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Microbial Outlook for the Bioremediation of Crude Oil Contaminated Environments

Rachel Passos Rezende, Bianca Mendes Maciel,
João Carlos Teixeira Dias and Felipe Oliveira Souza
Universidade Estadual de Santa Cruz
Brazil

1. Introduction

The oil and petrochemical industry in the course of the exploration, production, refining and transportation operations required for the commercialization of oil and its derivatives have contaminated with toxic wastes the environment, which requires urgent remediation. The biological treatment of these wastes aims the rational exploitation of microorganisms that use hydrocarbons as the source of carbon and energy. This biotechnological strategy includes one of the processes most commonly used by industries today: *bioremediation*. Bioremediation makes possible the recovery of oil contaminated sites through biostimulation, which increases the hydrocarbon-degrading ability of native microorganisms, thereby transforming or mineralizing the pollutants.

The study of microbes in bioremediation systems makes possible the selection of microorganisms with potential for the degradation and production of compounds with biotechnological applications in the oil and petrochemical industry. These applications include the use of biosurfactants for advanced oil recovery, cleaning of tanks, and the dispersion of oil spills on water environments. The current concern for the environment has stimulated research on biosurfactants, which are considered to be more acceptable because of their higher biodegradability and lack of toxicity compared with chemical synthesized surfactants.

The study of native microbial diversity is fundamental to understand the roles played by microorganisms in contaminated sites and for determining their interactions with the environment. Such information can generate economic and strategic benefits in several areas.

2. Oil bioremediation

Petroleum is a complex mixture of aliphatic, alicyclic, aromatic hydrocarbons, and smaller proportions of heteroatom compounds, such as sulfur, nitrogen, and oxygen. Crude oil also contains organometallic complexes containing nickel and vanadium in much smaller proportions compared to the other constituents; however these organometallic compounds are problematic during crude oil refining (Head et al., 2003). The susceptibility of these compounds to biodegradation varies with the molecular concentration (molecular weight),

composition, and electronic stability (Chosson et al., 1991). For instance, it is difficult for microorganisms to use long-chain alkanes with several branches as a carbon source because of the increased hydrophobicity of the molecule, which makes the molecule more resistant to oxidization during microbial assimilation. Thus, in these situations the biodegradation rate is reduced. Another relevant factor is the bioavailability of nutrients, particularly of nitrogen, phosphorus, and trace elements. The lack of such nutrients in ancient oil reservoirs explains their continued existence, without any significant degradation.

Different microorganism species have different enzymatic abilities and preferences for the degradation of oil compounds. Some microorganisms degrade linear, branched, or cyclic alkanes. Others prefer mono- or polynuclear aromatics, and others jointly degrade both alkanes and aromatics.

Although the constituent atoms in oil molecules generally provide nutrients for microorganisms, these same molecules can be bioinhibitors because of their intrinsic toxic properties. The lighter alkanes, for example, those in the C_5 to C_9 range, are toxic to many organisms because of their solvent effects, which tend to break the structure of microbial lipid membranes. Aromatic hydrocarbons, particularly molecules with more than five condensed aromatic rings called polycyclic aromatic hydrocarbons (PAHs), are especially harmful to microorganisms. These molecules are lipophilic and interact with the cell membrane.

Several methods can be employed to remove oil wastes and derivatives from soil and water. These include physical (spray, vapor extraction, stabilization, solidification), chemical (photo-oxidation, dissolution, detergent use), and biological methods (bioremediation). All these methods can be used in the treatment of contaminated sites depending on the priorities and circumstances of each case. Physical treatments have the advantage of separating contaminants without destroying or chemically modifying them but also have limitations. Most of these techniques are too costly to be implemented on a large scale and require continuous monitoring and control to achieve optimal performance. In addition, they do not usually result in complete contaminant destruction, removal, or degradation. Biological processes, in contrast, have the promise of complete contaminant removal and are usually simpler and less expensive than other alternatives.

Bioremediation is an alternative process that involves accelerated natural biodegradation. Bioremediation techniques can be performed either *in-situ* or *ex-situ*. In *in-situ* processes, the biological remediation is conducted at the contaminated site, whereas in *ex-situ* processes, the contaminated medium is extracted and processed at off-site purification facilities. Usually, bioremediation techniques involve biostimulation, i.e., the addition of nutrients (mainly nitrogen, phosphorus, and potassium), and the adjustment of environmental conditions (pH, temperature, moisture, aeration) to facilitate native microbial growth. Microorganisms with the ability to quickly degrade specific contaminants can also be added in a technique known as bioaugmentation. Thus, bioremediation can be defined as a process that exploits microbial diversity and metabolic versatility to transform chemical contaminants into less-toxic products. This alternative offers significant advantages over other processes because the costs are reduced and local pollution is minimal.

Among the various treatments methods for the removal of oily wastes, *landfarming* is the most widely used, as this method makes possible to transform pollutants into products that

are less toxic to humans and the environment. Landfarming consists on the *ex-situ* treatment of the oily sludge waste by means of biodegradation using native microorganisms. Initially, the soil is distributed over a large flat surface area above an impervious layer. The oily sludge landfarm is divided into cells containing soil, usually at different treatment stages, in which the oily wastes are placed for biodegradation (Figure 1).

In oily sludge landfarm systems, the soil is periodically aerated by plowing and enriched with nutrients to stimulate native aerobic microbial metabolism, thereby favoring biodegradation (Ausma et al. 2003). This soil is treated until the contaminant concentrations are below or at acceptable limits as established by environmental control agencies.

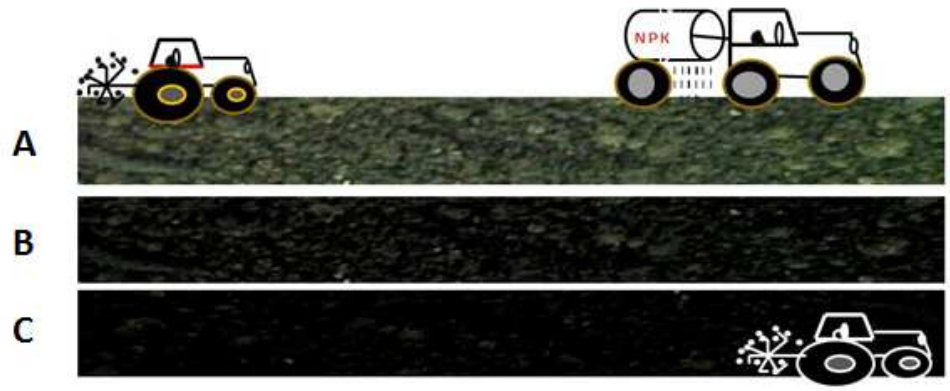


Fig. 1. Schematic representation of a *landfarm*. **(A)** Landfarm cell during the plowing process that promotes aeration and biostimulation by nutrient addition. (B) Representation of a landfarm cell at equilibrium. **(C)** Representation of a landfarm cell newly inoculated with oily sludge during the aerobic metabolism-promoting plowing process.

Biopiling is another technique that is increasingly applied to biological waste treatment by the petrochemical industry (Figure 2). Biopiles involve the transfer of contaminated soil into cells suspended above the ground, where organic contaminants are degraded by aerobic microbial activity. This technique is different from landfarming because it allows control over the levels of moisture, pH, temperature, oxygen, nitrogen, and phosphorus. Thus, the nutritional and physical-chemical soil conditions ideal for bioremediation are maintained. In biopiles, the mass transfer efficiency, i.e., the transfer efficiency of air, water, and nutrients, makes it possible to obtain better results in contaminant reduction. Structural materials that can be used to increase mass transfer in biopiles include sand, straw, wood chips, sawdust, and dry manure (Seabra 2005). To improve aeration in biopiles, air pipes are used for artificial ventilation or soil can be raked periodically. Aeration determines treatment success because aerobic degradation processes are more efficient than anaerobic processes. Biopiles are very efficient when applied to sandy soils if compared to clay soils because clay soils can form agglomerates that reduce soil permeability, thereby making the mass transfer difficult (Seabra, 2005).

As in landfarms, the microbial metabolism in biopiles is stimulated by the addition of nutrients whose concentrations must be monitored, as nutritional excess can inhibit

microbial activity (Khan et al., 2004). Metabolic activity is constantly monitored by measuring the reduction of contaminants via chromatography. Biopiles are simple to implement and advantageous because they require smaller areas and relatively short treatment compared with other techniques such as landfarms.

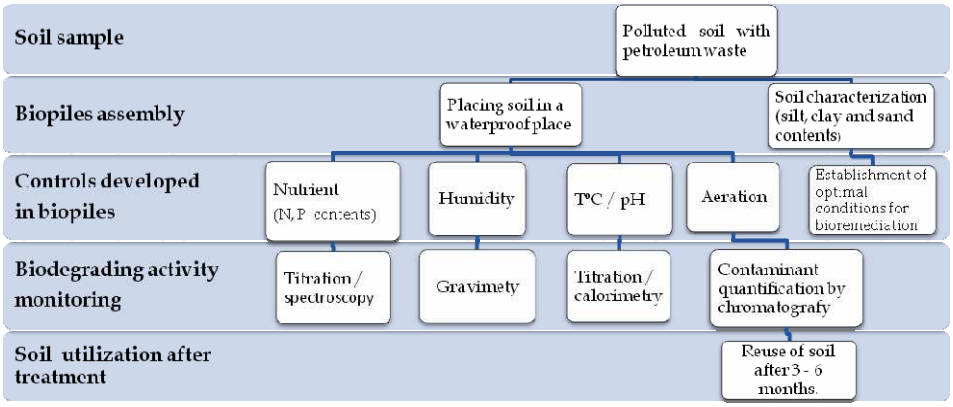


Fig. 2. Schematic representation of the structure and application of the biopile technique (Silva, 2004, adapted).

3. Biosurfactants

Compounds with surface-active properties synthesized by microorganisms are called biosurfactants and consist of metabolic byproducts from a wide variety of bacteria, fungi, and yeasts. Biosurfactants are amphipathic molecules composed of hydrophobic and hydrophilic groups that are able to spontaneously associate between liquid interfaces (oil/water and water/oil) with different degrees of polarity. As a result, the surface and interfacial tensions are reduced, and large molecular aggregates, called micelles, are formed. These properties give biosurfactants the following attributes: detergency, emulsification, lubrication, foaming ability, solubilization, and phase dispersion.

Micelle formation is associated with the critical micellar concentration (CMC) of the surfactant. Below its CMC, the surfactant is predominantly in monomer form, but when the concentration approaches the CMC, a dynamic equilibrium leads to micelle formation, with an individual micelle typically representing an association of 30 to 200 monomers (Maniasso, 2001). The CMC depends on the surfactant structure and is commonly measured to assess the surface-active efficiency in oil solubilization between the aqueous phases (Desai & Banat, 1997).

The activity of biosurfactants is commonly quantified by measuring the change in surface and interfacial tensions and by calculating the CMC. The surface tension of distilled water is 72 mN/m and the addition of a surfactant reduces its surface tension to 30 mN/m. Most biosurfactants render a surface tension in aqueous systems of approximately 30 mN/m and an interfacial tension in oil-water systems of approximately 1 mN/m. The CMC of biosurfactants is estimated by continuously increasing the concentration of biosurfactants in aqueous solutions and measuring the surface tension of the aqueous system after each

addition of biosurfactant using a tensiometer. The CMC is reached when the surface tension value levels off. Thus, the CMC for most biosurfactants ranges from 1-2,000 mg/L (Desai & Banat, 1997; Nitschke & Pastore, 2002).

Because biosurfactants possess significant advantages over chemically synthetized surfactants, they have been widely studied and used in various industries, such as the petrochemical industry, in the food industry, as additives in the manufacture of cosmetics, for biological control, in medicinal therapy, in bioremediation, and several other applications (Banat et al., 2000; Desai & Banat, 1997; Fiechter, 1992; Karanth et al., 1996; Makkar & Cameotra, 2002; Nitschke & Pastore, 2002; Ron & Rosenberg, 2001). The advantages of biosurfactants include low toxicity, high biodegradability, tolerance to temperature, pH, and salinity; high foaming activity, excellent surface and interfacial activity (they decrease surface tension at low concentrations), production from renewable substrates; and their potential to be structurally modified by genetic engineering or biochemical techniques. Furthermore, there is a large and chemically diverse group of biosurfactants with distinct physical properties. The broad diversity enables biosurfactants to be better selected for specific applications.

Hydrocarbon-degrading microorganisms play a key role in bioremediation, and biosurfactant production from these processes is important. There are two mechanisms by which biosurfactants enhance the hydrocarbon degradation rate. First, biosurfactants can solubilize hydrophobic compounds between the micelle structures, effectively increasing hydrocarbon solubility and their availability to the cell. Second, biosurfactants can cause the cell surface to become more hydrophobic because of an increased cell surface association with the hydrophobic substrate (Al-Tahhan et al., 2000).

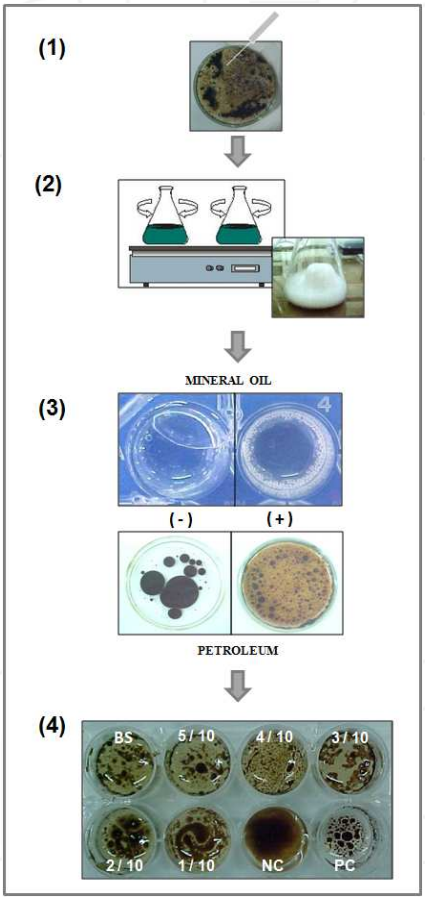
3.1 Selection of biosurfactant-producing microorganisms

The increasing industrial interest in microbial surfactant production has motivated many researchers to study and develop fast and safe methods for the selection of biosurfactant-producing microorganisms. Among these methods, the drop-collapse test (Jain et al., 1991) deserves special attention because it is fast, practical, and reproducible. Youssef et al. (2004) compared different methods for detecting potential biosurfactant-producing microorganisms, and the drop-collapse test proved to be a reliable method for the selection of microorganisms. In this method, a small amount of water is applied to a hydrophobic compound. In the absence of surfactants, a drop forms because the polar water molecule is repelled by the hydrophobic surface. However, if a surfactant is added to the water, the accompanying interfacial tension reduction results in dispersion (collapse) of the water drop on the hydrophobic surface. Based on this principle, the drop-collapse test can be used to select potential biosurfactant-producing microorganisms.

Bodour and Miller-Maier (1998) developed a modified drop-collapse test method for the quantitative analysis of biosurfactants. In this technique, a standard concentration curve for each surfactant is calculated. Drops of a known surfactant solution at various concentrations are placed on a solid and flat surface. After one minute, the diameter of each drop is measured, and a standard curve of drop diameter (Y axis) as a function of surfactant concentration (X axis) is plotted. As the surfactant concentration increases, the drop diameter also increases until the CMC is reached, at which point the diameter remains

constant. This curve is used to determine the surfactant concentration from unknown samples by comparing the diameters found with the standard drop diameter. The authors demonstrated that this method had greater sensitivity and reproducibility than surface tension measurements.

Biosurfactant-producing microbial strains can be selected by isolating native microorganisms from environments naturally contaminated with oil. In this case, the drop-collapse technique can be used for screening purposes. Figure 3 illustrates a simplified method for the isolation and selection of potential biosurfactant- producing microorganisms.



*DM composition: 2% (w/v) agar, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) K_2HPO_4, 0.1% (w/v) NH_4NO_3, 0.05% (w/v) $MgSO_4$, saturated $FeSO_4$ solution and saturated $CaCl_2$ solution added to the medium at 0.001% (v/v), pH 7.2. **EM composition: 0.5% w/v beef extract, 1% w/v peptone, 0.5% w/v NaCl, and pH 7.0 (Li et al., 2000)

Fig. 3. Schematic representation of an experiment for the selection of biosurfactant-producing microorganisms. **(1)** Isolation of microorganisms able to grow in degradation

medium (DM*) containing 2% petroleum as the sole carbon source. **(2)** Microbial colonies isolated from the DM medium are inoculated into 100 mL flasks containing 5 mL of enrichment medium (EM**) and incubated at room temperature (28°C ± 2°C) under constant agitation at 130 rpm for five days. At this stage, it is possible to observe the formation of foam produced by the biosurfactant. **(3)** To select microorganisms that exhibit surfactant activity, the drop-collapse method is performed in mineral oil and petroleum after the fifth day of growth. Then 5-mL aliquots of supernatant from each microbial suspension grown in EM are placed in the center well of an ELISA plate containing 1.8 mL of each type of oil previously deposited. The results are visually determined after 1 minute. In positive samples, there is a collapse of the oil drop. The photographs on the left depict the negative control (water and oil), and the pictures on the right indicate the test samples that were positive for biosurfactant production. **(4)** A quantitative analysis of biosurfactant production in petroleum with the drop-collapse test after increasing dilutions (5/10, 4/10, 3/10, 2/10, and 1/10) of supernatants from microbial cultures in sterile distilled water. BS. – Pure biosurfactant (undiluted) with added petroleum; NC - Negative control (water and oil); PC - Positive control with 1% SDS and petroleum.

3.2 Microbial growth kinetics related to biosurfactant production

Because of the amphipathic structure of the surfactant molecules, two biochemical pathways are necessary for biosurfactant production. One pathway is related to the synthesis of the hydrophobic portion, and the other is related to the synthesis of the hydrophilic portion of the molecule. These pathways produce a hydrocarbon and a carbohydrate, respectively. Several pathways, involving specific enzymes, are used for the synthesis of these two precursor groups. In many cases, the regulatory genes for specific enzymes are the first to be expressed, and despite the diversity among biosurfactants, there are some common characteristics in their synthesis and gene expression regulation. The study of molecular genetics in biosurfactant production is still new and many aspects remain unclear. However, the genetic mechanisms for the synthesis and regulation of rhamnolipid and surfactin in *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively, have been extensively studied (see review by Sullivan, 1998).

Generally, the genes required for biosurfactant production are controlled by *quorum sensing* systems. By definition, a *quorum sensing* system is a mechanism by which bacterial cells regulate specific genes in response to critical concentrations of signal molecules produced with increasing cell density (Diggle et al., 2002). Therefore, a high cell density is necessary for biosurfactant production to occur. Thus, signal molecule production becomes sufficient for binding to specific autoinducers and consequent gene activation. However, this mechanism does not explain biosurfactant production in environments contaminated with hydrocarbons, where the cell density is presumably low but biosurfactant production is high. In these environments, each cell creates its own microenvironment within each micelle, stimulating biosurfactant production independent of increased cell density (Sullivan, 1998).

Genetic, nutritional, and environmental factors are all related to biosurfactant production. Each microorganism requires an appropriate medium for growth. Thus, the choice of the ideal medium for biosurfactant production depends on the microbial strain used. Therefore, the best conditions for an individual strain's development must be assessed.

Among the nutritional factors that influence biosurfactant production, the available sources of carbon and nitrogen in the medium are crucial. Production can also be increased with the addition of hydrophobic compounds, such as aliphatic and even aromatic hydrocarbons, to the culture medium during the stationary growth phase (Desai & Banat, 1997; Déziel et al., 1996). However, some microorganisms produce biosurfactants only when consuming hydrocarbons, i.e., only after all of the soluble carbon in the medium has been consumed. In such cases, the use of hydrocarbons is essential for the acceleration of biosurfactant production. Nitrogen metabolism is also directly related to biosurfactant production, especially among denitrifying microorganisms. It should be noted that for some bacteria, the culture medium composition may interfere with the biosurfactant chemical composition (Desai & Banat, 1997; Zhang & Miller, 1995).

The kinetics of biosurfactant production depends on the substrate used for microbial growth. Biosurfactant production may occur during the microbial active growth period or it may take place during the stage of limited growth. In the first case, biosurfactants production begins simultaneously with microbial growth when the substrate is used. In contrast, production under limited growth conditions occurs only during substrate scarcity (Desai & Banat, 1997; Maciel et al., 2007;). Figure 4 shows different stages of bacterial growth and the corresponding kinetics for biosurfactant production.

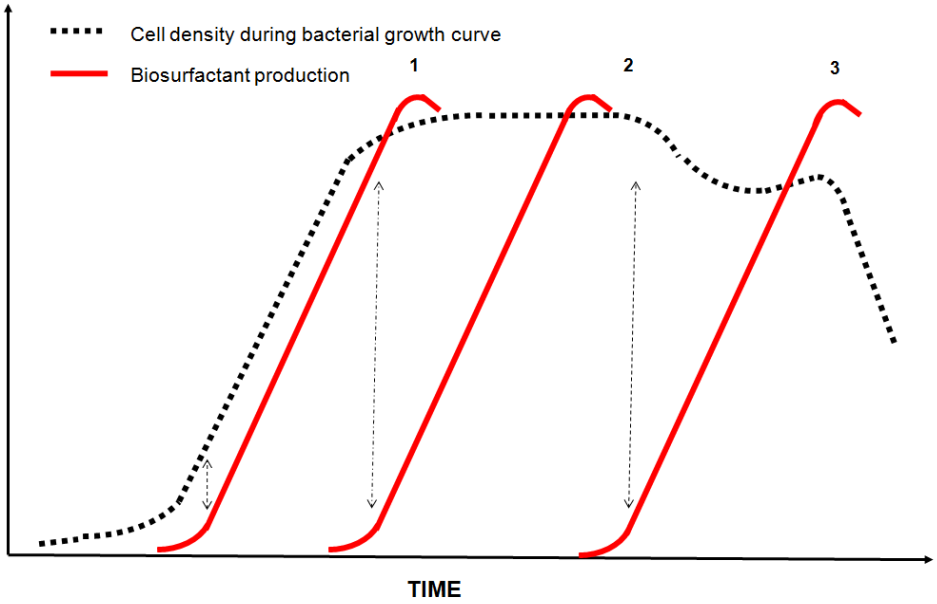


Fig. 4. Schematic illustration of the three different stages of bacterial growth and the corresponding kinetics for biosurfactant production. **(1)** Biosurfactant production associated with microbial growth in *Acinetobacter calcoaceticus*. The arrow indicates the start of biosurfactant production associated with the onset of the logarithmic phase in the bacterial growth curve. Biosurfactant production peak occurs in the early stationary phase in the bacterial growth curve. **(2)** Biosurfactant production in *Pseudomonas aeruginosa* under limited

growth conditions. The arrow indicates the onset of biosurfactant production associated with the late logarithmic phase and early stationary phase of the bacterial growth curve. The peak of biosurfactant production occurs at the end of the stationary phase in the bacterial growth curve. **(3)** This biosurfactant production curve corresponds to different bacterial isolates under nutritional stress. These bacteria include the *Achromobacter xylosoxidans, Cellulosimicrobium cellulans, Alcaligenes* sp., and the *Achromatium oxaliferum*. The arrow indicates the start of biosurfactant production associated with the end of the stationary phase and the beginning of the decline phase for the bacterial growth curve. Biosurfactant production under these conditions causes a prolongation of the bacterial culture survival, which reflects a second stationary phase during the bacterial growth curve.

Environmental factors, such as pH, temperature, agitation, and oxygen availability, should be also carefully adjusted in biosurfactant production to provide the best conditions for microbial growth. In general, the optimum pH range for biosurfactant production is between 6.5 and 8.0, although biosurfactant activity remains stable over a wide pH range. Temperature and aeration should also be carefully adjusted because most biosurfactant-producing bacteria grow well at room temperature (28°C ± 2°C), and heat treatment can alter the stability of some biosurfactants. However, some bacteria, such as thermophilic bacilli, are capable of producing biosurfactants above 40°C without any alterations in stability (Banat, 1993) and therefore can be used in deep-water oil recovery. The aeration rate should also be adjusted depending on the microorganism.

3.3 Economic viability of biosurfactant applications

Economic viability is often a major constraint for biotechnological processes, especially in biosurfactant production. Biosurfactants have to compete with surfactants of petrochemical origin in three aspects: cost, functionality, and production capacity to meet the needs of the application. A high production cost is incompatible with microbial-enhanced oil recovery (MEOR), which requires large amounts of biosurfactants. Some factors may decrease the production cost in these cases: (i) selection of microorganisms adapted to the polluted area, (ii) decreased biosurfactant recovery cost, and (iii) microbial growth on more affordable substrates (Makkar & Cameotra, 2002).

To minimize production costs, agro-industrial waste products have been used as substrates for biosurfactant production. These waste products include post-harvest waste from cassava, soybeans, beets, sweet potatoes, potatoes, sorghum, wheat, and rice; husks from soybeans, corn, and rice; sugar cane and cassava bagasse; coffee industry waste products (pulp, husk, and coffee grounds); industrial wastes from the juicing of apples, grapes, pineapples, bananas, and carrots; candy manufacturing waste products; and other substrates such as sawdust, corn grain, tea manufacturing waste, and chicory roots (Makkar & Cameotra, 2002; Pandey et al., 2000). Other substrates have been suggested for biosurfactant production, such as syrup, whey, and distillery waste products (Makkar & Cameotra, 2002).

4. Application of microbiological culturing and metagenomic methods focused on oil degradation

To allow adaptation to various environments, microorganisms have a rich taxonomic, metabolic, physiological, and molecular diversity in nature. Therefore, studies based on the

isolation of microorganisms that biodegrade oil and its fractions are certainly of great importance. In contaminated environments, such as oily sludge landfarming or biopile soil, different microorganisms with different metabolic pathways and desirable biodegradation or bioconversion attributes can be found. In these studies, the test compound, in this case a hydrocarbon should be the energy source in the culture medium to exert a selection pressure on microorganisms with the potential to metabolize it. Bioprospecting studies can be then conducted with the aim of exploiting potential bioremediation or bioconversion biotechnology.

Studies on the impact of environmental changes on microbial populations and their activities have often been restricted to evaluating basic parameters including the total number of microorganisms, microbial biomass, respiration rate, and total enzyme activities involved in organic carbon and nitrogen mineralization. Currently, these studies can be conducted using methods based on DNA sequence analysis, especially analysis of the 16S rDNA gene in bacteria and archaea and the 18S gene in fungi. These genes can be amplified by PCR (Polymerase Chain Reaction) and subsequently characterized by electrophoresis using different molecular techniques such as ARDRA (Amplified Ribosomal DNA Restriction Analysis), T-RFLP (Terminal Fragment Length Polymorphism), RAPD (Random Amplification of Polymorphic DNA), RISA (Ribosomal Intergenic Space Analysis), DGGE/TGGE (Denaturing Gradient Gel Electrophoresis / Temperature Gradient Gel Electrophoresis, and SSCP (Single-Strand Conformation Polymorphism to obtain microbial community profiles (Ranjard et al., 2000; Kozdrój & Van Elsas, 2001, Konstantinidis & Tiedje, 2007, Maciel et al. 2009).

In DGGE (denaturing gradient gel electrophoresis), for example, DNA fragments with the same size but with different sequences can be separated. Thus, each band formed during gel electrophoresis represents a single operational taxonomic unit (OTU), i.e., a single microorganism. Thus, changes in environmental microbial diversity can be observed with the DGGE banding profiles. Separation is based on the decreased electrophoretic mobility of a partially denatured double-stranded DNA molecule in a polyacrylamide gel containing an increasing linear denaturing gradient (a mixture of urea and formamide). One of the primers used in the PCR reactions must contain a G-C clamp in its sequence to prevent complete denaturation of the double-strands. Fragments with GC-rich sequences need higher denaturing gradients so that AT-rich sequences can separate the double-strands completely (Figure 5).

However, high-impact molecular studies of microbial communities and the development of metagenomic libraries are dependent on DNA extraction. This critical step of the procedure should be the first and most important step for such studies. The success of the evaluation is directly related to the type and quality of DNA extraction (Maciel et al., 2009). There are several techniques to extract the total DNA from environmental samples; however, no method is universally applicable, as each type of sample requires an individually optimized extraction method. Four important variables must be considered during DNA extraction: the amount of extracted DNA, purity, DNA integrity, and representativeness. The extraction of total DNA from a sample is necessarily a balance between rigorous extraction, which is required for representation of all microbial genomes in the sample (direct lysis); minimal DNA fragmentation; and avoidance of co-extracted contaminants.

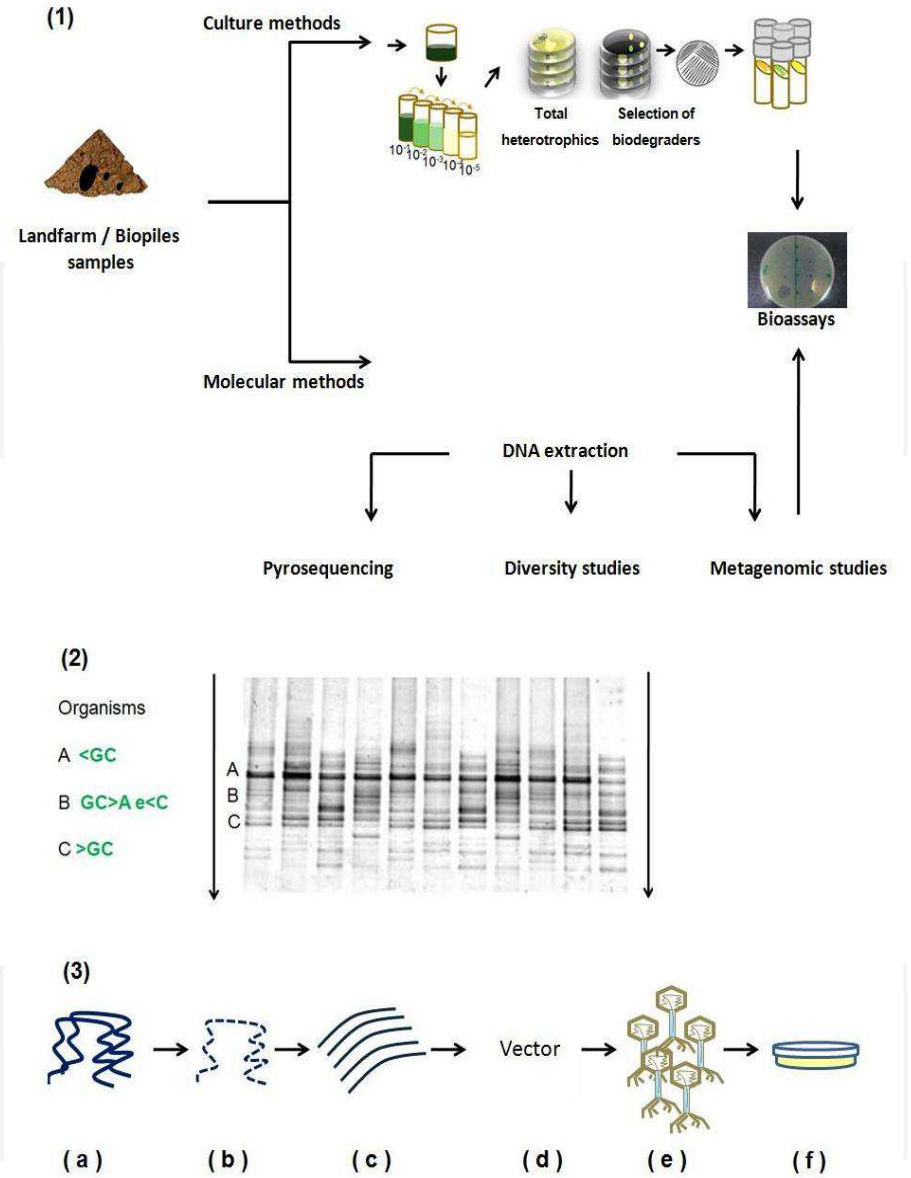


Fig. 5. Application of molecular and culture techniques for the study of microorganisms from *landfarming* and biopiles soils with biotechnological potential. **(1)** Culturing methods - After soil homogenization, a small aliquot is serially diluted and cultured in media for counting of the total number of heterotrophic microbes and in medium containing the hydrocarbon test compound as sole carbon source for biodegrader selection. The

microorganisms are then isolated in pure culture and stored for bioprospecting studies (bioassays). Molecular methods - Different molecular methods can be applied after DNA extraction, depending on the purpose: sequencing methods are used to study genome structure, microbial diversity methods are used to assess environmental impact on microbial communities, and metagenomic studies are used to prospect genes of biotechnological interest (bioassays). **(2)** An example of a microbial diversity study by the DGGE technique (denaturing gradient gel electrophoresis). Photograph of a DGGE were the OTU (operational taxonomic unit) in A has a standard denaturing gel pattern different from that of B and C because B and C have a higher GC content (guanine and cytosine). **(3)** Schematic representation of steps for metagenomic library construction (modified from http://www.epibio.com): (a) extraction of total DNA from contaminated soil sample, (b) DNA fragmentation by restriction enzymes, (c) tip repairing to obtain DNA fragments with appropriate sizes to be used in the vector, (d) inserting the DNA fragments into a vector system (fosmid), (e) packaging of the recombinant vector in envelope proteins from *Lambda* bacteriophage, and (f) transformation into competent *E. coli* cells.

The rational exploitation of the microbial potential is now possible not only through culturable microorganisms but also with molecular strategies, where it is possible to clone microbial DNA extracted from different environments in *Escherichia coli* hosts. These strategies, combined with general metagenomic naming, use BAC (*bacterial artificial chromosome*), cosmid, or fosmid vectors for cloning of different-sized inserts and require the use of different methods for host cell transformation (Yung et al., 2009; Singh et al., 2009). Most libraries constructed in vectors that contain large DNA fragments (40-100 Kb) obtained directly from environmental microbial communities, such as soil, sediment, or sludge, are used to investigate the expression of genes involved in the synthesis or metabolism of certain compounds (Rondon et al., 2000; Singh et al., 2009).

After the DNA extraction, the library construction involves DNA fragmentation by restriction enzymes or mechanical breaking, DNA fragment insertion into a suitable vector system, and transformation of recombinant vectors into a suitable host (Figure 5). The vector choice depends on the DNA quality, average insert size, number of host vector copies, and testing strategy. Libraries with large DNA inserts (~ 20 Kb) are constructed with cosmid, fosmid, and BAC vectors, among others. Libraries with smaller inserts (less than 10 Kb) are constructed with plasmid vectors (Handelsman, 2004).

Fosmid vectors fit fragments of up to 45 Kb, offering excellent gene coverage. This vector is particularly suitable when the genes to be cloned are organized in large operons of up to 25 Kb, as is the case for microbial genes that encode the biosurfactant synthesis. After vector ligation, the vector and insert assembly are encapsulated using an envelope protein from the *Lambda* bacteriophage. The resulting particles are adsorbed to the host cells and are responsible for introducing the DNA. Cloning efficiency must be verified by plating serial dilutions generated with the phage capsids and *E. coli* cells. Generally, this plating is performed in LB medium with chloramphenicol added as a selective antibiotic. After collection and storage, the clones can be tested for degradation of innumerous aromatic compounds.

It is important to note that most metagenomic studies aim to isolate the genes responsible for biosurfactant synthesis and to use culture media that favor the production of this

secondary metabolite (Franzetti et al. 2008; Pornsunthorntawee et al., 2008). However, as discussed earlier, most of the genes for surfactant molecules are regulated by *quorum sensing* systems that trigger the synthesis with increasing cell density, thus delaying the process. To circumvent this limitation, cloning and heterologous expression can be performed using vectors with constitutive promoters, such as the T7 promoter from the pCC2FOS vector, which eliminate gene regulation by the *quorum sensing* system and therefore may be a promising alternative for biosurfactant production on an industrial scale.

5. Conclusion

The search for culturable microorganisms with desirable attributes for the effective bioremediation of crude oil contaminated environments and the successful biodegradation of hydrocarbons remains the main focus for biotechnology research, because of the natural metabolic ability found in impacted environments. However, no single microbial species is capable of degrading all oil components; thus, a consortium between strains is required. In addition, most microorganisms are not culturable, and those that may exhibit a diverse catabolic potential are still unknown. The use of culture-independent methods has shown a vast microbial diversity in these environments. Metagenomics offers an alternative to the exploitation of heterologous gene expression important in degradation processes. It is worth noting that there are two sides to biotechnology the culture- and the culture-independent methods and that each complements the other.

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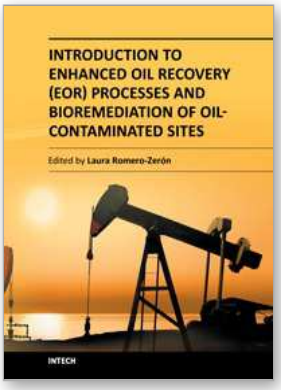

Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites

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This book offers practical concepts of EOR processes and summarizes the fundamentals of bioremediation of oil-contaminated sites. The first section presents a simplified description of EOR processes to boost the recovery of oil or to displace and produce the significant amounts of oil left behind in the reservoir during or after the course of any primary and secondary recovery process; it highlights the emerging EOR technological trends and the areas that need research and development; while the second section focuses on the use of biotechnology to remediate the inevitable environmental footprint of crude oil production; such is the case of accidental oil spills in marine, river, and land environments. The readers will gain useful and practical insights in these fields.

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Phone: +385 (51) 770 447
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821