petroleum derivatives.

Many important processes influence the destination of hydrocarbons in the environment. Among these are sorption, volatilisation, abiotic transformation (chemical or photochemical), and biotransformation [7]. Sorption and volatilisation do not destroy contaminants, but, instead, they only accumulate or transport them to another location. Abiotic chemical transformations involving organic contaminants are usually slow, while photochemical reactions are insignificant in most environments [5, 7, 8]. Because microorganisms are directly involved in biogeochemical cycles as key drivers of the degradation of many carbon sources, including petroleum hydrocarbons, the furthered understanding and application of petroleum biodegradation is a matter of great interest.

The presence of a high enzymatic capacity allows microbial communities to degrade complex hydrocarbons [9]. This capacity to modify or decompose certain pollutants, such as petroleum, summarises the importance of enzymes in the bioremediation process. Their genetic diversity contributes to the metabolic versatility of microorganisms for the transformation of contaminants into less-toxic final products, which are then integrated into natural biogeochemical cycles [9]. The chief benefit of the contaminant-degrading process is the complete mineralisation of compounds, as well as biomass formation [10–12]. Many biotic and abiotic factors can influence the effectiveness of petroleum contaminant biodegradation, including the presence and activity of petroleum-degrading microorganisms in the environment, competitiveness, availability and concentration of petroleum and nutrients, salinity, and temperature, among others [5].

3. Aerobic and Anaerobic Degradation of Petroleum and Petroleum-Degrading Enzymes

Numerous microorganisms, such as bacteria, cyanobacteria, green algae, and fungi, are capable of degrading different components of petroleum under different environmental conditions (e.g., aerobic and anaerobic conditions at varied salinities and pHs). The enzymatic apparatus provides these capabilities to microorganisms. Petroleum degradation occurs gradually by sequential metabolism of its compounds. The genes involved in degrading petroleum enzyme production may be located on chromosomal or plasmid DNA [13].

Biodegradation of hydrocarbons, both aliphatic and aromatic compounds, may occur under anaerobic or aerobic conditions [3]. Under aerobic conditions, oxygenase enzymes introduce oxygen atoms into hydrocarbons (monooxygenases introduce one oxygen atom to a substrate while dioxygenases introduce two). The anaerobic degradation is catalysed by anaerobic bacteria, such as sulphate-reducing bacteria, using different terminal electron acceptors [3].

Aerobic catabolism of hydrocarbons can be faster, due to the metabolic advantage of having the availability of O_2 as an electron acceptor [2]. The final product of the oxidation of saturated aliphatic hydrocarbons is acetyl-CoA, which is catabolised in the citric acid cycle, together with the production of electrons in the electron transport chain. This chain is repeated, further degrading the hydrocarbons, which are normally fully oxidised to CO_2 [1]. Aromatic hydrocarbons, such as benzene, toluene, xylene, and naphthalene, can also be degraded in aerobic conditions. The degradation of these compounds usually serves as an initial step in the formation of catechol or a structurally related compound. Once formed, catechol can be degraded, resulting in compounds that can be introduced into the citric acid cycle. Also these compounds can be completely degraded to CO_2 [1, 2].

Alkane hydroxylases are alkane-degrading enzymes that are distributed among many different species of bacteria, yeast, fungi, and algae [14]. Furthermore, van Beilen and Funhoff [14] proposed three categories of alkane-degrading enzyme systems: C1–C4 (methane to butane, oxidised by methane-monooxygenase-like enzymes), C5– C16 (pentane to hexadecane, oxidised by integral membrane nonheme iron or cytochrome P450 enzymes), and C17+ (longer alkanes, oxidised by essentially unknown enzyme systems). They then reported the compositions, cofactors, substrate ranges, and presence of the main groups of alkane hydroxylases (soluble methane monooxygenase (sMMO), particulate methane monooxygenase (pMMO), AlkB-related alkane hydroxylases, eukaryotic P450 (CYP52, class II), Bacterial P450 oxygenase system and dioxygenase (CYP153, class I). These authors also noted that microorganisms that are able to degrade alkanes can contain multiple alkane hydroxylases and can thus consume different substrate ranges. As already cited by van Hamme and colleagues in 2003 [3], to date, one of the most studied alkane degradation pathways is that described for *Pseudomonas putida* Gpo1, encoded by the OCT plasmid [15, 16]. In this case, the conversion of an alkane into an alcohol is first mediated by a membrane monooxygenase, soluble rubredoxin, and rubredoxin reductase [3]. van Hamme and colleagues [3] presented a model for alkane metabolism in gram-negative bacteria and described the locations and functions of the *ALK* gene products.

The catechol dioxygenase class of bacterial iron-containing enzymes is an example of an enzyme class involved in the degradation of aerobic aromatic hydrocarbons. These enzymes are able to catalyse the addition of molecular oxygen atoms to 1,2-dihydroxybenzene (catechol) and its derivatives, with subsequent cleavage of the aromatic ring [1–3]. Enzymes like catechol dioxygenases that are involved in aromatic ring cleavage are responsible for the wide variety of microorganisms capable of degrading aromatic compounds [13].

Despite the fact that petroleum degradation under aerobic conditions occurs faster than under anaerobic conditions, it is important to note that anaerobic degradation is also essential to the bioremediation process because in several cases the environmental conditions can include limitations of the oxygen availability, such as in mangroves, aquifers, and sludge digesters [5]. In anaerobic metabolism, generally, aromatic compounds are converted into benzoyl-CoA, which is target of the benzoyl-CoA reductase (BCR) action [17]. Depending on the environmental conditions, different

terminal electron acceptors can be used, such as nitrate, sulphate, and Fe (III); generally, the degradation pathways converge to benzoyl-CoA [2].

4. Bioremediation Applications

According to Nyer [18], the term "bioremediation" refers to all biochemical reactions of natural attenuation, which includes all biotic and abiotic processes used to reduce contaminant levels. "Biodegradation" is the primary mechanism to reduce biodegradable contaminants. This method offers low risks to contaminated sites, and it is an alternative with a favourable cost-benefit ratio for treatment [7, 8].

When feasible, bioremediation is usually applied after the use of physical and chemical methods and natural attenuation. It can be a slow process because its kinetics may be conditioned to various factors, such as temperature, salinity, microbial diversity, and C:N:P ration, among others [5]. Bioremediation techniques were improved after the spill of 41 million litres of petroleum from the *Exxon Valdez* in Alaska in 1989. More than 10 million dollars were spent on studies sponsored by the Exxon company on bioremediation from 1993 to 1997, and many patents were generated [5, 19].

The characterisation of petroleum-degrading strains and their metabolic pathways serves to improve bioremediation approaches. Bioremediation can occur either naturally or by the use of bioaugmentation (whole cell introduction) or biostimulation approaches (use of nutrients or conditions to stimulate the native microbial community) [5, 20]; isolated enzymes may also be used to transform the contaminant into less-toxic or nontoxic compounds [3, 5, 20].

Many authors have described bioaugmentation and biostimulation approaches to restore different petroleum-contaminated sites; both are accepted options for minimizing the impact of petroleum spills [5]. These approaches must be carefully studied and planned for each type of contaminant and environmental condition, as both present advantages and disadvantages. For instance, bioaugmentation success depends on the competitiveness of the inoculated strains in different environments [20]. Genetically modified organisms (GMOs) can also be used to improve petroleum degradation efficiency, but other limitations may complicate the procedure, such as problems with international legislation [20, 21]. In both cases (GMO or wild-type strains), the potential impacts of introducing degrading microorganisms in the presence of indigenous microbes must be evaluated [5, 20–22]. Considering biostimulation, it is only useful to be applied in environments where indigenous petroleum-degrading microorganisms are present. A search for alternative bioremediation strategies is crucial to increase their effectiveness in different locations.

Biocatalysis is opening new paths toward improving the development of products and processes to reduce industrial costs and the generation of toxic subproducts and, consequently, the impact on the environment. Both enzymatic bioremediation and new clean energy production are contributing to minimising fossil fuel damages [20]. Enzymatic remediation can be simpler than working with whole organisms. Some advantages, including the enzymatic

potential, can be increased in laboratory conditions [23]. The use of isolated enzymes does not generate toxic byproducts [24] and whole cell competitiveness is not necessary [20].

Sutherland and colleagues [23] summarised the main aspects to be considered, from search to production, in enzymatic bioremediation. First, for an enzyme to be selected for a bioremediation application, it needs to have the capacity to degrade the target contaminant into less-toxic products. It is also important to search for enzymes that do not depend on cofactors, which would increase process costs at the commercial level. After screening, the next step is to identify the gene encoding the selected enzyme and, if necessary, improve enzymatic production. Commercial companies produce their enzymes via large-scale industrial fermentation; unlysed cells are removed during downstream processing. The authors also noted that the purification of enzymes from other soluble materials in the fermented liquor is not required for environmental remediation, which can facilitate the production process and reduce costs, but they highlighted that shelf-life and environmental stability must be evaluated to ensure effectiveness of the enzyme against the contaminant. The steps and considerations outlined by Sutherland and colleagues [23] can be extrapolated to the bioremediation of any contaminant; their report describes how to produce an enzymatic bioremediation agent for different applications (Figure 1).

Polycyclic aromatic hydrocarbons (PAHs) are mutagenic, cytotoxic, and carcinogenic organic chemicals. PAHs are widely distributed in the environment as a result of the incomplete combustion of organic matter, emission sources, automobile exhaust, domestic matter, and other factors [25]. The enzymatic remediation of PAHs has been proposed by many authors [25, 26]. PAH degradation under aerobic conditions involves the oxidation of the aromatic ring by specific dioxygenases, as described above, and a complete biotransformation into CO_2 and water [26]. As we have previously described, the BTEX compounds (benzene, toluene, ethylbenzene, and xylene) can be degraded in both aerobic and anaerobic conditions by microorganisms such as sulphate reducers.

As an example of enzymatic bioremediation, PAH detoxification can be achieved by the use of laccases [27] (enzymes capable of catalysing the oxidation of phenols, polyphenols, and anilines, coupled to the 4-electron reduction of molecular oxygen to water) [28]. A great advantage of the enzymatic bioremediation of xenobiotics that are either hydrophobic or poorly soluble in aqueous solutions, such as PAHs, is that enzymatic oxidation can occur in the presence of organic solvents [27]. A disadvantage is that the relevant enzymes can be unstable, inhibited, or denatured in organic solvents. In the work of Bulter and colleagues [29] laccase was expressed from *Myceliophthora thermophila* (MtL) in *Saccharomyces cerevisiae*, using directed evolution, and extensively improved laccase expression.

Recently, Scott and colleagues [30] successfully reported an initial field trial with an enzyme-based product, based on the enzyme TrzN, demonstrating that the technology can efficiently remediate water bodies contaminated with
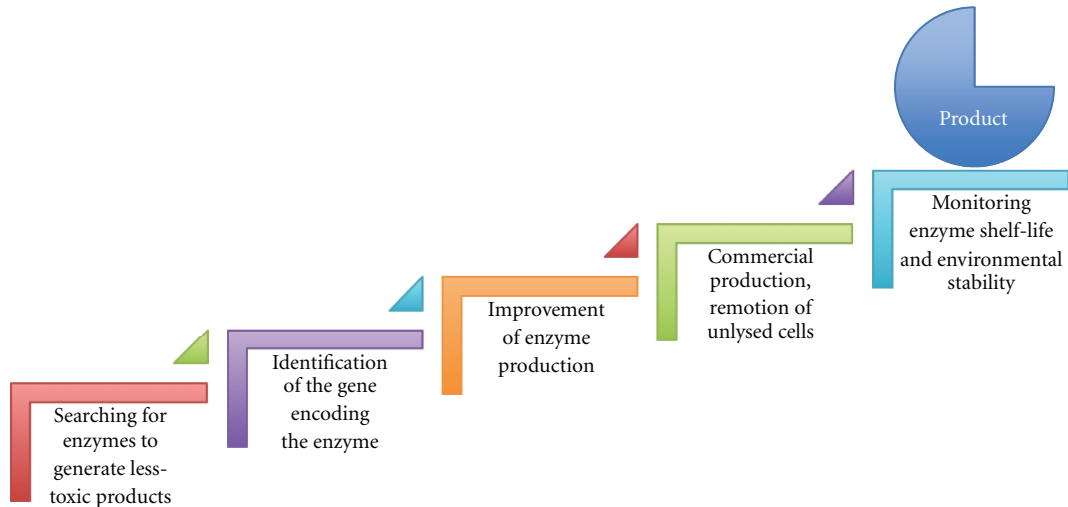
FIGURE 1: General steps proposed by Sutherland and colleagues [23] to get from bioprospection to an enzymatic bioremediation product.

herbicides. However, few field studies with enzymatic bioremediation are currently available.

Whitely and colleagues [26] cited that until 2004, there were over 1000 described enzymes involved in the biodegradation of aromatic systems (organic pollutants or otherwise).

It has been reported that worldwide sales of environmental biotechnology products for the US manufacturers, including microorganisms, enzymes, microbial blends, and nutrients, totalled U.S. $153.87 million by 2006 [31]. The estimations for increased sales of microbial blends were higher than the estimations for isolating microorganisms and enzymes because the latter have limited market potential [32].

Despite the advantages of enzymatic bioremediation, there are also limitations and features required for enzymatic remediation which restricts its applicability to a few enzyme classes [30]. Bioremediation enzymes must be adapted to relatively specific environmental conditions and must be rather independent of cofactors [20, 23].

In fact, to date, the U.S. EPA (Environmental Protection Agency) has currently listed (2011) 20 bioremediation agents and only one pure enzyme additive. The product, Petroleum Spill Eater II, is described by the producer as a "bioremediation agent (biological enzyme additive (previously listed as a nutrient additive))," with a 5-year shelf-life [32]. The producer indicated a reduction of 36.9 and 33.6% of alkanes and aromatics, respectively, after 7 days, and reduction of 89.8 and 89.6% 28 days after Petroleum Spill Eater II application, which represent great reductions over a short period of time.

Generally, enzymatic bioremediation limitations are still basically related to high costs; enzyme production typically generates a low yield of enzymes, and enzyme stability must often be optimised in the field.

5. Molecular Biology, Metabolic Engineering and Future Prospects

Despite all the advantages related to enzymatic bioremediation, high production costs, low yields, and enzymatic inhibition are some of the problems that must be overcome.

Many production improvements are necessary to avoid nonprohibitive processes. Therefore, molecular tools are being widely explored to provide competitive enzymatic bioremediation products. Molecular tools allow us to detect genes related to degrading enzymes in environmental samples or isolates, thus serving as powerful tools for bioprospection. Furthermore, DNA engineering can considerably improve enzyme yield with lower costs [20].

Enzymatic bioremediation improved with molecular tools can be particularly suitable for situations where rapid remediation is required [23]. Alcalde and colleagues [20] reported that recent studies of protein engineering, metagenomics, and proteomics are effectively contributing to cost reduction, minimising chemical use and also improving cost-benefit ratios. The use of molecular tools for biocatalysis applications can also help solving the problem of GMO use in the environment [20]; for instance, if the production of a modified enzyme is performed *in vitro*, it is not necessary to introduce the modified organism into the natural environment.

Many PCR primers that target genes related to petroleum-degrading enzymes, both in aerobic and anaerobic conditions, have already been described (Table 1). The utilisation of these already-characterised primers may facilitate environmental screening of degrading abilities and may help to evaluate the potentials of microbial isolates. More primers can be described for specific pathways or to improve the comprehensiveness of known primers using available databases.

The benefits provided by molecular tools can open unlimited windows of opportunity, as it is possible to detect genes from cultivable or noncultivable organisms (using metagenomics) and to express these genes in cultivable organisms, using enzymes that were not yet described. For instance, the use of fosmid and cosmid shotgun metagenomic libraries offers a great improvement to the bioprospection of new enzymes. The possibility of identifying and using genes from yet-undescribed microorganisms increases possible enzyme targets from about 0.1 to 1%

TABLE 1: Examples of references describing or modifying primers to amplify genes involved in petroleum degradation.

Target	Function	References
Aerobic degradation		
Catechol 2,3-dioxygenase genes	Degradation of aromatic compounds	[33, 34]
ALKA and/or *ALKB* genes	Encode enzymes related to alkane degradation	[35–39]
Anaerobic degradation		
bamA gene	Encodes 6-OCH-hydrolases (last step of the route of dearomatization of benzoyl-CoA)	[40–42]
bssA gene	encodes the α subunit of benzylsuccinate synthase (BSS), which starts the anaerobic degradation of toluene and xylene	[43–45]

of microbial cells (consisting of cultivable microbial cells in environmental samples), including all available DNA in that sample [46]. Molecular tools also allow us to increase expression levels manipulating not only physiochemical conditions (optimal conditions), but also the organisms at a genetic level, to improve enzyme production in many different conditions, for instance, improving the efficiency and speed of the petroleum degradation, decreasing the time of the remediation process. Genetic manipulation would be also useful to allow or improve the petroleum degradation in extreme environments, such as cold or hypersaline sites. The use of free extremozymes would be advantageous in these environments, since it avoids some of the limitations of the bioremediation using whole cells in extreme conditions, such as microbial competitiveness.

The advances in high-throughput "omics" techniques are improving the study of microbial ecology, including biodegradation processes, for instance, identifying and quantifying bacterial enzymes responsible for aromatic hydrocarbon metabolism [47].

6. Conclusions

Considering that bioremediation remains a field with much work to be done, with few extremely effective field applications due to the extremely diverse conditions found in different ecosystems, the development of alternative or complementary strategies is continually encouraged.

Despite the fact that in many cases the costs are still prohibitive, enzymatic bioremediation can provide real benefits to the environment, avoiding the conditions that are required for whole-cell applications, especially in extreme environments. Furthermore, enzymatic effectiveness can be improved *in vitro* also using molecular tools, such as DNA engineering, to generate super bioremediators, which can present advantages in field.

Enzymatic bioremediation also influences other biological areas, such as medicine, since Rittmann and Schloendorn [48] proposed the idea of "medical bioremediation," based on and inspired by environmental bioremediation principles. Medical bioremediation studies have proposed the utilisation of one or several microbial enzymes to degrade intracellular accumulators that impair cellular function and viability and cause diseases such as atherosclerosis, macular degeneration, and neurodegenerative diseases. Medical bioremediation could be effective enough to eliminate intracellular accumulators from affected cells.

Our experience with whole cells indicates that bacterial consortium is a better alternative for the degradation of diverse and complex petroleum compounds. Likewise, the use of enzyme mixture is probably a more suitable tool for use against petroleum contamination in the environment because specific enzymes for recalcitrant and toxic compounds can be applied together.
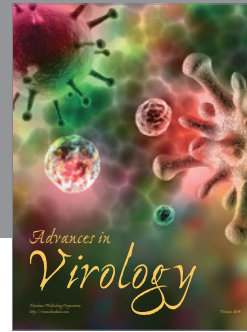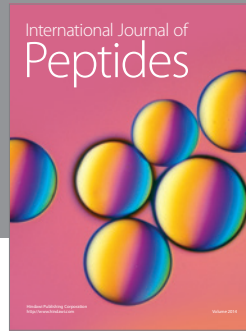
Acknowledgments

References

[1] M. T. Madigan, J. M. Martinko, P. V. Dunlap, and D. P. Clark, *Brock Biology of Microorganisms*, Benjamin Cummings, 12th edition, 2010.

[2] B. Cao, K. Nagarajan, and K. C. Loh, "Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches," *Applied Microbiology and Biotechnology*, vol. 85, no. 2, pp. 207–228, 2009.

[3] J. D. van Hamme, A. Singh, and O. P. Ward, "Recent advances in petroleum microbiology," *Microbiology and Molecular Biology Reviews*, vol. 67, no. 4, pp. 503–549, 2003.

[4] H. L. Ehrlich, *Geomicrobiology*, Marcel Dekker, New York, NY, USA, 1995.

[5] H. F. Santos, F. L. Carmo, J. E. Paes, A. S. Rosado, and R. S. Peixoto, "Bioremediation of mangroves impacted by petroleum," *Water, Air, and Soil Pollution*, vol. 216, no. 1–4, pp. 329–350, 2011.

[6] H. F. Santos, J. C. Cury, F. L. do Carmo et al., "Mangrove bacterial diversity and the impact of petroleum contamination revealed by pyrosequencing: bacterial proxies for petroleum pollution," *PLoS One*, vol. 6, no. 3, Article ID e16943, 2011.

[7] A. Korda, P. Santas, A. Tenente, and R. Santas, "Petroleum hydrobcarbon bioremediation: sampling and analytical techniques, in situ treatments and commercial microorganisms currently used," *Applied Microbiology and Biotechnology*, vol. 48, no. 6, pp. 677–686, 1997.

[8] M. A. C. Crapez, A. L. N. Borges, M. G. S. Bispo, and D. C. Prereira, "Tratamento para derrames de petróleo," *Ciência Hoje*, vol. 30, no. 179, pp. 32–37, 2002.

[9] M. Alexander, *Biodegradation and Bioremediation*, Academic Press, San Diego, Calif, USA, 1994.

[10] R. M. Atlas, "Microbial hydrocarbon degradation-bioremediation of oil spills," *Journal of Chemical Technology and Biotechnology*, vol. 52, no. 2, pp. 149–156, 1991.

[11] C. D. Cunha and S. G. F. Leite, "Gasoline biodegradation in different spetroleum microcosms," *Brazilian Journal of Microbiology*, vol. 31, no. 1, pp. 45–49, 2000.

[12] K. Watanabe and N. Hamamura, "Molecular and physiological approaches to understanding the ecology of pollutant degradation," *Current Opinion in Biotechnology*, vol. 14, no. 3, pp. 289–295, 2003.

[13] J. B. Broderick, "Catechol dioxygenases," *Essays in Biochemistry*, vol. 34, no. 11, pp. 173–189, 1999.

[14] J. B. van Beilen and E. G. Funhoff, "Alkane hydroxylases involved in microbial alkane degradation," *Applied Microbiology and Biotechnology*, vol. 74, no. 1, pp. 13–21, 2007.

[15] J. B. van Beilen, M. G. Wubbolts, and B. Witholt, "Genetics of alkane oxidation by *Pseudomonas oleovorans*," *Biodegradation*, vol. 5, no. 3-4, pp. 161–174, 1994.

[16] J. B. van Beilen, S. Panke, S. Lucchini, A. G. Franchini, M. Rothlisberger, and B. Witholt, "Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes," *Microbiology*, vol. 147, no. 6, pp. 1621–1630, 2001.

[17] A. Hosoda, Y. Kasai, N. Hamamura, Y. Takahata, and K. Watanabe, "Development of a PCR method for the detection and quantification of benzoyl-CoA reductase genes and its application to monitored natural attenuation," *Biodegradation*, vol. 16, no. 6, pp. 591–601, 2005.

[18] E. K. Nyer, *Groundwater and Spetroleum Remediation: Pratical Methods and Strategies*, Ann Arbor Press, Ann Arbor, Mich, USA, 1998.

[19] P. C. S. Seabra, J. V. Bomtempo, and N. O. Costa, "Biotechnology in the petroleum industry: 1988–1997," Technical Bulletin 42, PETROBRAS, Rio de Janeiro, Brazil, 1999, http://wwwv2.petrobras.com.br/boletim/Boletim_42_1_4/3Biotec.PDF.

[20] M. Alcalde, M. Ferrer, F. J. Plou, and A. Ballesteros, "Environmental biocatalysis: from remediation with enzymes to novel green processes," *Trends in Biotechnology*, vol. 24, no. 6, pp. 281–287, 2006.

[21] D. Paul, G. Pandey, J. Pandey, and R. K. Jain, "Accessing microbial diversity for bioremediation and environmental restoration," *Trends in Biotechnology*, vol. 23, no. 3, pp. 135–142, 2005.

[22] R. S. Peixoto, R. F. Silva, and A. S. Rosado, "Biorremediação de ambientes contaminados com petróleo e seus derivados," *Microbiologia in Foco*, vol. 8, pp. 17–30, 2009.

[23] T. D. Sutherland, I. Horne, K. M. Weir et al., "Enzymatic bioremediation: from enzyme discovery to applications," *Clinical and Experimental Pharmacology and Physiology*, vol. 31, no. 11, pp. 817–821, 2004.

[24] L. Setti, G. Lanzarini, and P. G. Pifferi, "Whole cell biocatalysis for an petroleum desulfurization process," *Fuel Processing Technology*, vol. 52, no. 1–3, pp. 145–153, 1997.

[25] S. K. Samanta, O. V. Singh, and R. K. Jain, "Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation," *Trends in Biotechnology*, vol. 20, no. 6, pp. 243–248, 2002.

[26] C. G. Whiteley and D. J. Lee, "Enzyme technology and biological remediation," *Enzyme and Microbial Technology*, vol. 38, no. 3-4, pp. 291–316, 2006.

[27] M. Alcalde, T. Bulter, M. Zumárraga et al., "Screening mutant libraries of fungal laccases in the presence of organic solvents," *Journal of Biomolecular Screening*, vol. 10, no. 6, pp. 624–631, 2005.

[28] M. Smith, F. Thurston, and D. A. Wood, "Fungal laccases: role in delignification and possible industrial applications," in *Multi-Copper Oxi- Dases*, A. Messerschmidt, Ed., pp. 201–224, World Scientific Publishing, Singapore, 1997.

[29] T. Bulter, M. Alcalde, V. Sieber, P. Meinhold, C. Schlachtbauer, and F. H. Arnold, "Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution," *Applied and Environmental Microbiology*, vol. 69, no. 2, pp. 987–995, 2003.

[30] C. Scott, S. E. Lewis, R. Milla et al., "A free-enzyme catalyst for the bioremediation of environmental atrazine contamination," *Journal of Environmental Management*, vol. 91, no. 10, pp. 2075–2078, 2010.

[31] http://www.bccresearch.com/report/BIO019A.html.

[32] U.S. EPA, "States Environmental Protection Agency National Contingency Plan Product," 2011, http://www.epa.gov/oswer-oe1/content/ncp/product_schedule.

[33] M. B. Mesarch, C. H. Nakatsu, and L. Nies, "Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR," *Applied and Environmental Microbiology*, vol. 66, no. 2, pp. 678–683, 2000.

[34] K. M. Anderson, L. Jaquinod, M. A. Jensen, N. Ngo, and R. W. Davis, "A novel catechol-based universal support for oligonucleotide synthesis," *Journal of Organic Chemistry*, vol. 72, no. 26, pp. 9875–9880, 2007.

[35] L. G. Whyte, C. W. Greer, and W. E. Inniss, "Assessment of the biodegradation potential of psychrotrophic microorganisms," *Canadian Journal of Microbiology*, vol. 42, no. 2, pp. 99–106, 1996.

[36] A. Vomberg and U. Klinner, "Distribution of *alkB* genes within n-alkane-degrading bacteria," *Journal of Applied Microbiology*, vol. 89, no. 2, pp. 339–348, 2000.

[37] T. Kohno, Y. Sugimoto, K. Sei, and K. Mori, "Design of primers and gene probes for general detection of alkane-degrading bactéria," *Microbes and Environments*, vol. 17, no. 3, pp. 114–121, 2002.

[38] K. Kloos, J. C. Munch, and M. Schloter, "A new method for the detection of alkane-monooxygenase homologous genes (*alkB*) in soils based on PCR-hybridization," *Journal of Microbiological Methods*, vol. 66, no. 3, pp. 486–496, 2006.

[39] L. Wang, W. Wang, Q. Lai, and Z. Shao, "Gene diversity of CYP153A and AlkB alkane hydroxylases in oil-degrading bacteria isolated from the Atlantic Ocean," *Environmental Microbiology*, vol. 12, no. 5, pp. 1230–1242, 2010.

[40] A. Aroonsri, S. Kitani, S. Uk. Choi, and T. Nihira, "Isolation and characterization of *bamA* genes, homologues of the c-butyrolactone autoregulator-receptor gene in *Amycolatopsis mediterranei*, a rifamycin producer," *Biotechnology Letters*, vol. 30, no. 11, pp. 2019–2024, 2008.

[41] K. Kuntze, Y. Shinoda, H. Moutakki et al., "6-Oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolases from obligately anaerobic bacteria: characterization and identification of its gene as a functional marker for aromatic compounds degrading anaerobes," *Environmental Microbiology*, vol. 10, no. 6, pp. 1547–1556, 2008.

[42] M. Staats, M. Braster, and W. F. Röling, "Molecular diversity and distribution of aromatic hydrocarbon-degrading anaerobes across a landfill leachate plume," *Environmental Microbiology*, vol. 13, no. 5, pp. 1216–1227, 2011.

[43] H. R. Beller, S. R. Kane, T. C. Legler, and P. J.J. Alvarez, "A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene," *Environmental Science and Technology*, vol. 36, no. 18, pp. 3977–3984, 2002.

[44] S. Botton, M. van Harmelen, M. Braster, J. R. Parsons, and W. F. M. Röling, "Dominance of *Geobacteraceae* in BTX-degrading enrichments from an iron-reducing aquifer," *FEMS Microbiology Ecology*, vol. 62, no. 1, pp. 118–130, 2007.

[45] C. Winderl, S. Schaefer, and T. Lueders, "Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker," *Environmental Microbiology*, vol. 9, no. 4, pp. 1035–1046, 2007.

[46] M. Zeyaullah, M. R. Kamli, B. Islam et al., "Metagenomics–an advanced approach for non- cultivable micro-organisms," *Biotechnology and Molecular Biology Reviews*, vol. 4, no. 3, pp. 49–54, 2009.

[47] S. J. Kim, O. Kweon, and C. E. Cerniglia, "Proteomic applications to elucidate bacterial aromatic hydrocarbon metabolic pathways," *Current Opinion in Microbiology*, vol. 12, no. 13, pp. 301–309, 2009.

[48] B. E. Rittmann and J. Schloendorn, "Engineering away lysosomal junk: medical bioremediation," *Rejuvenation Research*, vol. 10, no. 3, pp. 359–365, 2007.