Chapter 28
Biosurfactant-Assisted Bioaugmentation in Bioremediation

Chinmay Hazra, Debasree Kundu, and Ambalal Chaudhari

Abstract Surface active compounds (SACs) are basically amphipathic in nature, which alter the properties of fluid interfaces, partition at interface between fluid phases leading to formation of micro-emulsion and impart better wetting, spreading, foaming and detergent traits, thereby rendering them as most versatile process chemicals to be utilized in surfactant-enhanced bioremediation practices. Use of chemical surfactants as an additive, however, warrant (i) toxicity, (ii) carcinogenicity, (iii) non-biodegradibility, (iv) bioaccumulation and (v) inconsistent performance with slow desorption kinetics. Therefore, attention has been focused on alternative amphiphilic surfactants of biological origin, which have predilection for interfaces of dissimilar polarities (liquid-air/liquid-liquid) and are soluble in both organic (non-polar) and aqueous (polar) milieu. The mechanisms of biosurfactant-assisted bioaugmentation in bioremediation include: (i) lowering of interfacial tension, (ii) biosurfactant solubilization of hydrophobic contaminants, and (iii) the phase transfer of pollutants from soil-sorbed to *pseudo*-aqueous phase. Hence, microbial surfactants have potential attributes as alternative to synthetic surfactants.

This article reviews key aspects of microbial tensioactives for applications in bioremediation and biodegradation of environmental pollutants with focus on properties and physiological roles, followed by its laboratory, field demonstrations and full-scale applications. Finally, it is concluded with a concise appraisal on *in situ* and *ex situ* biosurfactant-assisted bioaugmentation, along-with impediments and future challenges.

Keywords Biosurfactant • Bioaccumulation • Bioaugmentation • Biosparging

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28.1 Introduction

Pollution is an inadvertent introduction of elements, compounds, or energy into the environment at concentrations that impair its biological functioning or present an unacceptable risk to humans or other target species in the environment. In tandem with phenomenal growth in manufacturing, processing, handling of chemicals and human interference have instigated tremendous pollution of the environment with wide variety of recalcitrant/persistent, hazardous and toxic anthropogenic organic pollutants. Several of these hydrocarbon chemicals have been obtained from petroleum; some synthesized in large volume and substantially differs in chemical structure from natural organics. Many of these chemicals have high degree of carcinogenic potential to bio-accumulate in living system, recalcitrant to biodegradation and persistent in the eco-system for longer duration to cause severe environmental problems. Due to possible potential threats, pollution have now been the most serious environmental concerns in the world and yet, in many respects, least understood. Such concerns have realized the necessity of advanced approach to de-contaminate the affected environment. For this reason, pollution has received the wider consciousness as a part of the burgeoning 'greening' of society (Kundu et al. 2010a, b).

A wide variety of hazardous and toxic hydrocarbons appeared to be the most ubiquitous and priority pollutants due to its application as (i) petroleum derivative fuels and (ii) synthetic precursors in large volumes. Each year, about 1,680,000 gal (~40,000 barrels) of crude oil is spilled from pipeline failure and more than 200,000 from underground storage tanks in the US causing major environment hazards (Huesemann 2004). Oil spill alone accounts for ~ 15% of all pollution incidents in England with about nine incidents per day (Environment Agency 2006) resulting in one million tonnes of oil spillage into terrestrial ecosystems every year (Stroud et al. 2007; Ripley et al. 2002).

Several other hydrocarbons including trichloroethylene (TCE), poly-chlorinated bi-phenyls (PCB), poly-cyclic aromatic hydrocarbons (PAH), benzene, nitro-aromatics, etc. are synthesized in large volumes, released into the environment deliberately, persist in the environment for prolong time, and identified as most hazardous priority pollutants. It is followed by trace metals contamination of the environment. The local concentrations of these pollutants depend on the rate at which the compound is released, its stability, mobility in the environment and its rate of biotic and abiotic removal. Despite of stringent enforcement of regulations, several countries have simply ignored it in order to keep pace with the economic and industrial growth (Dua et al. 2002). Technologies from physical to biological removal approaches for contaminants have been discussed in earlier reviews about their feasibility and economic aspects. However, conventional approaches for treatment of pollutants are not only technically challenging but also cost-intensive and hence, increasing consideration has been focused on development of alternative, economical and reliable biological amelioration.

28.2 Bioamelioration: Restoring Eco-habitats

Microbial communities with great biodiversity, catabolic potential, significant role in nutrient cycling have been explored for the biodegradation of toxicants through (i) catabolic genes and enzymes (Khomenkov et al. 2008) and (ii) acclimation strategies, viz. (a) capacity to tailor the cellular membrane for necessary biological functions (de Carvalho et al. 2009), (b) production of surface-active biosurfactants (Ron and Rosenberg 2002) and (c) potential efflux pumps to overcome passively internalized toxicants (Van Hamme and Urban 2009; Van Hamme et al. 2003). However, the magnitude of microbial degradation of toxicants depend on (i) environment (pH, temperature etc.), (ii) nutrient availability and oxygen, (iii) microbial interactions, (iv) cellular transport properties, (v) degree of acclimation, (vi) chemical complexicity of toxicants and (vii) chemical partitioning in medium. Knowledge pertinent to these bottlenecks of bioremediation can provide tools to (i) optimize, (ii) control key parameters and (iii) make the process more reliable. Hence, fresh spurt in bioremediation processes has recognized microbial potential for decontamination of toxicants. Now, bioremediation is considered as a safe, less expensive approach for removal of toxicants.

Amelioration technologies can be divided into two groups based on the physical location of the remedial action: (i) *in situ* remediation, where treatment of the contaminated media takes place by actions in its actual location in the subsurface, and (ii) *ex situ* remediation, wherein contaminated media is removed from the site for subsequent treatment in an above ground treatment facility (on-site) or disposal elsewhere (off-site) (Gerhardt et al. 2009). Ultimate objectivity of both technologies is to degrade organic chemicals to concentrations below the permissible limits established by regulatory authorities and preferably to undetectable levels (Kulkarni and Chaudhari 2007). Amelioration technologies for *in situ* removal of contaminants include: soil washing, soil vapor extraction, landfarming, composting, biopiles, bioventing, bioslurping and biosparging which are time tested and generally cost-effective. In case of *in situ* technologies viz. (i) natural attenuation (NA) and enhanced natural attenuation (ENA), (ii) biostimulation, (iii) bioaugmentation and (iv) phytoremediation are being heralded for biodegradation (Bombach et al. 2010). The choice of a remedial strategy for a contaminated site entails economical and environmental consequences for the local, regional, and global environment.

Microbial remediation has distinct merits over physico-chemical removal methods which include: (i) least expensive, (ii) flexibility and adaptability to edaphic conditions, (iii) environmentally benign and eco-friendly, (iv) better public acceptability as achieve complete degradation of pollutants without collateral damage to the eco-system, and (iv) on site implementation, indeed often *in situ*, and with dilute or widely diffused contaminants. These environmentally compatible features rendered remediation services market represent 4% of the US$ 213 billion annual environmental industry market, which supports the expansion in near future (Ward 2004).

28.3 Bioaugmentation: An Emerging Trend

Bioaugmentation, involve the use of degradative microbial consortia (Dejonghe et al. 2001) or the augmentation of catabolically-relevant organisms to hasten remediation (Thompson et al. 2005). On the contrary, biostimulation encompasses application of indigenous microbe(s) adapted to the contaminated environment of the site that is being treated (Tyagi et al. 2010). Such approach is not always effective and may need a much longer time because of the scarcity of indigenous microbe(s) capable of degrading high concentrations of the pollutants. Hence, bioaugmentation treatment has been regarded as a promising technology (El Fantroussi and Agathos 2005) for sites that (i) do not have sufficient microbial cells or (ii) the native population do not possess the metabolic apparatus necessary to catabolize the toxicants under concern (Tyagi et al. 2010).

Thematically, bioaugmentation entails two approaches: (i) allochthonous, wherein foreign microbes (single/consortia) are introduced into the target site (soil, sand, and water) and (ii) autochthonous bioaugmentation i.e. use of microbes indigenous to the sites to be decontaminated (Vogel and Walter 2001; Ueno et al. 2007). Practical utility of allochthonous technology is limited because of (i) complex environments to potent microbes, particularly when isolated from sites other than the ones being decontaminated, (ii) effective bioaugmentation in the early phases due to high population density of allochthonous microbes, (iii) rapid loss of dominance of single strains and (iv) poor public acceptability for foreign and genetically engineered microbes (Hosokawa et al. 2009). On the other hand, autochthonous makes use of indigenous microbial consortia or, better adapted isolates to the historically/artificially contaminated environments (Hosokawa et al. 2009).

Inspite of success, application of allochthonous/ autochthonous bioaugmentation (Hosokawa et al. 2009) appeared limited due to problems like (i) adaptation of inoculated microorganisms, (ii) insufficiency of substrate, (iii) competition between introduced and indigenous biomass, (iv) use of other organic substrates in preference to the pollutant, and (v) predation protozoa in food chain (Tyagi et al. 2010).

Alternative bioaugmentation approaches to increase the prevalence and activity of exogenous microorganisms and/or genes following introduction into the contaminated environment include: (i) bioaugmentation with cells encapsulated in a carrier, (ii) gene bioaugmentation where added inoculants transfer remediation genes to indigenous microorganisms, (iii) rhizosphere bioaugmentation where the microbial inoculant is added to the site along with a plant that serves as a niche for the inoculant's growth, and (iv) phyto-augmentation, where the remediation genes are engineered directly into a plant for use in remediation without a microbial inoculants (Gentry et al. 2004). These approaches are still at experimental level. For efficient use of bioaugmentation, due consideration to variety of parameters are required.

Hence, seeding alone should be accompanied by suitable physical and environmental alterations so as to enhance the bioavailability of polllutants (Leahy and Colwell 1990; Gentry et al. 2004).

28.4 Chemical Surfactants: A Solution to Pollution?

Based on the initial realization of enhanced benefit of biodegradation rate, synthetic surfactant enjoyed the coveted role as an external biostimulation factor. Chemical surfactants are basically amphipathic compounds, which partition at interface between fluid phases leading to formation of micro-emulsion, to impart better wetting, spreading, foaming and detergent traits, rendering them as most versatile process chemicals (Mulligan 2005). Use of petrochemical-based surfactants as mobilizing agents for soil flushing and washing, mostly employs synthetic surfactants with hydrophobic parts of paraffins, olefins, alkylbenzenes, alkylphenols and alcohols; the hydrophilic part is usually a sulphate, a sulphonate, or a carboxylate (anionic surfactants), a quaternary ammonium (cationic surfactants), polyoxyethylene, sucrose, or polypeptide (non-ionic) group.

More often than not, from environmental and industrial perspectives, applications of these compounds are discouraged due to the following reasons: (i) disruption of cellular membranes by interaction with lipid components, (ii) reactions of surfactant molecules with proteins essential to the functioning of the cell (Helenius and Simons 1975), (iii) inhibitory effect especially in concentrations above the critical micelle concentration (CMC) due to reduced availability of micellar substrates (Volkering et al. 1998), (iv) negative effects caused by (a) depletion of minerals or oxygen, (b) toxicity of surfactant intermediates than the parent compounds (Holt et al. 1992), (c) preferential degradation of the surfactant, slowing the pollutant degradation (Tiehm 1994), (v) decreased microbial mobility, and (vi) lowered bioavailability by inhibiting bacterial attachment, dispersing soil colloids causing clogging of pores, or interfering with the natural interactions of microbes with the pollutant.

In order to alleviate potential risks and increased environmental awareness among the consumers, cost and public-regulatory perception of sustainable harmony with global environment, oleochemical surfactants viz. natural biosurfactants are seen as a better alternative to the existing chemical surfactants for bioaugmentation tool (Hazra et al. 2010a).

28.5 Biosurfactant: A Balancing Act

Threaded through the theme of molecular commonality, amphipathic molecules characterized by hydrophobic and hydrophilic, or non-polar and polar regions, are common and essential due to life's aqueous milieu. At the cellular and physiological level, single and multi-cellular life forms evolved amphipathic lipid bilayers, transmembrane sensory proteins, electron transport chain proton and sodium motive pumps, flagellar motors and internal membranes to create a controlled environment for biomolecular synthesis which is, among other things, the basis of heredity. These signature features extend to what may be described as biosurfactant/bioemulsifier

produced to modulate and facilitate diverse physical, chemical and behavioural activities within and without the cell.

Biosurfactants are amphiphilic (amphipathic) surface-active agents of biological origin, which comprise both hydrophilic (head) and hydrophobic groups (tail), have predilection for interfaces of dissimilar polarities (liquid-air/liquid-liquid) and are soluble in both organic (non-polar) and aqueous (polar) milieu. Several unique properties like (i) reduction in the surface tension (ST), interfacial tension (IFT) and CMC, (ii) stabilization of emulsions, (iii) promotion of foaming, (iv) induction of flocculating action, (v) increasing wetting, spreading and penetrating action(s), (vi) enhancement of microbial growth and metal sequestration, (vii) rapid biodegradability, (viii) lower toxicity and (ix) environment-friendly 'green' characteristics render them as most possible alternatives to chemical counterparts (Mukherjee et al. 2006; Singh et al. 2007; Rahman and Gakpe 2008; Vardar-Sukan and Kosaric 2009; Roane et al. 2009; Mulligan 2009; Abdel-Mawgoud et al. 2010; Banat et al. 2010; Hazra et al. 2010a, b; Satpute et al. 2010a, b).

28.5.1 Basis of Biosurfactant: A Lucrative Background

Bergström et al. (1946a, b) reported an oily glycolipid produced by *Pseudomonas pyocyanea* (now *P. aeruginosa*) after growth on glucose that was named pyolipic acid and whose structural units were identified as L-rhamnose and β-hydroxydecanoic acid (Hauser and Karnovsky 1954; Jarvis and Johnson 1949). Jarvis and Johnson (1949) further elucidated the structure of a rhamnolipid isolated from *P. aeruginosa* and showed that it was composed of two β-hydroxydecanoic acids linked through a glycosidic bond to two rhamnose moieties, with the two β-hydroxy fatty acid portions linked through an ester bond while the disaccharide portion contained a putative 1,3-glycosidic linkage. Further, Edwards and Hayashi (1965) identified α-1,2-glycosidic linkage between the two rhamnose moieties through periodate oxidation and methylation. On this basis, they chemically described this rhamnolipid as 2-O-α-1,2-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (di-rhamnolipid). This was the first discovered glycolipid containing a link between a sugar and a hydroxylated fatty acid residue (Shaw 1970).

Among glycolipid surfactants, the best-known compounds are rhamnolipids, trehalolipids, sophorolipids and mannosylerythritol lipids (MELs), which contain mono- or disac-charides, combined with long-chain aliphatic acids or hydroxyaliphatic acids. Rhamnolipids from *Pseudomonas aeruginosa* are currently commercialised by Jeneil Biosurfactant, USA, mainly as a fungicide for agricultural purposes or an additive to enhance bioremediation activities. Sophorolipids, on the other hand, are produced mainly by yeasts, such as *Candida bombicola* (also known as *Torulopsis bombicola*), *Centrolene petrophilum, Candida apicola* and *Rhodotorula bogoriensis*, while MELs are produced by *Pseudozyma* yeasts, *Pseudozyma aphidis, Pseudozyma antarctica* and *Pseudozyma rugulosa* (Banat et al. 2010). Besides, cyclic lipopeptides (mainly surfactin and iturin) are produced by a number of *Bacillus* species

as antibiotic molecules. High-molecular-weight polymers RAG-1 emulsan, an amphiphilic polysaccharide produced by *Acinetobacter calcoaceticus* RAG-1, is the only commercially available bioemulsifier at present (Suthar et al. 2008).

28.5.2 Classification and Types

Primarily, biosurfactants include low- and high-molecular weight compounds (Table 28.1); the former are generally glycolipids or peptidyl-lipids (lipo-peptides), while the latter are (lipo) polysaccharides, lipo-proteins or their combinations (Desai and Banat 1997). Alternatively, low or high molecular weight biosurfactants are categorized into (i) glycolipids, (ii) lipo-amino acids and lipo-peptides, (iii) lipo-proteins and lipo-polysaccharides and (iv) phospholipids, mono- and di-glycerides and free fatty acids (Kulkarni et al. 2007). As per Neu (1996), low-molecular-weight compounds are called biosurfactants, such as lipopeptides, glycolipids, proteins and high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins are collectively termed as bioemulsans (Rosenberg and Ron 1997) or bioemulsifiers (Smyth et al. 2010).

28.5.3 Why Biosurfactant?

Biosurfactants are gaining prominence by virtue of commercial applicability due to unique attributes like (i) feasible fermentative production using economical renewable resources, (ii) functionality in ppm quantities under extreme conditions (temperature, pH, and salinity), (iii) specificity of application, (iv) potential for tailoring to suit specific applications and (v) better foaming useful in mineral ore processing, besides the above-mentioned attributes. Due to this wide array of applications, ranging from biotechnology to environmental clean-up, biosurfactants have become versatile commodity in technical applications (Banat et al. 2010; Hazra et al. 2010a).

In recent years, biosurfactants have emerged as key metabolites in the rapidly growing biotechnology industry, owing to their multi-faceted functions as utility commodity in wide array of industrial applications (Banat et al. 2010; Satpute et al. a, b). They are potentially useful for (i) emulsification, (ii) emulsion polymerization, (iii) phase dispersion and (iv) de-emulsification. Many of these biosurfactants also possess therapeutic and biomedical properties viz. (i) antibacterial, (ii) antifungal, (iii) algicidal, (iv) antiviral activities, (v) anti-fibrin clotting and (vi) anti-adhesivity action against several pathogens (Cameotra and Makkar 2004; Singh and Cameotra 2004; Rodrigues et al. 2006). Environmentally, substitution of biosurfactant for chemical surfactants reduces the life-cycle of CO_2 emissions by 8%. On this basis, it is estimated that 1.5 million tonnes of CO_2 emissions were avoided (Patel 2004; Rahman and Gakpe 2008). According to Frost and Sullivan, microbial surfactants find most promising applications in oil spill bioremediation of sites contaminated

Table 28.1 Microbial biosurfactants: a brief profile

Microbes	Tensiometric properties: ST (mN m^{-1})	CMC	IFT (mN m^{-1})	Structure
1. **Glycolipid: (a) Rhamnolipid**				
Pseudomonas aeruginosa	29	–	0.25	1. One or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid
Pseudomonas sp.	25–30	0.1–1	1	
Serratia rubidea	30	10	1–3.5	2. Hydroxyl group of one of the acids forms glycosidic linkage with reducing end of rhamnose; OH group of the second acid forms hydroxyl ester
Teratogenococcus sp.	–	8–10	–	
Rhodococcus erythropolis	32–36	1 4	14–17	
P. putida	–	–	–	3. Predominantly rhamnosyl-L-rhamnosyl-β-hydroxyl decanoate and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate are referred to as rhamnolipids 1 and 2, respectively
Renibacterium salmoninarum	–	–	–	
Pseudoxanthomonas sp.	–	–	–	
Burkholderia sp.				
Cellulomonas cellulans				
(b) Trehalolipid				
Arthrobacter paraffineus	–	–	–	Disaccharide trehalose linked at C-6 and C-6 to α- branched β-hydroxyl mycolic acid
Corynebacterium sp.	–	–	–	
Mycobacterium sp.	38	3	15	
Neisseria erythropolis	30	20	3.5	
Nocardia sp.	–	–	–	
Rhodococcus ereythropolis	–	–	–	
Torulopsis bombicola	33	–	1.8	
(c) Sophorolipid				
Candida apicola	–	–	–	Carbohydrate sophorose coupled to a long chain hydroxyl fatty acid by glycosidic linkage and occurs as a mixture of macrolactones and free acid
C. bogoriensis	–	–	–	
C. bombicola	31	22	-	
T. bombicola				
C.batistae				
Wickerhamiella domercqiae				
Rhodotorula bogoriensis				
(d) Cellobiose lipids				
Ustilago maydis	–	–	–	–
(e) Polyol lipids				
Rhodotorula glutinus	–	–	–	–
R. graminus				
(f) Diglycosyl diglycerides				
Lactobacillus fermentii	–	–	–	–
(g) Lipopolysaccharides				
Acinetobacter calcoaceticus	–	–	–	–

(continued)

Table 28.1 (continued)

Microbes	Tensiometric properties: ST (mN m^{-1})	CMC	IFT (mN m^{-1})	Structure
RAG1				
Candida lipolytica	–	–	–	
Pseudomonas sp.	–	–	*	
(h) Glucose lipids				
A. borkumensis	29	–	–	Anionic glucose lipids with tetrameric oxyacyl side chain with chain lengths of one/two of the four β-hydroxy fatty acids
C. borgoriensis	–	–	–	
2. Lipopolypeptides- peptide lipids: (a) Serrawettin				
Serratia marcesens	25–28	20	12	Lipid linked to a polypeptide chain
(b) Viscosin				
Pseudomonas fluorescens	25–28	18	–	Lipid attached to a polypeptide chain
(c) Surfactin				
Bacillus subtilis	29	15	12	Cyclic hepta-peptide coupled to a fatty acid chain via lactone ring
B. pumilis	27	10	–	
(d) Lichenysin (Type A and B)				
Bacillus licheniformis	27.9	–	0.36	Molecular weight 1,006 to 1,034 Da; Similar to surfactin
(e) Subtilisin				
Bacillus subtilis	27	–	–	Similar to surfactin
(f) Gramicidins				
Bacillus licheniformes	27	–	–	1. Cyclo-symmetric decapeptide antibiotic 2. Exist as rigid ring with ornithine side chain 3. A two moles of gramicidin form a stable complex with 1 mole of ATP
(g) Polymyxins				
B. polymyxa	27	–	–	Lipodecapeptide antibiotic with amino acid 3 through 10 form cyclic octapeptide
(h) Arthrofactin				
Arthrobacter sp.	–	–	–	–
(i) Ornithine lysine peptides				
Gluconobacter, Streptomyces, Thiobacillus				
(j) Sulfonylipids				
Corynebacterium alkanolyticum	–	–	–	–
Thiobacillus thioxidans	–	–	–	
(k) Protein complex				
M. thermoautotrophium	–	–	–	–

(continued)

Table 28.1 (continued)

Microbes	Tensiometric properties: ST (mN m^{-1})	CMC	IFT (mN m^{-1})	Structure
3. Polymeric surfactants: (a) Emulsan				
Bacillus licheniformes	27.9	–	–	High molecular weight hetero-polysaccharide protein containing repeating trisaccharide of N-acetyl -D-galactosamine, N-acetylgalactosamine uronic acid and an N-acetyl amino sugar.
Bacillus sp.	–	–	–	
A. calcoaceticus BD413	–	–	–	
P. fluorescens				
(b) Liposan				
Acinetobacter calcoaceticus (RAG1)	–	–	–	Poly-anionicamphiphatic hetero-polysaccharides
A. calcoaceticus A2	–	–	–	
C. lipolytica	–	–	–	
(c) Mannosylerythreitol- lipid A				
Candida bombicola	0.1	–	–	Consists of 83% hetero-polysaccharide and 17% protein with carbohydrate portion (glucose, galactose, galactosamine and galacto-uronic acid).
C. lipolytica	–	–	–	
C. tropicalis	–	–	–	
Pseudozyma sp.	–	–	–	
(d) Alasan				
A. radioresistens KA53	–	–	–	Protein polysaccharide (1 MDa); Contains covalent bound alanine
(e) Lipomannan				
Acinetobacter radioresistens	–	–	–	Consists of 44% mannose and 17% protein.
Acinetobacter sp.	–	–	–	
Candida tropicalis	–	–	–	
Saccharomyces cerevisiae	–	–	–	
Myroides				
(f) Streptofactin (Extracellular hydrophobic peptide)				
Streptomyces tendae	39.4	36.4	–	1. Mixture of structurally related peptide (MW 1.003 to 1.127 Da) 2. Allows erection of aerial hyphae
4. Particulate biosurfactants: (a) Vesicles				
Acinetobacter calcoaceticus	–	–	–	–
(b) Whole microbial cells				
Cyanobacteria	–	–	–	–
(c) Waxy matter				
Pseudomonas marginalis	–	–	–	–
(d) Biosur PM				
Pseudomonas maltophila	–	–	–	–

(continued)

Table 28.1 (continued)

Microbes	Tensiometric properties: ST (mN m^{-1})	CMC	IFT (mN m^{-1})	Structure
(e) Fatty acids (Corynomycolic acids, Spiculisporic acids etc.)				
Arthobacter parrafineus	–	–	–	–
	–	–	–	
Capnocytophaga sp.	–	–	–	
Corynebacterium lepus	1	30	–	
	–	–	–	
	–	–	–	
Nocardia erythropolis	–	–	–	
Pennicillium spiculisporum	–	–	–	
Talaromyces trachyspermus				
Acinetobacter sp.				
Thiobacillus thiooxidans				
Halomonas				
Antarctobacter				
Marinobacter				
5. Insecticide emulsifier				
P. tralucida	–	–	–	–
6. Thermophilic emulsifier				
B. stearothermophilus	–	–	–	–
7. Acetyl-heteropolysaccharide				
S. paucimobilis	–	–	–	–
8. Food emulsifier				
C. utilis	–	–	–	–
9. Sulfated polysaccharide				
H. eurihalinia	–	–	–	–
10. PM-factor				
P. marginalis	–	–	–	–
11. Emulcyan				
Phormidium J-1	–	–	–	–

with hydrocarbons and oil-contaminated tanker clean-up, removal of crude oil from sludge, enhanced oil recovery, and recovery of other organic pollutants and heavy metals due to broad range of functional properties (Mulligan 2005). As of now, biosurfactant occupy about 10% of the total world production (nearly ten million ton per year) (Van Bogaert et al. 2007).

28.5.4 Significance of Biosurfactant in Bioaugmentation

Structural uniqueness of biosurfactant resides in the co-existence of a hydrophilic (a sugar or peptide) and a hydrophobic (fatty acid chain) domain in the same molecule,

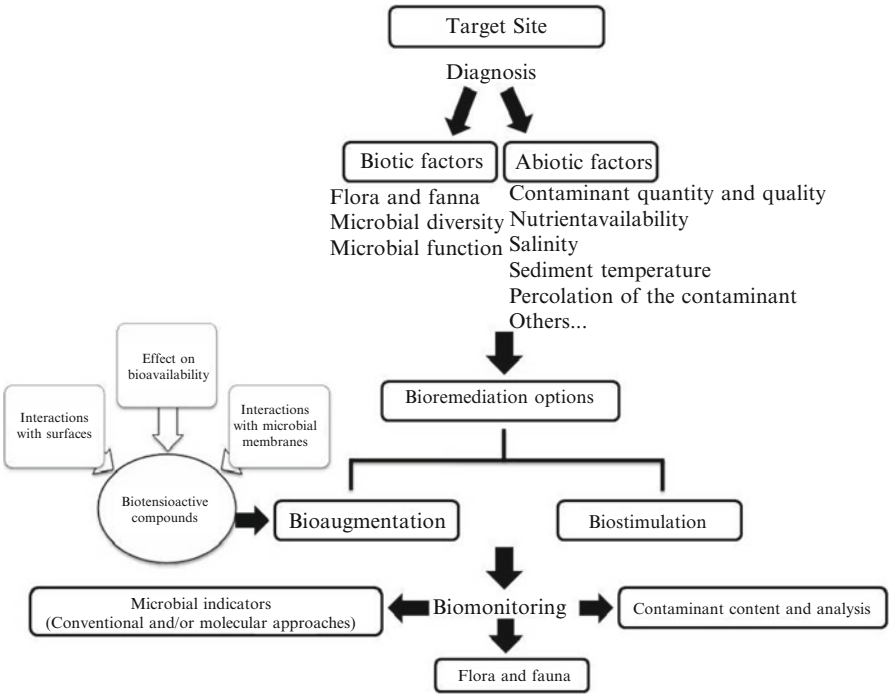

Fig. 28.1 Possible approach of biosurfactants in bioaugmentation: a *bird's eye view*

which allows them to occupy the interface of mixed phase systems (e.g., oil/water, air/water, oil/solid/water) and consequently, to alter the forces governing the equilibrium conditions. It constitutes the pre-requisite for a broad range of surface activities to take place including emulsification, dispersion, dissolution, solubilization, wetting and foaming (Perfumo et al. 2010a, b). Moreover, biosurfactants seem to confer an essential evolutionary advantage allowing microbes to grow under specific conditions as evidenced by their wide distribution across the eubacterial and archeal domains. The natural roles of biosurfactants have been claimed to increase the surface area of hydrophobic, water-insoluble growth substrates, increasing their bioavailability by increasing the apparent solubility or desorbing them from surfaces and regulating attachment and detachment of microorganisms to and from surfaces (Fig. 28.1). Thus, the net effect of a biosurfactant on biodegradation depends on the benefits that result from enhanced solubility of target compounds versus the reduction in direct adhesion of bacteria to those compounds.

Although biosurfactants reportedly enhance bioavailability of hydrophobic organic compounds (HOCs)/persistent organic pollutants (POPs), understanding the term 'bioavailability' is complicated because of a number of interpretations in the literature. For this purpose, Semple et al. (2004) proposed two linked definitions, bioavailability and bioaccessibility. A bioavailable compound is defined as 'a compound which is freely available to cross an organism's membrane from the

medium the organism inhabits at a given point in time'. A bioaccessible compound is described as 'a compound which is available to cross an organisms' membrane from the environment it inhabits, if the organism has access to it; however, it may either be physically removed from the organism, or only bioavailable after a period of time' (Semple et al. 2004). Thus, water-solubility and hence, bioavailability raises several issues pertinent to bioaccessibility and biodegradation of aromatic and aliphatic HOCs/POPs in soil: (i) is the emphasis on the aqueous phase and passive uptake actually relevant to the very low water soluble hydrocarbons? (ii) does the readily desorbed fraction adequately describe the size of a bioaccessible fraction? (iii) are there different modes of biodegradation? The bioavailability of hydrophobic organic pollutants can be enhanced by biotensioactives through the following six mechanisms:

Emulsification of Non-aqueous Phase Liquid Pollutant

As mentioned above, surfactants can decrease the interfacial tension between an aqueous and a non-aqueous phase. This may lead to the formation of micro-emulsions or, with energy input, to the formation of macro-emulsions, resulting into an increase in the contact area, enabling improved mass transport of the pollutant to the aqueous phase and in mobilization of sorbed liquid-phase pollutant.

Enhancement of Apparent Solubility of the Pollutant

The so called 'solubilisation' is caused by the presence of micelles. Hydrophobic organic compounds dissolve mainly in the core of the micelles, whereas more hydrophilic molecules, such as mono-aromatic compounds, may be present in the core and the shell of the micelles. The transport of micellar hydrocarbon to the aqueous phase can be very rapid due to the small size of the micelles, but it is not clear whether 'solubilised' hydrocarbons are directly available to the degrading microorganisms.

Facilitated Transport of the Pollutant

This term covers several different processes, such as the interaction of a pollutant molecule with single surfactant molecules, the interaction of surfactants with separate-phase or sorbed hydrocarbons (both as single biosurfactant molecules and as micelle-like aggregates at surfaces), mobilization of pollutant by swelling of the organic matrix, and mobilization of pollutant trapped in soil ganglia caused by lowering the surface tension of the pore water in soil particles.

Solubilization

It involves the production of biosurfactants by microbes, which increase the concentration of hydrocarbons in the aqueous phase (Fig. 28.2). The solubilization of hydrocarbons by biosurfactants is widely reported (Banat et al. 2010; Perfumo et al. 2010a, b), where higher concentrations of hydrocarbons were found in the aqueous phase than was expected. Bouchez-Naitali and Vandecasteele (2008) noted the importance of solubilization while examining the biodegradation of hexadecane by a variety of bacteria strains. Similarly, Bai et al. (1997) found that the solubility of hexadecane in a 500 $mg \cdot L^{-1}$ rhamnolipid solution was

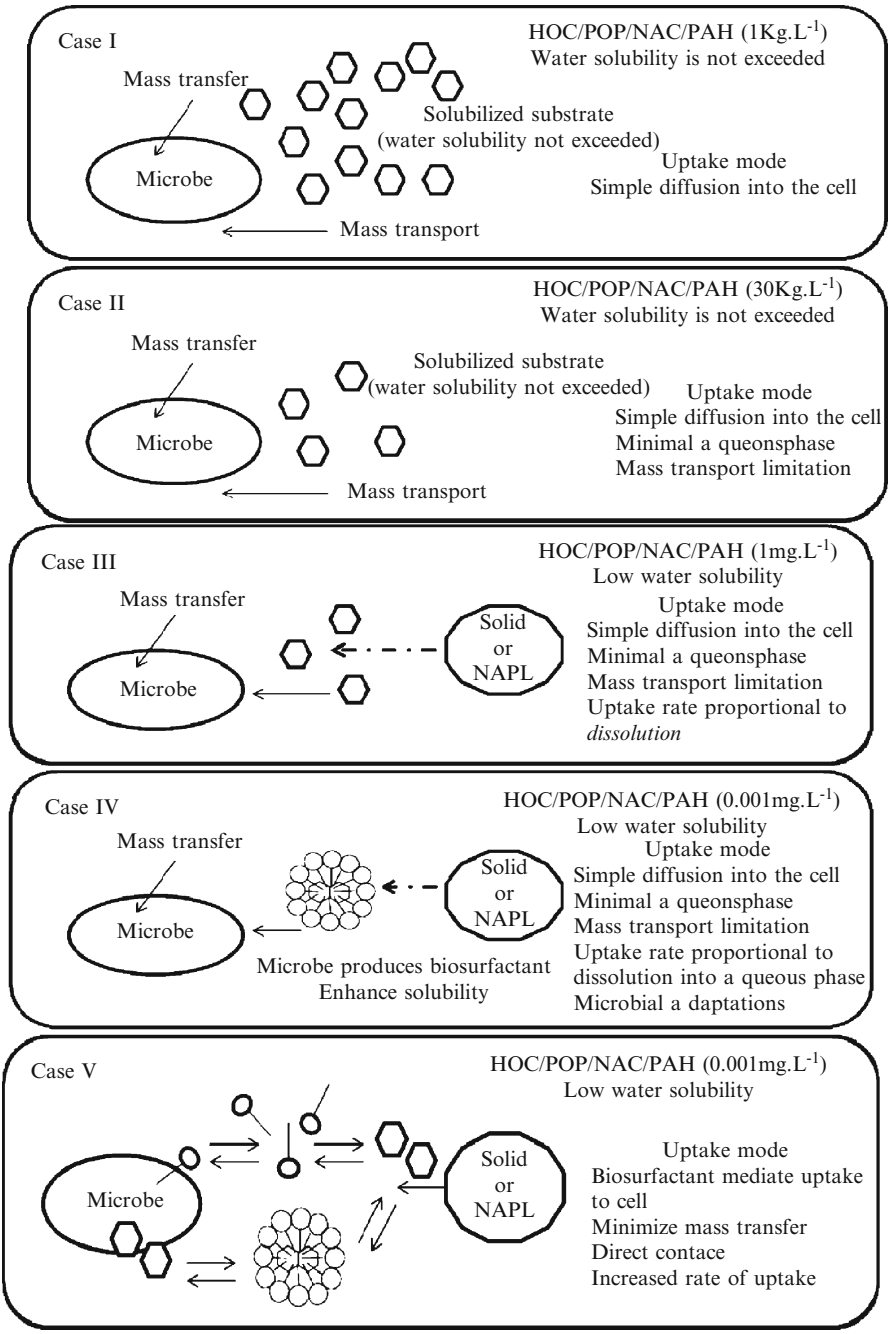

Fig. 28.2 Proposed involvement of biotensioactives for solubilization in bioremediation

19 mg·L^{-1} thereby, increasing hydrocarbon concentrations in the aqueous phase. Whyte et al. (1999) reported that invagination of hydrocarbons occurred, where inclusions of hydrocarbons in cells formed, followed by the uptake. Noordman et al. (2000) reported that the role of rhamnolipids was to mediate the mass transfer of hexadecane into cells, causing biodegradation.

Micellarization

Above the CMC, the formation of micelles s can partition hydrocarbon into the hydrophobic micellar core with increased apparent aqueous solubility. Supplementation of rhamnolipids above CMC, enhanced the apparent aqueous solubility of hexadecane, favoured biodegradation of hexadecane, octadecane, n-paraffins, creosotes and other hydrocarbon mixtures in soil and promoted bioremediation of petroleum sludges (Franzetti et al. 2010a). Biodegradation of chlorinated hydrocarbons can be enhanced by addition of glycolipids to the medium containing poly-chlorinated bi-phenyls. Similarly, pesticide biodegradation was promoted by surfactin (Awasthi et al. 1999).

Direct Contact

The interactions between bacteria, contaminants and biosurfactant can be interpreted from a functional perspective, considering that the main natural role attributed to biosurfactants is their involvement in hydrocarbon uptake (Perfumo et al. 2010b). Microbial surfactants can promote the growth of bacteria on hydrocarbons by increasing the surface area between oil and water through emulsification and increasing pseudosolubility of hydrocarbons through partitioning into micelles (Volkering et al. 1998). In direct contact, the bacterial cells adhere to the surface of the hydrocarbon and were crucial to the bacterial degradation of hexadecane. Direct contact can facilitate biosurfactants and bioemulsifiers to enhance adhesion between the cell wall and the accessible hydrocarbon. For example, the Gram-negative bacterium, *Acinetobacter* spp. is widely reported to produce biosurfactants / bioemulsifiers; thus, it has a hydrophobic exterior to allow cellular contact with the hydrocarbon. Additionally, some bacteria naturally have hydrophobic cell surfaces enabling cellular adhesion to hydrocarbons. Further, it was observed that uptake of the biosurfactant-coated hydrocarbon droplets occurred, suggesting a pinocytosis mechanism, a process not previously reported in bacterial hydrocarbon uptake systems (Cameotra and Singh 2009).

Changing Cell-Surface Hydrophobicity

Hydrophobic interactions play a role in the adherence of micro-organisms to a wide variety of surfaces. In particular, the hydrophobic nature of the bacterial surface has been cited as a factor in the growth of cells on water insoluble hydrophobic substrates such as hydrocarbons. In this case, cell contact with hydrophobic compounds is a requirement because the first step in aromatic or aliphatic hydrocarbon degradation is the introduction of molecular oxygen into molecules by cell-associated oxygenases. Uptake and utilization of water-insoluble substrates require specific physiological adaptations. Various microorganisms have developed different strategies of interaction with hydrophobic compounds. Two general types of

hydrocarbon-cell interactions, depending on the state and size of oil droplets relative to the size of microbial cells, have been postulated: specific adhesion of cells to larger oil drops and pseudosolubilization involving the cellular assimilation of emulsified small hydrocarbon droplets. The proposed role of biosurfactants in hydrocarbon uptake is the regulation of cell attachment to hydrophobic and hydrophilic surfaces by exposing different parts of cell-bound biosurfactants, thus changing cell-surface hydrophobicity (Franzetti et al. 2010a). This natural role through exogeneous (bio) surfactants supplementation can increase the hydrophobicity of degrading microbial cells and can facilitate easier access to hydrophobic substrates.

28.5.5 Biosurfactant-Assisted Bioaugmentation: Laboratory Scale Studies

Feasibility studies are a prerequisite for any planned intervention that usually revolves around screening, followed by tailoring of a competent microbial formula for a particular site. The initial screening/selection step usually based on the metabolic potential of the microorganism and also on essential features to enable the cells to be functionally active and persistent under the desired environmental conditions. The best approach for selecting competent microbes is primarily based on the prior knowledge of the microbial communities inhabiting the target site (Thompson et al. 2005). In the case of co-contaminated sites, e.g. contaminated with both high metal concentrations and organic pollutants, the microbial population ability to degrade the organic compounds may be inhibited by the co-contaminants (Roane et al. 2001). The proposed strategies, in such cases, have involved the use of multicomponent systems such as a microbial consortium, which truly represent a real environment than model – based on single-component systems (Ledin 2000). From an applied perspective, application of microbial consortium rather than a pure culture for the bioremediation is more advantageous as it provides the metabolic diversity and robustness needed for field applications (Rahman et al. 2002; Nyer et al. 2002). Table 28.2 summarizes the use of biosurfactants to stimulate hydrophobic organic contaminant biodegradation.

Apparently, the combination of bioaugmentation, biostimulation, and biosurfactant addition, depending on the characteristics of the contaminated site, might be a promising strategy to speed up bioremediation (Baek et al. 2007). However, any such planned intervention must be followed by ecotoxicity and quality studies of the contaminated site to ascertain that it has regained its natural biological activity and integrity (Hamdi et al. 2007; Liu et al. 2010a).

28.5.5.1 Biosurfactant in Oil Spills

Conservative estimates revealed that approximately $0.8 \pm 0.4\%$ of the total worldwide production of petroleum eventually reaches the oceans. As per National

Table 28.2 Biosurfactant-aided bioremediation: laboratory studies[a]

Compound(s)	Surfactant	Medium	Organism	Biodegradation kinetics[b]	Reference
14–16 C alkanes, pristane phenyldecane, and naphthalene	Sophorose lipid	Liquid	Mixed population	+	Oberbremer et al. (1990)
Mixture of alkanes and aromatics	Biosurfactant	slurry reactor	mixed culture	Stimulation and emulsification	Oberbremer et al. (1990)
Crude oil	Emulsan	Liquid	*Acinetobacter calcoaceticus* RAG-1	0	Glaser (1991)
Octadecane	Rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Zhang and Miller (1992)
Hexachlorobiphenyl	Rhamnolipid Emulsan	Soil	*P. aeruginosa Acinetobacter calcoaceticus* RAG-1	+	Van Dyke et al. (1993)
Aliphatic and aromatic hydrocarbons	Rhamnolipid	Soil	*P. aeruginosa* UG12	+	Scheibenbogen et al. (1994)
Mixture of alkanes and naphthalene	Rhamnolipid and oleophilic fertilizer	Soil	*P. aeruginosa*	+	Churchill et al. (1995)
Metals, phenanthrene, and PCBs	Rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Miller (1995)
Naphthalene	Rhamnolipid	Soil	*P. aeruginosa* 19SJ	+	Providenti et al. (1995)
Phenanthrene, pyrene, and B[*a*]P	Sodium dodecyl sulfate and rhamnolipid	Soil	*P. aeruginosa* UG2	–	Deschenes et al. (1996)
4,4,9-dichlorobiphenyl	Rhamnolipid	Soil	*P. aeruginosa*	+	Robinson et al. (1996)
Crude oil	Biosurfactant	Liquid		0	Kanga et al. (1997)
Naphthalene and methyl naphthalene	Glycolipid and Tween 80	Liquid	*Rhodococcus* sp. H13A	+	Kanga et al. (1997)

(continued)

Table 28.2 (continued)

Compound(s)	Surfactant	Medium	Organism	Biodegradation kinetics[b]	Reference
Hexadecane and kerosene oil	Crude surfactin	Soil	*Bacillus subtilis* ATCC 2423	+	Makkar and Cameotra (1997)
Naphthalene and phenanthrene	Rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Zhang et al. (1997)
Phenanthrene, fluoranthene, pyrene, B[*a*]P, and pentachlorophenol	Rhamnolipid	Soil	*P. aeruginosa* #641	+	Awashti et al. (1999)
Endosulfan	Lipopeptide	Soil and liquid	*B. subtilis* MTCC 1427	30–45% enhanced degradation	Awasthi et al. (1999)
Phenanthrene, fluoranthene, and pyrene	Alasan	Liquid	*Acinetobacter radioresistens* KA53	+	Barkay et al. (1999)
Endosulfan	Crude surfactin	Soil	*Bacillus subtilis* ATCC 2423	+	Straube et al. (1999)
Phenanthrene and cadmium	Rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Maslin and Maier (2000)
Aliphatic and aromatic hydrocarbons	Crude surfactin	Seawater	*Bacillus subtilis* O9	+	Moran et al. (2000)
Phenanthrene and hexadecane	Rhamnolipid	Soil	*P. aeruginosa* UG2	+	Noordman et al. (2000)
Naphthalene and cadmium	Mono-rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Sandrin et al. (2000)
Phenanthrene	Sophorolipid	Soil	*Candida bombicola* ATCC 22214	+	Schippers et al. (2000)
Toluene, ethyl benzene, and butyl benzene	Di-rhamnolipid	Liquid	*Pseudomonas*	+	McCray et al. (2001)
Phenanthrene slurries	Rhamnolipid	Soil	*P. aeruginosa* UG12	+	Makkar and Rockne (2003)

Hexachlorobiphenyl	Rhamnolipid	Soil slurries	*P. aeruginosa* UG12	+	Makkar and Rockne (2003)
Phenanthrene and pyrene	Rhamnolipid	Soil-aqueous	*P. aeruginosa* ATCC 9027	Enhanced cell surface hydrophobicity, solubilization and transport	Jennings and Tanner (2004)
Hexadecane	Peptidyl-lipid	Liquid	*Bacillus* sp. JF2	High cmc micelles forms; increases dissolution	Jennings and Tanner (2004)
Naphthalene and Phenanthrene	Rhamnolipid	Silica-sand	*P. aeruginosa*	Sorption and transport in silica sand	Chen et al. (2005)
Phenanthrene	Rhamnolipid	Liquid	*P. putida*	Increased soil-sorbed PAHs desorption, solubilisation, and dissolution	Martinez-Toledo et al. (2006)
Petroleum hydrocarbons	Rhamnolipid	Liquid	*P. aeruginosa*	Rhamnolipid-induced cell surface hydrophobicity	Song et al. (2006)
Hexadecane	Biosurfactant	Liquid	*P. aeruginosa* GL1	Increased cell hydrophobicity, emulsification, pseudosolubilization	Bouchez-Naitali and Vandecasteele (2008)
Naphthalene, pyrene and benzo(a)pyrene	Emulsan	Liquid	*Acinetobacter calcoaceticus* BU03	Increased solubilization and uptake	Zhao and Wong (2009)
Pyrene	Rhamnolipid	Liquid	*Pseudomonas* sp. LP1	Increased solubilization and uptake	Obayori et al. (2009)
Phenanthrene	Lipopeptide	Liquid	*P. putida* PCL1445	Augment alkane bioavailability and thus degradation rate	Tecon and van der Meer (2009)
Tetrachloroethylene (PCE) and trichloroethylene (TCE)	Rhamnolipids and surfactin	Liquid	*P. aeruginosa* (MTCC 2297) *B. subtilis*	Increased bioavailability to enhance the biodegradation process	Albino and Nambi (2010)

(continued)

Table 28.2 (continued)

Compound(s)	Surfactant	Medium	Organism	Biodegradation kinetics[b]	Reference
Phenanthrene	Rhamnolipid	Batch soil slurry	*P. putida* ATCC 17484	Emulsification, solubilization and transport	Gottfried et al. (2010)
2-methylnaphthalene, hexadecane and pristane	Sophorolipid	Liquid	*C. bombicola* ATCC 22214	Surfactant solubilization and enhancement	Kang et al. (2010)
Phenol	Mono-rhamnolipid	Liquid	*C. tropicalis*	Surfactant solubilization and enhancement	Liu et al. (2010b)

[a]Studies found in the literature where biosurfactants were used to stimulate hydrophobic organics biodegradation by defined cultures

[b]+: beneficial effect defined as a significant increase in biodegradation rate and/or extent; −: detrimental effect; 0: no effect

Research Council (2003) about 1.3 million tonnes of crude oil is released into the marine environment each year and over 5.6 million tonnes of oil have been released in the environment since 1970 (Cho et al. 2006). Notable examples of massive oil spillage since Arabian Gulf War (1991–1992) include (i) Amoco Cadiz oil spill in Brittany coastal waters in 1978, (ii) Exxon-Valdez spill in the Prince William Sound in 1989, (iii) Haven spill on the coast of Italy in 1991, (iv) over 105 t of petroleum released in the Gulf waters, (v) Nakhodka tanker oil spill (1997) off the Oki Islands in the Sea of Japan, (vi) San Jorge tanker spill (1997) on the shores of Punta Del Este in Uruguay and (vii) Nissos Amorgos spill (1997) in the Maracaibo Channel in the Gulf of Venezuela (viii) release of about 11 million barrels of crude, (ix) pollution in more than 1,280 km coastline of Kuwait and Saudi Arabia and (x) British Petroleum's accidental spills off Gulf of Mexico and (xi) oil spills (2010) off Mumbai shore lines. Microbial system with biosurfactant activity have been recruited for removal of oil spillage at field scale levels.

Laboratory investigations have indicated that rhamnolipids were able to degrade (i) hexadecane, heptadecane, octadecane and nonadecane in seawater upto 47%, 58%, 73% and 60%, respectively (Shafeeq et al. 1989), (ii) n-paraffins, (iii) tetradecane, hexadecane and pristine, (iv) naphthalene, anthracene, phenanthrene, fluorine, 2,2′,5,5′-tetrachloro-biphenyl and 3,3′,4,4′,5,5′-hexachloro-biphenyl, (v) recovery of hydrocarbons to 25–70% and 40–80% in silt-loam soil and sandy-loam soil upon application of 5 gL^{-1} of rhamnolipid, (vi) recovery of aliphatic and aromatic hydrocarbons to 36% and 40%, respectively using a 0.08% mixture of rhamnolipids and (vii) 100% of C8-C11, 83–98% of C12-C21, 80–85% of C22-C31 and 57–73% of C32-C40 of petroleum sludge (Rahman et al. 2002). Further, *in situ* field experiments demonstrated that a 1.0% rhamnolipid solution yielded (a) twofold oil recovery at 10–80°C, (b) 3.5 times more recovery at 40^0 C, (c) 1 min contact time in the Prince William Bay after Alaskan Exxon-Valdez oil spills or oil-contaminated desert sand in Kuwait. Tang et al. (2007) enhanced crude oil biodegradability proportional to rhamnolipid production by *P. aeruginosa* ZJU after preservation in an oil-containing medium. Nitschke et al. (2009) removed 67% of 10% (w/w) of crude oil using 0.1% (w/v) rhamnolipid. Biosurfactant-producing microbes are currently being exploited in British Petroleum's spill off Gulf of Mexico and accidental spill off Mumbai shorelines in India in a joint effort by National Institute of Oceanography (N.I.O) and The Energy Research Institute (TERI), New Delhi.

28.5.5.2 Biosurfactant in Microbial Enhanced Oil Recovery (MEOR)

The concept of MEOR technology was poorly scaled up from laboratory-based studies (1980) to field applications (1990) due to failure of existing EORs to attend problems viz.: (i) low permeability of some reservoirs, (ii) high viscosity of oil leading to poor mobility, (iii) high IFT between the water and oil, (iv) high capillary forces retaining the oil in the reservoir rock, (vi) hazardous implications of chemical surfactants, (vii) high costs, (viii) difficult to dispose undesirable residues, (ix) only

30–50% oil recovery and (x) adsorption of surfactants on the surface of the reservoir by rock-oil-brine ternary interactions. As per National Institute of Petroleum and Energy Research (Dehradun, India), about (i) 27% of the oil reservoirs (600 reservoirs containing over 12 billion barrels of unrecoverable oil) and (ii) 40% of the oil-producing carbonate reservoirs in the US may be suitable for MEOR (Singh et al. 2007). At $100 per barrel, the entrapped but retrievable oil is valued at $32 trillion in the US alone and over $500 trillion worldwide. As of now, more than 400 MEOR tests have been conducted in the US alone.

A 3 year study (2004–2007) by the US Dept. of Energy (USDE) showed that just 250 mg L^{-1} rhamnolipids was sufficient to recover 42% of otherwise entrapped oil from sand-pack. The potential application of the biosurfactants produced by the thermo- and halo-tolerant species of *Bacillus licheniformis* JF-2 and *Bacillus subtilis* have been explored for enhanced oil recoveries in laboratory columns and reservoirs with oil recoveries from 9.3% to 62% (Perfumo et al. 2010b; Singh et al. 2007). Similarly, oil recovery was significantly elevated by 30% from underground sandstone using trehalolipids from *Nocardia rhodochrus* (Franzetti et al. 2010b). Flooding strata with suspensions of *Bacillus, Desulfovibrio, Clostridium, Micrococcus, Pseudomonas, Arthrobacter, Peptococcus, Microbacterium,* and other microorganisms of different taxonomic groups has been recommended. Injection of biosurfactants and bacteria such as *Pseudomonas aeruginosa, Xanthomonas campestris, B. licheniformis* and *Desulfovibrio desulfuricans* along with nutrients showed increase in oil recovery by 30–200%. Pornsunthorntawee et al. (2008) demonstrated that *P. aeruginosa* SP4 biosurfactants removed 57% oil effectively compared to three synthetic surfactants using sand-packed column. Further, a 70% bioremediation and bioreclamation rate of a slop-oil contaminated site has been achieved with emulsan®, a commercial biosurfactant. Moreover, it reduces the viscosity of Boscon heavy crude oil from 200,000 to 100 cP, and facilitated pumping of heavy oil to 26,000 miles through a commercial pipeline. Application of rhamnolipids and surfactin in this area is also visibly encouraging due to (i) possibility of 95% recovery of crude oil, (ii) retaining 100% hydrocarbon content, (iii) comparable American Petroleum Institute (API) values of extracted crude oil and the API range of standard crude, (iv) production of 5,550 barrels of saleable crude from about 750 m^3 of sludge and (v) recovery of cleaning costs by selling recovered crude oil at US $ 1,00,000–1,50,000/storage tank. Kuwait Oil Company has examined 90% oil recovery with biosurfactants for crude oil storage tank clean up.

Although off-site biosurfactant production is the most common practice in MEOR, its potential has not been fully analysed yet due to its high cost. The prospect for strategy like reducing the costs by genetically improved strains (Wang et al. 2007) are probably quite poor since the production of rhamnolipids in *Pseudomonas* is regulated through the quorum sensing system and hence, genetic intervention is difficult (Banat et al. 2010).

Several *in situ* applications of MEOR are reported at field scale level (Sen 2008), but none has clarified whether (i) introduced microorganisms can actually be effective in oil recovery or (ii) they are out-competed by indigenous bacteria (Wang et al. 2008; Banat et al. 2010), and (iii) predictability of biosurfactant-based MEOR

process performance (Banat et al. 2010). Thus, effective MEOR application requires substantial research on a case-by-case basis and the associated costs to minimize uncertainties for MEOR application.

28.5.5.3 Biosurfactant Enhanced Bioremediation: Organics

Interaction of bio-amphiphiles with contaminated soil (which contains at least six phases: bacteria, soil particles, water, air, immiscible liquid and solid contaminants) result into partioning of pollutants among different states: solubilised in the water phase, absorbed to soil particle, sorbed to cell surfaces and as a free/insoluble phase (Banat et al. 2010). Biosurfactants added to this system can interact with both the abiotic particles and the bacterial cells.

High-molecular-weight biosurfactants (bioemulsifiers) have great potential for stabilizing emulsions between liquid hydrocarbons and water, thus increasing the surface area available for bacterial biodegradation. However, they have been rarely tested as enhancers of hydrocarbon biodegradation in bioremediation systems, and contrasting results are reported in the literature (Banat et al. 2010; Franzetti et al. 2010a, b). Emulsan from *Acinetobacter* RAG-1 are known to degrade oil, aromatic and paraffinic hydrocarbons. Alasan, a bioemulsifier produced by *Acinetobacter radioresistens* KA53 effectively emulsifies a wide range of hydrophobic compounds, long chain alkanes, aromatics, PAHs, paraffins and crude oil. While polymeric biosurfactants like sphingans (from *Sphingomona*s strains) and biosurfactant from *Halomonas eurihalina* are able to adsorb to PAHs, but it is unclear about enhancement of apparent substrate solubility and therefore, the mass transfer to the cells.

For low-molecular-weight biosurfactants, above the CMC, a significant fraction of the hydrophobic contaminant partitions in the surfactant micelle cores. In some cases, it increases in the bioavailability of contaminants for degrading microorganisms. Rhamnolipids above CMC, enhanced the (i) apparent aqueous solubility of hexadecane, (ii) biodegradation of hexadecane, octadecane, n-paraffins, creosotes and other hydrocarbon mixtures in soil and (iii) bioremediation of petroleum sludges (Rahman et al. 2002), chlorinated hydrocarbons (PCBs) and pesticides (Singh et al. 2007). Almost double hydrocarbon recovery (from 25% to 70% and 40% to 80%) from contaminated soil using rhamnolipids from *P. aeruginosa* has also been demonstrated. Glycolipid biosurfactants have also been shown to enhance the hydrocarbon removal (from 80% to 90–95%) from soil; furthermore, the biosurfactant was reported to increase hydrocarbon mineralization by twofold and shorten the adaptation time of microbial populations to fewer hours. Recently, uptake, solubilization and biodegradation of 2-chlorobenzoic acid (75%), 3-chlorobenzoic acid and 1-methyl naphthalene (60%) by 0.5% rhamnolipids from *Pseudoxanthomonas* sp. PNK-04 has been examined by Nayak et al. (2009). External addition of a 0.1% commercial rhamnolipids increased (i) both growth and green fluorescent protein (GFP) expression of *Burkholderia sartisoli* RP037 and (ii) phenanthrene bioavailability compared to non-amended control (Tecon and van der Meer 2009). Further, Gottfried

et al. (2010) showed that rhamnolipid with salicylate or glucose in liquid solution increases the apparent aqueous solubility of phenanthrene, and overall degradation by 20% compared to solutions containing only salicylate or glucose. For solubilization of chlorinated solvents in surfactant enhanced aquifer remediation, Albino and Nambi (2010) reported 2.06 and 8.36 Weight Solubilization Ratio (WSR) with rhamnolipids for tetra-chloro-ethylene and tri-chloro-ethylene, respectively. Yuan et al. (2010) used rhamnolipids to accelerate aerobic degradation of tetrachlorobisphenol-A (TCBPA) in sediment samples. Besides being used in remediation of soil and water (Mulligan 2009), rhamnolipids are persistent enough to remain in soil for periods useful for phytoextraction (Wen et al. 2009). Average removal efficiency of PAHs by rhamnolipid-enhanced multi-technique phytoremediation reached to 60.5% and 251.8% vis-a-vis phytoremediation itself (17.19%) (Zhang et al. 2010). Henry and Abazinge (2009) used micro-encapsulated rhamnolipids in ε-polycaprolactone microparticles to optimize the formulation factors and achieved 100% release after 30 days in different release media. The presence of rhamnolipids (300 mgL^{-1}) increased the EC_{50} of phenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,5-trichlorophenol by about 12%, 19%, 32% and 40%, respectively (Chrzanowski et al. 2009). Kulkarni (2005) opened a new gateway towards rhamnolipid-assisted bioremediation of nitroaromatics by enhanced biodegradation of p-nitrophenol upto 300 ppm with 0.8 $ml{\cdot}L^{-1}$ of rhamnolipid. Similarly, Singh et al. (2009) found more than 98% degradation of chlorpyrifos (0.01 $g\ L^{-1}$) using rhamnolipid (0.1 $g\ L^{-1}$) as compared to 84% in control experiment after 120 h incubation.

Interestingly, the release of LPS by *Pseudomonas* spp. induced by sub-CMC levels of rhamnolipids allowed a more efficient uptake of hexadecane by rendering the cell surface more hydrophobic. It has been reported that rhamnolipid produced by *P. aeruginosa* UG2 facilitated the hydrocarbon uptake of the producer strain and increased the degradation of hexadecane but failed to stimulate the biodegradation of hexadecane by *Acinetobacter lwoffii* RAG1, *R. erythropolis* ATCC 19558, *R. erythropolis* DSM 43066 and BCG112 (Noordman and Janssen 2002). Zhong et al. (2008) showed that the adsorption of di-rhamnolipid biosurfactants on cells of *B. subtilis, P. aeruginosa* and *Candida lipolytica* depended on the physiological status of the cells and was species specific. Furthermore, the biosurfactant adsorption affected the cell-surface hydrophobicity and its physiological state by rhamnolipid concentration. The effect of exogenous rhamnolipids on cell-surface composition of *P. aeruginosa* NBIMCC 1390 studied by Sotirova et al. (2008) showed that (i) about 22% reduction of total cellular LPS content above the CMC, and (ii) changes in the bacterial outer membrane protein composition without affecting the LPS component below the CMC. But Chang et al. (2009) demonstrated that the cell-surface hydrophobicity was enhanced by the accumulation of different fatty acids at the cell surface during growth on hydrocarbon in *R. erythropolis* NTU-1. A significant correlation between the modification of the cell surface by saponins and the degree of hydrocarbon biodegradation was reported by Kaczorek et al. (2008). In addition, Wang and Mulligan (2009) observed the effect of ammonium ion concentration and pH on the potential application of rhamnolipid and surfactin for enhanced biodegradation of diesel. Similarly, a lipopeptide and protein-starch-lipid

produced by two strains of *P. aeruginosa* significantly favoured the solubilization and metabolism of phenanthrene, pyrene and fluorine concomitant with growth (Bordoloi and Konwar 2009).

Lipopeptides from hydrocarbon-degrading bacilli act to (i) solubilize and emulsify the substrate, (ii) modulate the bacterial hydrophobicity, and (iii) adsorb onto the cell surface alternately exposing the cyclic peptide (hydrophilic) or the fatty acid tail end (hydrophobic). The contribution of lipopeptides is not merely limited to hydrocarbon access but may confer an evolutionary advantage to the producing bacteria in response to prevailing environmental conditions and substrate availability.

Biosurfactant production was shown a key characteristic of alkane-degrading bacteria, for which it serves to augment alkane bioavailability and thus, degradation rate. A similar effect has been observed for the bacterial degradation of polycyclic aromatic hydrocarbons (PAHs), but it appears that biosurfactant production is not an essential trait of PAH degraders. The external addition of biosurfactants, however, is believed to increase the solubilization of PAHs from non-aqueous phase liquids (NAPLs) and solid particles. Yet, an augmentation of PAH solubilization is not necessarily associated with an equivalent increase of its bioavailability to microorganisms, and hence, the nature of biosurfactants effect on PAH degradation rate are complex (Banat et al. 2010). In fact, it might be possible that molecules dissolved in micelles are actually less available to certain bacteria than freely dissolved molecules, when these are incapable of releasing the molecules from the micelles. Also, biosurfactants themselves might be used as a preferential substrate by microorganisms, which would lead to a reduction of the degradation rate of the HOCs. While *in situ* biosurfactant production has been reported as easier and more cost efficient than external addition (Mulligan 2005). However, it may lead to numerous secondary effects that have no role in increasing availability of organics. On the other hand, augmentation of organic compounds and solubilization is not necessarily associated with an equivalent increase of its bioavailability to microbes. Reports on the efficacy of surfactants on bioremediation have, however, been mixed, inconclusive and remains inoscuous.

28.5.5.4 Biosurfactant Enhanced Bioremediation: Heavy Metals

Annual worldwide release of heavy metals reached (a) 939,000 t for copper, (b) 783,000 t for lead, (c) 1,372,000 t Zn and (d) 22,000 t for cadmium leading to agricultural land contamination to the tune of 10,000 ha in Germany and 100,000 ha in the US and Europe. Rhamnolipid and surfactin facilitate partitioning of metal-surfactant complexes in an aqueous phase and their subsequent removal from soil in the washing process, alleviate heavy metal toxicity and enhance degradation of organic pollutants through heavy metal complexation.

In batch scale, rhamnolipids were found to (i) possess high affinity for lanthanum, (ii) complex with Cd^{++} at binding capacity of 0.2 Cd/rhamnolipid molecule, (iii) have better stability constant ($\log K = 6.89$ and 8.58) for Cd^{++} and Pb^{++},

respectively as compared to oxalic acid, citric acid and SDS, (iv) preferentially complex with Cd^{++}, Pb^{++} and Hg^{++} by mono-rhamnolipid (RL-1), (v) remove 19.5 and 35.1% Zn^{++} and Cu^{++}, respectively, using a 12% rhamnolipid solution, and (vi) desorb Zn^{++} and Cu^{++} from contaminated soil with 12.6% oil and grease content. In column studies, 0.1% di-rhamnolipid solution facilitated 13-fold higher removal of Cr^{++} from the heavy metal-spiked soil, whereas removal of Pb^{++}, Cu^{++} and Cd^{++} was 10, 14 and 25 -fold higher, respectively (Juwarkar et al. 2008). Studies performed by Massara et al. (2007) showed that rhamnolipids (i) remove Cr (III) mainly from the carbonate and oxide/hydroxide portions of the kaolinite, and (ii) reduce close to 100% of the extracted Cr(VI) to Cr(III) over a period of 24 days. It was also found to improve the utilization of zinc (Zn) fertilizers by plant roots in Zn-deficient soils and in solution culture (Stacey et al. 2008). Jordan et al. (2002) examined the chelant-assisted phytoextraction of Cu, Pb and Zn by maize (*Zea mays*) and saltbush (*Atriplex numilaria*) from a soil contaminated by mine tailings using rhamnolipid application. Subsequently, Johnson et al. (2009) observed the effect of 43 and 347 μM of rhamnolipid (along with several other chelants) to improve Cu accumulation by Indian mustard (*Brassica juncea*) and ryegrass (*Lolium perenne*) in hydroponic culture and found (i) negligible toxicity of rhamnolipid to plant shoot growth, and (ii) enhancement of metal uptake. On the contrary, Wen et al. (2010) suggested that rhamnolipid in the soil contaminated by Cd and Zn remain long enough to promote metal phytoextraction, yet not long enough to raise concerns regarding metal transport in the long term.

Similarly, combinatorial amendments of EDDS, rhamnolipid and citric acid resulted in the highest shoot metal levels (Cu and Cd), but also caused severe phytotoxicity in perennial ryegrass (*Lolium perenne*) with negative synergism (Gunawardana et al. 2010). Detrimental effects of rhamnolipids on copper uptake, biomass yield, and the translocation of copper from roots to shoots in *Brassica juncea* and *Lolium perenne* plant species have raised concern about possible role in metal (Johnson et al. 2009).

28.5.6 Biosurfactant-Assisted Bioaugmentation: Field Case Studies

A successful process in the laboratory-controlled conditions does not imply similar success in an uncontrolled environ. Bioaugmentation and biostimulation studies at laboratory, simulated field and *in situ*, are very few so far. It can provide insight about the microbes and their growth requirements, before any on-site intervention for decontamination is carried out. Rosenberg et al. (1992) optimized conditions for bioremediation of crude oil using a combination of bioaugmentation and biostimulation technique in the laboratory, thereby successfully implementing the same for field and *in situ* beach remediation. While Gallego et al. (2007) performed laboratory, pilot, and full scale experiments to select nutrient sources, surfactants, and

other bioremediation amendments for *in situ* bioremediation of spilled oil. Some field remediation studies using bioaugmentation and biosurfactant are presented in Table 28.3. The published results of application of biosurfactants in field scale bioremediation processes are often limited to the statement that they were used to overcome bioavailability limitations without further evidence for beneficial effects of their addition.

28.5.7 Confined Systems and Real-Case Studies: Bridging the Gap

Despite its long-term use in bioremediation, bioaugmentation of contaminated sites with microbial cells continues to be a source of controversy within environmental microbiology. From an applied perspective, successful laboratory studies concerning bioremediation do not necessarily lead to reproducible *in situ* decontamination (El Fantroussi and Agathos 2005; Tyagi et al. 2010). This impending gap between laboratorial trials and on-field studies may be due to several factors influencing the remediation process: (i) strain selection, (ii) indigenous microbial ecology, (iii) type of contaminants, (iv) environmental constraints, and (v) the procedures used for the introduction of the remediation agents. Thus, contrasting effects of biosurfactant application are a result of the poorly understood complexity of interactions between soil/sediment, pollutant, surfactant and microorganisms in different environments. The recent observations that single biosurfactants can have contrasting effects on the degradation of organic pollutants and may further explain why applications of biosurfactants have yielded inconclusive results. There is certainly a need to design an optimal surfactant/biodegrader/target environment combination and to further unravel the underlying complex interactions. Thus, the current knowledge about the optimization of degradation by unknown metabolic communities on site through the addition of biosurfactants remains a futile approach. The combination of surfactant production with degradative capabilities in a single bacterial strain may offer advances for *in situ* bioremediation, but further insights into the genetic organization and regulation of surfactant production are essential.

28.6 Biosurfactant and Bioaugmentation: An Economical Perspective

Existing non-biological remediation technologies seem to be economically non-viable due to (i) impractical cost (US$ 750 billion) and time (about 30 years) of physico-chemical processes, (ii) escalating cost estimates (US$ 30 billion) and hundreds of years of work for organo-metallics, (iii) overburden project estimation of soil

Table 28.3 Biosurfactant-aided bioremediation: field trials

Technique/process	Contaminants removed	Groups and location	Reference
Biostimulation using oleophilic fertilizer and biosurfactant-producing organisms	Oil	Exxon Valdez oil spill, Alaska, USA	Pritchard et al. (1992)
Bioaugmentation and biostimulation using hydrophobic fertilizer as N and P source along with enriched microbial culture	Crude oil	Zvulon beach oil spill, Israel	Rosenberg et al. (1992)
Bioaugmentation and biostimulation with inorganic mineral nutrients and/ or inoculation of biosurfactant-producing microbes	Light crude oil contaminating a sandy beech	Delaware, USA	Venosa et al. (1996)
Low concentration of EPA and FDA approved biosurfactants	Residual wood treating oil (DNAPLs)	Surtek, Inc. Mobility controlled surfactant flushing	USEPA (1998)
Biostimulation using liquid inorganic and slow release mineral fertilizer	Crude oil and heavy fuel oil degradation	Sea Empress oil spill, UK	Swannell et al. (1999)
Biostimulation using forced aeration and nutrient supplementation	Spill simulation (light crude oil and fuel oil) at mature mangroves	Gladstone, Australia	Duke et al. (2000)
Oil spills and bioremediation strategy applied; Bioaugmentation using commercial Biosurfactant producing microbial culture	Heavy crude oil degradation	Nakhodka oil spill, Japan	Tsutsumi et al. (2000)
1% food grade sorbitan mono-oleate (3.8 $L.min^{-1}$ for 3.8 days)	TCE (DNAPL)	US DOE gaseous diffusion pint, Paducah, NY	Mulligan et al. (2001)
Biosurfactant: single phase microemulsion	DNAPL (PCE and perchloroethylene)	Dover AFB, Dover DE	Mulligan et al. (2001)
Biostimulation using oleophilic fertilizer	High molecular weight n-alkanes, alkylcyclohexanes, and benzenes, and alkylated PAHs	Prestige oil spill, Spain (2006)	Jimenez et al. (2006)
Surfactant enhanced pilot-scale *in situ* flushing of biosurfactant	Diesel, kerosene, and lubricating oil	Pusan, Korea	Paria (2008)
Biostimulation using fertilizers (urea and di-ammonium phosphate) and surfactant; moisture content and temperature amendments	Petroleum hydrocarbons degradation in polar desert	Tanquary fiord, high Arctic	Sanscartier et al. (2009)
Biostimulation of manure oily sludge-contaminated soil with biosurfactant	Total petroleum hydrocarbon (TPH) and PAHs	China	Liu et al. (2010b)

excavation in Europe (US$ 270–460 t^{-1}) and US (US$ 324–552 m^{-3}), and (iv) unrealistic demands of incineration (US$ 1.7 trillion; US $7,000/citizen). Comparatively, cost for application of bioremediation approach reduced to US$ 75 billion (US$ 14 billion $year^{-1}$) or 5–170 £ t^{-1} soil.

Due to these facts, biotensioactives-based bioaugmentation technologies as preferred *in situ* remediation techniques have attracted commercial interest. Emerging formulations and products are gaining attention because of successful application, thereby claims for rapid decontamination rates. However, these products are not panacea and need to be evaluated according to the requirements of the site before implementation. As of now, rhamnolipids are commercially available from Jeneil Biosurfactant Inc. (USA), Ecover (France) and Rhamnolipid holdings Inc., (USA), while sophorolipids are currently offered as sophoron™ from Saraya (Japan) and Soliance (France). The current production price of sophorolipids amounts to 2–5 € kg^{-1} whereas rhamnolipids cost US $ 5–20 kg^{-1}; at 20 m^3 US $20 kg^{-1}; when produced at 100 m^3 scale, it costs US $ 5 kg^{-1}, against ethoxylate or alkyl polyglycoside [US $1–3 kg^{-1}].

In bioremediation studies supported by Exxon company from 1993 to 1997 (spill of 41 million litres of petroleum from the Exxon Valdez in Alaska in 1989), has (i) spent > US$ 10 million dollars, (ii) generated seven patents, and (iii) made bioremediation second only to enhanced oil recovery during the first years of its implementation. The distribution of patents in specific areas of the biosurfactants oil industry includes 17 patents in soil and water bioremediation and 20 in enhanced oil recovery (Santos et al. 2011).

28.7 Future Directions and Concluding Remarks

A most sustainable alternative is an integrated approach more focused on (i) economical feasibility, (ii) ensuring protection to the environment and (iii) acceptable by stakeholders and the society in general. At present, biosurfactant-aided bioaugmentation hold the promise of epitomizing *in situ* bioremediation. The gap between R&D and the application of biotensioactives in bioremediation options is partly due to a lack of awareness by regulators and problem owners, a lack of expertise and knowledge by service providers. It would be naive to believe that by simply picking the ‘right’ biosurfactant-producing microbe(s) or manipulating the right field parameter, bioaugmentation will suddenly become as reliable and predictable as engineered systems. Development of a tailor-made additive mixture suitable for extreme reservoir conditions, consisting of a combination of suitable microbial strains, nutrients, biosurfactants and buffering agents in appropriate proportions, may foster a further productive line of research. Based on these considerations, a deeper understanding of microbial degradation abilities, together with their metabolic networks as well as their cellular resistance and adaptation mechanisms, will bring out a variety of appropriate microbial formula tailored for decontamination of a specific site.

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