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Microbial Bioremediation of Fuel Oil Hydrocarbons in Marine Environment

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ABSTRACT

Pollution in marine environment due to heavier petroleum products such as high-speed diesel is known to take from days to months for complete natural remediation owing to its low volatility. For the survival of marine flora and fauna, it is important to control pollution caused by such recalcitrant and xenobiotic substances. Several petroleum hydrocarbons found in nature are toxic and recalcitrant. Therefore, pollution due to high-speed diesel is a cause of concern. The natural dispersion of high-speed diesel, a slow process, is attributed to an overall combined effect of physico-chemical and biological processes which take months for complete dispersion. History of marine oil spill bioremediation indicates limited laboratory studies. But experiences from various oil spill management and field trials indicate important role of bioremediation, where, biodegradation of hydrocarbons through microbial mediators plays a major role in pollutant oil dispersion. These microbial mediators such as bioemulsifiers and fimbrae, help in emulsification, dispersion, allowing attachment of bacteria to oil layers, followed by substrate-specific enzymatic biodegradation in water.

Keywords: Pollution, pollution control, marine environment, oil slick, oil spill, bioremediation, oil spill management, microbial mediators, bioemulsifiers, fimbrae, oil dispersion, microbial biodegradation

1. INTRODUCTION

Hydrocarbon pollution of marine ecosystem due to refined and crude fuel oil is always a cause of concern[1]. Pollution due to light petroleum products like gasoline is treated efficiently by natural physico-chemical factors. However, heavier fuels like diesel take months for complete natural remediation due to their low volatility. Therefore, it often requires other techniques for environmental management. For the survival of diversified life forms, it is important to control pollution of recalcitrant and xenobiotic substances. Several petroleum hydrocarbons found in nature are toxic and recalcitrant. These are known to affect ecological balance and are becoming a threat to the diversity of life forms. In addition, there are issues concerning economy of the affected zone.

In the last four decades, several incidences of crude oil spills have resulted in heavy pollution of seawater, affecting marine organisms such as fishes, birds, etc, in and around the sea where the oil spill occurred (Tables 1 and 2). Therefore, it is important to control oil pollution in both marine and terrestrial environment.

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Table 1. Important international incidents of oil spills

Year	Incident	Quantity of oil spill (Mega tonne)	Oil type	Place
1978	Amoco Cadiz	0.20	Crude oil	Along Brittany coast
1989	Exxon Valdez	0.04	Crude oil	Prince William sound
1991	Haven (caught fire) and sank onboard with oil	0.14	Crude oil	Coast of Italy
1991	Gulf war	0.82	Crude oil	Kuwait
1993	Braer (released)	0.08	Crude oil	Coastal water of Shetland islands

Table 2. Major incidents of oil spills in and around Indian Coastal waters

Year	Incident*	Quantity of oil spill (mega tonnne)	Oil type	Place
1991	MT Jaybola	692	Fuel oil	Gulf of Manner
1991	Zakir Hussain	40,000	Crude oil	Bombay High
1992	SCI Hommi Bhanha	10,000	Crude oil	54NM West Kochi
1992	Albert Ekka	10,600	Fuel oil	Madras harbour
1993	Mearsk Navigator	40,000	-	Off Nicobar island
1993	BHM Platform	6000	Crude oil	Mumbai High
1994	Innovative II	1400	Crude oil	Off Saccramento Pt.
1994	Sea Transporter	1025	Crude oil	Off Auguda Goa
1999	MV Pacific	500	Fuel oil/diesel	Mul Dwarka

* Oil spills less than 500 tonne are not included in the table.

2. HISTORY OF MARINE OIL SPILL BIOREMEDIATION

History of marine oil spill bioremediation indicates limited laboratory studies and field trials[2,3]. It is reported that interest in containment of marine oil spill gained importance following the Amoco Cadiz oil spill in 1978. Also for the first time, it became clear that biodegradation of hydrocarbons plays a major role in natural oil removal[4].

In this study, agricultural runoff water containing fertilisers was found to accelerate the biodegradation. However, the first pilot-scale study was initiated at Spits Bergen, Norway, in 1976. In this study, microbial response to crude oil spill was monitored[5]. Two years later, agricultural fertilisers were added to this site and a ten-fold increase in the marine oil degradation rate was recorded.

Experiments conducted at Baffin island to assess the effect of mineral fertilisers on biodegradation of Lago Medio crude oil buried under gravel and sand were discussed by Sendstad[6], *et al*. A five-fold increase in the marine oil degradation rate was inferred as compared to untreated area.

All the above field trials, on a small or pilot scale, indicated the need for a liquid fertiliser that allows slow release of nitrogen and phosphorus, and as such, these formulations remain attached to the oil to be degraded.

Inipol EAP22 is one such fertiliser developed by Elf Aquitaine[7], which forms an oil external microemulsion that contains nitrogen and phosphorous nutrients. Its outer oil surface is known to stick to pollutant oil residues, and therefore, it is described as an oleophillic fertiliser.

In an experimental oil spill at Norway, inipol-EAP22 was first compared with water-soluble agricultural fertiliser. A three-fold increase in alkane biodegradation of Statfjord oil was observed in both Inipol-EAP22 and agricultural fertilisers-containing sites as compared to the untreated areas. Although inorganic fertilisers are much cheaper, because of their high solubility, their concentration in the water column decreases very fast, limiting their use in water bodies and in marine environment. Further, excessive use of fertilisers is always a cause of concern for environmentalists. On the other hand, slow release formulations such as inipol, or Naval

Material Research Laboratory nutrient (Patent Application No.770/DEL/2004) remain attached to pollutant oil through their oleophillic component, and facilitate slow release of nutrients for sustained growth of oil-degrading bacteria.

Bioremediation on actual oil spill was first used in November 1985 of Ny Alesund, Spitsbergen, where above 88,000 litre of marine gas oil was spilled parallel to the shoreline[8,9]. Inipol was applied 4-5 months after the oil spill and a 6 to 9-time higher rate of biodegradation was observed as compared to the control.

The largest and the most extensively studied incident was of Exxon Valdez, Alaskan oil spill, in which a tanker ran aground on Blign Reef on 24 March 1989, spilling 1.8 million tonne of North Slope crude oil. Despite extraordinary efforts to contain the oil spill, tidal currents and winds caused significant portions of the oil to float ashore, covering 778 km of the shoreline in the gulf of Alaska.

On Knight island, bioremediation was approved for large-scale application on 1 August 1989 and by the summer, 118 km of shoreline had been treated. The cleanup workforce employed included over 11,000 workers, 1,400 boats, and 84 aircraft. By 1990, shoreline oil decreased substantially as a result of the cleanup exercise and natural cleansing[10].

In 1990, stand-alone bioremediation in combination with mechanical cleanup techniques was used. By 1991, oil was further reduced significantly and by May-June 1992, as per Joint Federal report, it was confirmed that all the oil had been removed from the shoreline. On 12 June 1992, the US Coast Guard and the state of Alaska officially declared that the cleanup had been concluded. This exercise, however, did not include the use of laboratory-grown culture of marine organisms.

In contrast to laboratory investigations, relatively few trials have been performed to test the effectiveness of bioremediation in the field. This is because such trials are difficult as well as expensive to conduct. Also, either experiments are carried out after a real oil spill incidence or permission has to be obtained to release oil into the environment for conducting field studies.

Field trials at the sea are virtually impossible[11] to control without containing the oil slick in someway as no control is possible over the distribution of oil. Experiments on oil-contaminated shoreline after an oil spill incidence are easier, but again, scientists have little choice in the type of beach, concentration, type of oil, degree of weathering, and emulsification. Therefore, the challenge is to deal with oil spills under the existing environmental conditions and try to design carefully-controlled experiments.

In controlled field trials, it is useful to select the location, oil type, concentration, and degree of weathering of the oil. The bioremediation studies under field conditions can be viewed as shoreline studies and open-water studies.

In shoreline studies, both the inorganic and the organic fertilisers are used for bioremediation of oil slick along with or without oil-degrading bacteria. The examples include the first field trial conducted at Spitsbergen, Norway, in 1976. In this study, 10 litre of non-weathered Forcados crude/m^2 was released onto a 10 m^2 test site. The inorganic fertiliser at a concentration of 1.2 per cent w/v stimulated oil biodegradation.

As confirmed by n-C17/ Pristane and n-C18/ Phytane ratios, and by the respiration rate of indigenous microorganisms, another study was conducted in Canada (Baffin island) during 1980-83. In this study, 45,000 litre of crude oil (medium gravity) was spilled. Short-and long-term effects of chemically-dispersed oil slick as well as effectiveness of other shoreline cleanup techniques, including *in-situ* burning, application of dispersant, solidification, mixing and bioremediation, were investigated. This exercise was undertaken in plots within the supertidal zone, where a commercial agricultural fertiliser (6.4 g of nitrogen/m^2) was used.

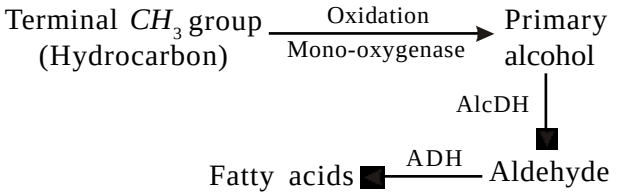
Another study by Lee and Levy[2,3] of Scotian shelf condensate (SSC) and Hibernia crude oil (HCO) was conducted on a sandy beach of Canada. This study used periodic addition of an agricultural fertiliser mixture having *N*:*P*:*K* ratio 10:1:0 composed of ammonium nitrate and granular superphosphate (without potassium). This was applied 2 weeks after the addition of oil to the sand beach (1.14 l/m^2).

A steady decline in plots treated with SSC and *HCO* over a period of 8-12 months was observed.

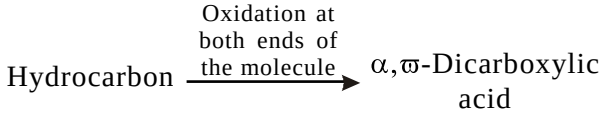
3. BIOCHEMISTRY OF FUEL OIL BIODEGRADATION

Biodegradation and bioremediation of diesel oil are important aspects and have gained importance in the past several years. Diesel fuel is known to be a mixture of *n*-paraffins, branched paraffins, cyclic-paraffins, and aromatic hydrocarbons. Normal and branched alkanes have been extensively studied for their microbial metabolism. Higher alkanes, often due to low volatility, require relatively longer time for their microbial degradation. In recent years, it has become clear that there are at least three basic modes of initial attacks on alkane molecules.

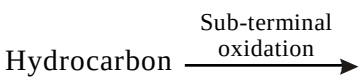
(i) The first one involves oxidation of one of the terminal methyl groups to a carboxylic acid which requires an enzyme called mono-oxygenase to form a primary alcohol. Successively involving two hydrogenation steps, alkanes are converted into an alcohol, aldehyde[12,13] and fatty acids[14] in the presence of alcohol dehydrogenase (AlcDH) and aldehyde dehydrogenase (ADH) as shown below:



(ii) The second mode involves oxidation at both ends of the molecule to form an alpha, omega-dicarboxylic acid.



(iii) The third mode is based on sub-terminal oxidation forming a ketone[15].



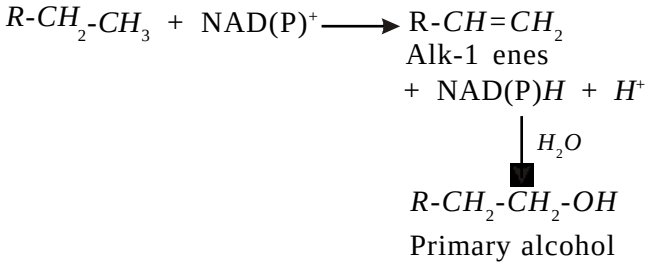
Among these modes of initial attack on alkanes, the first mode of attack is the most common where many enzymes, that catalyse initial terminal oxidation, have been studied extensively[16].

3.1 Mechanism of Alkane Oxidation

The mechanism of alkane degradation as per the available literature indicates various steps such as dehydrogenation, hydroxylation, and hydroperoxidation.

Step 1. *Dehydrogenation of alkanes*

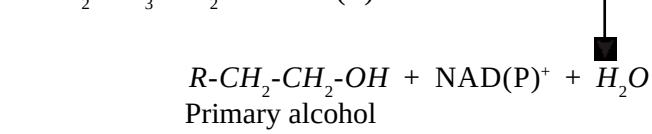
Dehydrogenation of alkanes to alk-1 enes, followed by hydration to form a primary alcohol was reported by various investigators[17-20].



Experimental support to this mechanism lacks rigorous enzymological proof. Formation of internal mono-hexadecanes by *Nocardia salmonicolor*, appears to be irrefutable[20].

Step 2. *Hydroxylation*

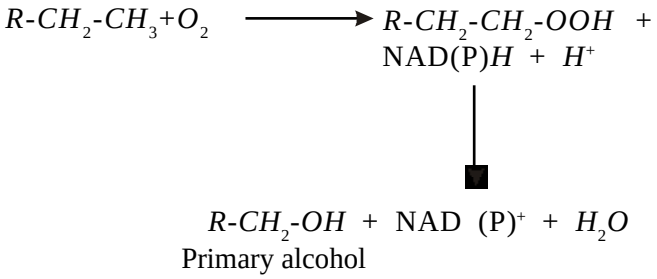
Gallo[2], *et al.* reported that hydroxylation step is catalysed by mixed function oxidases (mono-oxygenase). In this reaction, the alkane is converted to the primary alcohol in the presence of molecular oxygen and a reductant. One atom of oxygen gets incorporated into the alkane while the other atom is reduced to water.



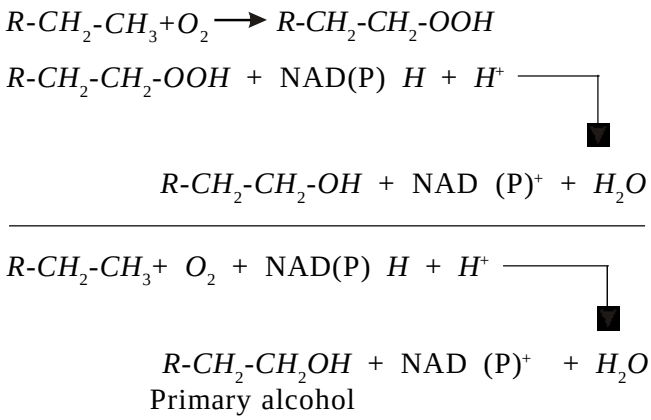
Step 3. *Hydroperoxidation*

This involves incorporation of molecular oxygen into the alkane by a dioxygenase yielding an *n*-

alkyl hydroperoxide, which in turn gets reduced to primary alcohol [14-21].



Various direct and indirect evidences largely support this mechanism. However, alkyl hydroperoxidase remain to be demonstrated in alkane-oxidising microorganisms. Further, since the hydroperoxide formation followed by reduction exhibits the same overall stoichiometry as that which occurs with the hydroxylases reaction, the distinction between the mechanism of hydroxylation and hydroperoxidation is mechanistically unresolved.



Although various steps in the above three mechanisms remain to be demonstrated and established, the role of molecular oxygen in alkane oxidation by microbes, such as bacteria, yeast, and fungi is established beyond doubt.

4. MEDIATORS IN FUEL OIL BIOREMEDIATION

In the aquatic environment, entry or attachment of bacteria to oil layers is considered important by many investigators for their effective utilisation. This attachment is dependent on various mediators such as bioemulsifiers, fimbrae, etc. Since dispersion of oil in the environment is a function of two phases and is dependent on surface-active compounds like bioemulsifiers produced by these organisms to form stable emulsions[22-24], role of biosurfactant and bioemulsifier is of paramount importance in oil bioremediation[15,25,26].

Several investigators have described the phenomenon of bacterial adherence to oil, oil-water interface, and considered it to be dependent on varying specificities of cell surface biochemical properties towards oil[27-29]. Further, it is reported that ability to biodegrade hydrocarbons is directly or indirectly influenced by the bacterial mediators affecting their adherence to oil or oil-water interface[30], but access to oil is also an important primary requirement for its degradation[31-33]. In view of these facts, bacteria having biochemical enzyme cascade to degrade oil along with high degree of adherence to oil, are considered more efficient at oil utilisation. Available literature indicates importance of studying enzymatic oil-degrading ability of such microbes along with oil adherence properties in relation to mediators such as bioemulsifiers[34,35].

4.1 Bioemulsifiers

The major classes of bioemulsifiers reported in the literature include glycolipids, lipopeptides, lipoproteins; fatty acids, phospholipids, neutral lipids; polymeric biosurfactants, and particulate biosurfactants.

4.1.1 *Glycolipid Biosurfactants*

This class of biosurfactants are very well studied. Their hydrophilic carbohydrate moiety in combination with hydrophobic moiety, ie long chain fatty acid or hydroxyaliphatic acids, forms excellent glycolipid biosurfactants. The best known examples include:

- *Rhamnolipid biosurfactants*: First described by Jarvis and Johnson[36], in *Pseudomonas aeruginosa* having one or two rhamnose linked to one or two molecules of β-hydroxy decanoic acid. It is described as rhamnolipid-1 (L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) and rhamnolipid-2 (L-rhamnosyl-β-hydroxydecanoyl-β- hydroxydecanoate). This

product is known to reduce surface tension of water from 72 mN/m to 25–30 mN/m in the presence of biosurfactant[37,38]. Critical micelle concentration reported for this substance range between 0.1–10.0 and interfacial tension is 0.25–1.00 mN/m[39,40].

- *Trehalolipids*: Several structural types are reported. These are made up of disaccharide trehalose linked at C6 and C6' to mycolic acids. These biosurfactants are commonly reported in *Mycobacteria*, *Nocardia*, and *Corynebacteria*. Similar biosurfactants are reported with slight variations in other organisms such as *Rhodococcus*, *Erythropolis* and *Arthrobacter* species. The surface tension observed with these biosurfactants in culture broth was in the range 25–40 mN/m and interfacial tension[41-43] was 1–5 mN/m.

- *Sophorolipids*: Glycolipid biosurfactants are mainly produced by yeasts such as *Torulopsis bambicola*, *Torulopsis petrophilum*, and *Candida bogoriensis*. These are formed of a dimeric carbohydrate sophorose linked to a long chain fatty acid. They reduce surface tension[44-47] to 30–33 mN/m.

4.1.2 *Lipopeptides & Lipoproteins*

Several cyclic lipopeptides described in the literature including antibiotics such as gramicidin and polymyxins are produced by some gram positive bacteria of genus *Bacillus* species. The most powerful biosurfactant known till date is surfactin. It is produced by *Bacillus subtilis* (ATCC21332) which lowers pure water surface tension[48] from 72 mN/m to 27.9 mN/m at a concentration as low as 0.005 per cent.

4.1.3 *Fatty Acids, Phospholipids & Neutral Lipids*

Large quantities of fatty acids, phospholipids, and neutral lipids are produced by both bacteria and yeast when grown on *n*-alkanes. The hydrophilic-lipophilic balance of such biosurfactants is directly related to hydrocarbon chain length. *Acinetobacter* species is known to produce such biosurfactants[49] (*n*-phosphatidylethanolamine). This material forms an optically clear microemulsion of alkane in water. *P. aeruginosa* (strain 44T1) is reported to produce 40-80 per cent (w/w) such lipids when grown on hexadecane and olive oil[50,51].

4.1.4 *Polymeric Biosurfactants*

These are high molecular weight biosurfactants and can be called bioemulsifiers. Well-known examples of this type include emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes. *Acinetobacter calcoaceticus* (strain RAG-1) produces a potent polyanionic amphipathic heteropolysaccharide bioemulsifier known as emulsan. Emulsan is known to emulsify hydrocarbons at concentrations as low as 0.001 per cent to 0.010 per cent. It is the most powerful emulsion stabiliser known today and resists inversion even at a water-to-oil ratio[52] of 1:4.

5. OIL POLLUTION CONTROL METHODS

5.1 Natural Means (Physico-chemical Factors)

5.1.1 *Evaporation*

Evaporation is one of the most important processes that removes volatile components of oil from the water surface. Components with boiling points lower than 200 ^{o}C will evaporate within 24 h. The loss of the more volatile components will cause the remaining oil to have higher viscosity, pour point, and flash point than the original one.

5.1.2 *Dissolution of Oil Components*

Some of the lower molecular weight components dissolve in water to a small extent. Although, a very small volume of the oil spill is lost as dissolved components, it is responsible for the acute toxicity to marine organisms.

5.1.3 *Photooxidation*

Exposure to the ultraviolet radiation of sunlight causes oxidation of more polar groups. This promotes formation of water-in-oil emulsion and slight increase in water solubility.

5.1.4 *Biodegradation*

Seawater contains many kinds of microorganisms that can use hydrocarbons as an energy source. In

case of an oil spill, the concentration of these microorganisms rises rapidly. However, the rate of biodegradation can be low and limited by factors such as the temperature, availability of oxygen, on nutrient sources such as nitrogen and phosphorus.

5.1.5 *Sedimentation*

Under turbulent conditions in shallow waters, some oil may stick to particles of sediment/silt and may sink.

5.1.6 *Water-in-oil Emulsification*

It is the most important process that causes spilled oil to persist at the sea surface. The formation of water-in-oil emulsions greatly increases the volume of pollutant, and the emulsion has a much higher viscosity than that of the original spilled oil.

5.1.7 *Natural Dispersion*

Breaking waves convert the oil slick into oil droplets with sizes ranging from 1 μm to 1000 μm in diameter. Due to vertical and horizontal turbulence of water, oil droplets less than 100 μm are repeatedly pushed back into the water column and can be considered permanently dispersed.

5.1.8 *Spreading & Drifting*

When oil is spilled on sea water, it spreads rapidly. A highly viscous oil spreads at a lower rate. This floating oil is transported to the sea surface under the influence of wind and water current. Oil spreads mainly in the downwind direction with very large variations in oil slick thickness.

5.2 Conventional Methods for Removal & Control of Floating Oil Pollution

Oil floating on the sea surface can be dealt with different options such as mechanical recovery, *in-situ* burning, chemical dispersion, and bioremediation.

5.2.1 *Mechanical Recovery*

Mechanical containment and recovery of oil is the most desirable option, but also the one most limited by wind, water current, and sea conditions. Containment is most often accomplished with commercially available booms. Skimmers and simple suction devices are used to recover oil from the sea surface. When skimmers and suction pumps are not effective in situations of small floating oil slicks, shallow waters, or inaccessibility, sorbents are used to recover spilled oil through adsorption or absorption (Table 3).

Chemicals commonly used in oil pollution control are adsorbents and their effective use involves other physical methods also. Use of some skimming devices is limited in recovering viscous oils. The rate of recovery for skimmers and the amount of storage available are often additional limiting factors. In case of sorbents, if these are not recovered completely, sorbents saturated with oil may spread the contamination further. Some sorbents absorb water and release oil as time passes and tend to sink. Complete separation of oil and sorbent is technically difficult and oil remains to a certain percentage in the sorbent. Generally, reuse of the sorbent is not a good option because of problems of storage after treatment and the requirement for

Table 3. Properties of some commonly used chemical sorbents

Sorbent	Maximum oil-adsorbing capacity g/g sorbent		Buoyancy after prolonged contact with oil on water
	High viscosity oil (3000 cSt* at 25° C)	Low viscosity oil (5 cSt* at 25° C)	
Inorganic			
Vermiculite	4	3	Sinks
Volcanic ash	20	6	Floats
Glasswool	4	3	Floats
Natural organic			
Corn cob	5-6	5	Sinks
Peanut husks	2-5	2	Sinks
Redwood fibre	6-12	6	Sinks
Wheat straw	2-6	2	Sinks
Peat moss	4-7	7	Sinks
Wood cellulose fibre	10-18	10	Sinks
Synthetic organic			
Polyurethane foam	70	60	Floats
Urea formaldehyde foam	60	50	Floats
Polyethylene fibre	35	30	Floats
Polypropylene fibre	20	7	Floats
Polystyrene powder	20	20	Floats

cSt*: Centistokes

additional personnel to process the oiled sorbent. For synthetic sorbents, although these are very efficient in recovery of oil, their disposal is a problem as these are not biodegradable.

5.2.2 *In-situ Burning*

This may be considered as a possible response option, provided there is no risk of fire spreading to cause injury to human beings or damage to the property. This approach has the following limitations:

- The oil slick must have a minimum thickness of 2 mm to burn on water.
- *In-situ* burning results in emission of air pollutants.
- Water-in-oil emulsions burn poorly.

5.2.3 *Chemical Dispersion*

Dispersants are chemical agents which alter the physical behaviour of oil on the sea surface. These consist of a mixture of surface-active agents dissolved in a solvent which assists penetration of the mixture into the oil. The surface-active agents reduce the surface tension of oil-in-water, so as to increase the rate of droplet formation, inhibiting coalescence, and facilitating natural degradation. Dispersed oil may degrade rapidly depending upon local conditions as compared to oil-in-a-surface slick, and in some circumstances, it will present less of a threat to the environment. If undegraded, both pollutant oil and chemical dispersants pose serious threat to the local flora and fauna, causing enhanced acute/chronic toxicity.

6. BIOREMEDIATION: WHY ? & HOW ?

Conventional treatments are no longer acceptable to the environmentalist and to the public with growing awareness, as these treatments only transfer the pollution, creating new waste, as in the case of incineration, which generates residues that are more toxic, non-biodegradable, and the process may contribute to greenhouse effects. This does not eliminate the problem of pollution completely. Chemical methods which involve adsorbents have their own limitations and their synthetic analogues, although more efficient, are less acceptable due to their non-biodegradable nature.

Bioremediation, which involves native microorganisms, can be used effectively for the control of such fuel oil pollution. Many naturally occurring microorganisms have the ability to utilise hydrocarbons as a sole source of carbon and are widely distributed in nature. The pollution control by such biological means is frequently being described as bioremediation. Many bacterial species play an active role in the process of petroleum fuel biodegradation. Some biodegradable hydrocarbons such as *n*-alkanes are known to be utilised by a large number of microorganisms as a sole source of carbon and energy[29,53-55]. Polycyclic aromatic hydrocarbons and cyclo-alkanes are mainly degraded by co-oxidation in the environment[56].

Rosenberg[57], *et al.* demonstrated the requirement of direct physical interaction of bacterial cells to oil-hydrophobic substrates for the initiation of biodegradation. Requirement of internalisation followed by degradation by intra- or extra-cellular enzymes was considered important by various investigators[35,58,59]. The process of internalisation is reported to be mediated through specific microbial biosurfactants and bioemulsifiers. It is reported that hydrocarbon internalisation is mediated by the formation of microemulsion, presumably by lowering interfacial tension[59]. Although, literature on the effect of surfactant on oil utilisation ability shows varying results, majority of reports indicate its important role in effective dispersion and degradation of pollutant oil in marine environment[60-63].

The surfactants are considered versatile process chemicals in many industries (soap, cosmetics, food, and agriculture) and are defined as amphiphillic molecules having both hydrophilic and hydrophobic moieties in a given molecule. Their amphiphillic nature renders excellent partitioning characteristics at the interface of two immiscible phases having varying degrees of polarity and hydrogen bonding. These properties confer them excellent ability to reduce interfacial and surface tensions. As a result, these display excellent dispersing, emulsifying, foaming, and detergent characteristics in their field of application.

The current worldwide market estimates indicate requirements of approx. $9.4 billion per annum[64] for these sufactants.

Till recently, most of the surfactants were derived chemically from the petroleum products. However, importance of microbial surfactants was realised recently due to their eco-friendly nature, application in environmental protection (bioremediation), diversity of nature, and scope of production by fermentation, in crude oil recovery, in healthcare, and in food industry[3,26,65-67]. Apart from these, microbial surfactants have known advantages over the chemical dispersants such as lower toxicity; higher biodegradability[64]; higher foaming[68]; high selectivity and specific activity at extreme temperature, p*H* and salinity[69]; better environmental compatibility[66]; and scope of synthesis from simple cost-effective carbon sources[67]. Due to their biological origin, these are frequently referred to as biosurfactants or bioemulsifiers (emulsion stabilisers).

Bioemulsifiers can be defined as a structurally diverse group of surface-active molecules of biological origin having ability to reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures. Like chemical surfactants, biosurfactants are known to display amphiphillic nature having a hydrophilic and a hydrophobic moiety. The hydrophilic moiety could be amino acids or peptides, anion, cation, mono, di, or polysaccharide, and the hydrophobic moiety are often represented by an unsaturated or saturated hydrocarbon component of the fatty acids[67].

Biosurfactants and bioemulsifiers, being relatively larger molecules than their chemical counterparts, are required to have excellent hydrophilic-lipophilic balance (HLB). The HLB values indicate whether a bioemulsifier will promote water-in-oil or oil-in water emulsion[34]. In view of these properties, biosurfactants can be better exploited in the bioremediation of floating oil in harbour waters along with oil-degrading bacteria and nutrient supplements.

7. COMBATING FLOATING OIL IN HARBOUR WATERS

7.1 NMRL'S Approach

To address the problem of floating oil in harbour waters, a bioremediation process has been developed which employs a consortium of five marine oil-degrading bacteria isolated from Mumbai harbour, bioemulsifier (patent filed, 2003), and nutrients (source of nitrogen and phosphorus, patent filed, 2004) produced by the process technology developed at the Naval Materials Research Laboratory, Ambernath. The basis of floating-oil bioremediation using this approach is explained in the schematic diagram (Fig. 1).

The three components, namely bioemulsifier, bacteria, and nutrients in the presence of mixed oxygen due to tidal currents facilitates effective dispersion and degradation of the pollutant oil. The bioemulsifier renders effective emulsification of oil-in-water column, allowing easy entry of hydrocarbon-utilising bacteria into the oil slick. These bacteria, through their membrane-bound enzymes of oxidoreductases class, degrade hydrocarbons and release carbon dioxide and water apart from generation of bacterial biomass (Fig.1).

The other primary and secondary metabolites, including surfactants and bioemulsifiers, further help to sustain emulsification and dispersion of oil on subsequent days. Further, this approach advocates supplementation with essential nutrients, such as nitrogen and phosphorus, for bacterial survival, and thus facilitating continuous availability of degradative enzymes and biosurfactants. Based on extensive evaluation in several field trials in association with various agencies such as Western Naval Command, Eastern Naval Command, and Coast Guard, this approach was found effective to combat pollutant oil effectively, depending upon the oil content on site during the initiation of bioremediation.

7.2 Research Work Undertaken at NMRL

Additionally, to understand molecular biology of the oil-degrading bacterial consortium, genomic

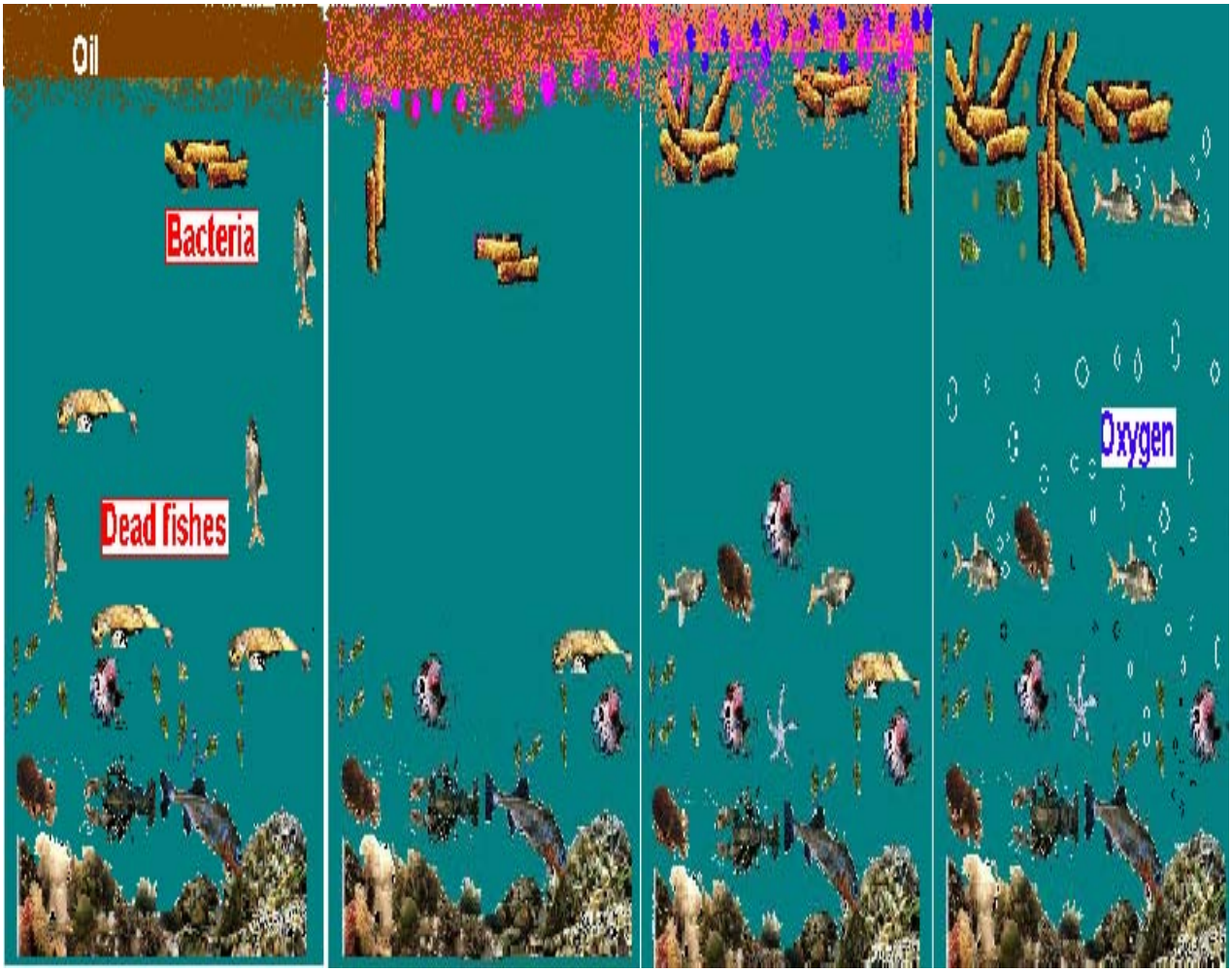


Figure 1. Schematic representation of bioremediation of floating oil in marine environment: (a) fuel oil pollution causing death of local flora and fauna, depletion of oxygen and light, (b) addition of bioemulsifier and nutrients facilitated emulsification of oil and growth of local oil utilizing bacteria, (c) addition of enriched consortium of oil-degrading bacteria along with bioemulsifier and nutrients facilitated fast emulsification, dispersion and degradation of pollutant oil, and (d) complete dispersion and degradation of oil achieved by bioremediation with nutrients, bioemulsifier and oil-degrading bacteria providing clean and healthy environment to marine organisms.

and plasmid DNA are being studied. Already, a few important genes have been detected in these bacteria encoding for enzymes of alkane degradation. Nevertheless, the complete operon and other genes are being studied. The gene (alkB) encoding for alkane mono-oxygenase, an important enzyme of the alkane hydroxylase complex, has been sequenced, reported, and submitted recently to Genbank USA (Accession No.:AY286497). The gene is found on an *p*OCT homologous plasmid which is being characterised.

7.3 Future R&D Work

Future of oil-spill bioremediation appears to be more promising as advent of biotechnology and information technology is facilitating development of more efficient, rapid, specific, and sensitive techniques for the bioremediation of recalcitrant and xenobiotic substances. For bioremediation of oil spills, the new emerging strategies include: (i) use of relevant enzymes immobilised on inert surfaces or outside bacteria for specific degradation of hydrocarbons,

(ii) use of more potent bioemulsifiers along with slow-release oleophilic fertilisers, (iii) use of genetically-modified omnipotent heterogeneous superbugs for efficient and fast remediation of hydrocarbons[70,71]. Therefore, new developments involving the above techniques hold a promising future for providing pollution-free marine environment.

REFERENCES

1. Leahy, J.G. & Colwell, R.R. Microbial degradation of hydrocarbons in environment. *Microbiological Reviews*, 1990, **54**, 305-15.

2. Lee, K. & Leavy, E. Field evaluations of marine oil spill bioremediation. Canadian Technical Report of Fisheries and Aquatic Sciences, No. 1442. April 1986.

3. Lee, K. & Leavy, E. *In* Aquatic toxicology and water quality management, edited by J.A. Nriagu. John Willey & Sons, New York. pp. 217-43.

4. Gundlach, E.R.; Boehm, P.D.; Marchand, M.; Atlas, R.M.; Ward, D.M. & Wolfe, D.A. *Science*, 1983, **221**, 122-29.

5. Sendstad, E. Enhanced oil biodegradation on the arctic shoreline. *In* Proceedings of the 7th Arctic and Marine Oil Spill Programme, Technical Seminar, June 12-14, 1984. Edmonton, Alberta, 1984. pp. 60-74.

6. Sendstad, E.; Hoddo, T.; Sveum, P. & Eimhjellen, K. Studies on a seven year old sea shorecrude oil spill on Spitsbergen. *In* Proceedings of the 5th Arctic and Marine Oil Spill Programme, Technical Seminar, June 15-17, 1982. Edmonton, Alberta, 1982. pp. 331-40.

7. Ladousse, A. & Tramier, B. Results of twelve year of research in spilled-oil bioremediation: INIPOL EAP-22. *In* Proceedings of the 1991 International Oil Spill Conference, March 4-7, 1991. San, Diego, CA, 1991. pp. 577-81.

8. Sveum, P. Accidently spilled gas oil in a shoreline sediment on Spitsbergen: Natural fate and enhancement of biodegradation. *In* Proceedings of the 10th Arctic and Marine Oil Spill Programme, Technical Seminar, June 9-11, 1987. Edmonton, Alberta, 1987. pp. 177-92.

9. Sveum, P. & Ladousse, A. Biodegradation of oil in the arctic: Enhancement of oil-soluble fertiliser application. *In* Proceedings of the 1989 Oil Spill Conference. American Petroleum Institute, Washington DC, 1989. pp. 439-46.

10. Jahns, H.O.; Bragg, J.R.; Dash, L.C. & Owens, E.H. Natural cleanup of shorelines following the Exxon Valdez oil spill. *In* Proceedings of the 1991 International Oil Spill Conference, March 4-7, 1991. San Diego, CA, 1991. pp. 167-76.

11. Swannell, R.P.J.; Kenneth, L & Madeleine. Field evaluation of marine oil spill biomediation. *Microbiological Reviews*, 1996, **60**, 342-65

12. Baptist, J.N.; Gholson, R.K. & Coon, M.J. Hydrocarbon oxidation by a bacterial enzyme system, Part I. Product of octane oxidation. *Biochim. Biophs. Acta*, 1963, **69**, 40-47.

13. Jurtshunk, P. & Cardini, G.E. The mechanisms of hydrocarbon oxidation by a *Corynebacterium* species. *CRC Crit. Rev. Microbiol.*, 1971, **3**, 239-89.

14. Singer & Finnerty. Microbial metabolism of straight-chain and branched alkanes. *In* Petroleum microbiology, Chapter 1, edited by Ronald M. Atlas. Macmillan Publishing Co, USA. pp. 1-59.

15. Rehm, H. & Reff, J. Mechanisms and occurrence of microbial oxidation of long chain. *Adv. Biochem. Engg.*, 1981, **19**, 175-15.

16. Markovetz, A.J. Subterminal oxidation of aliphatic hydrocarbons by microorganism. *CRC Crit. Rev. Microbiol.*, 1971, **1**, 225-38.

17. Chouteau, J.; Azoulay, E. & Senez, J.C. Anaerobic formation of n-hept-1-ene from n-heptane by resting cells of *Pseudomonas aeruhinosa*. *Nature*, 1962, **194**, 576-78.

18 Wagner, F.; Zahn, W. & Buhring, V. 1-Hexadecene an intermediate in the microbial oxidation on

n-hexadecane *in vivo* and *in vitro*. *Angew. Chem. Int. Ed. Engl.*, 1967, **6**, 359-60.

19. Izuka, H.; Iida, M. & Fujita, S. Z. Formation of *n*-decene-1 from n-decane by resting cells of Crugosa. *Zeitschrift for Allegemeine Mikrobiologie*, 1969, **9**, 223-26.

20. Abbott, B.J., & Casida, L.E. (Jr.). Oxidation of alkanes to internal mono-alkanes by a Nocardia. *Journal Bacteriol*ogy, 1968, **96**, 925-30.

21. Gallo, M.; Bertrand, J.C.; Roche, B. & Azouly, E. Distribution des enzymes et des cytochromes de cadida tropicalis culture aur alkanes. *Biochim. Biophys. Acta*, 1973, **296**, 624-38.

22. Zhang, Y. & Miller, R.M. Effect of rhamnolipid (biosurfactants) structure on solubilisation and biodegradation of *n*-alkanes. *Appl. Environ. Microbiol.*, 1994, **60**, pp. 2101-106.

23. Hanson, K.; Vikram, G.; Kale, C. & Desai, A.J. The possible involvement of cell surface and outer membrane proteins of *Acinetobacter* sp. A3, in crude oil degradation. *FEMS Microbiol. Lett.*, 1994, **122**, 275-80.

24. Liu, Z.; Jacobson, A.M.; & Luthy, R.G. Biodegradation of napthalene in aqueous non-ionic surfactant systems. *Appl. Environ. Microbiol.*, 1995, **61**, 45-151.

25. Worsey, M.J. & William, P.A. Metabolism of toluene and xylene by *Pseudomonas putida* (arvilla) Mt-2. Evidence for a new function of the TOL plasmid. *Journal Bacteriology*, 1975, **124**, 7-13.

26. William, P.A. Microbial Genetics relating to hydrocarbon degradation . *In* Developments in biodegradation of hydrocarbons, edited by R.J. Watkinson. Applied Science Publishers, London. pp. 135-64.

27. Kappeli, O. & Fiechter, A. Advances in bitechnology. *In* Proceedings of the 6th International Symposium on Fermentation, Vol. 1, edited by M. Moo Young, *Journal Bacteriology*, 1977, **131**, 917-21.

28. Rosenberg, M. Bacterial adgerance to hydrocarbons: a useful technique for studying hydrophobicity. *FEMS Microbiol. Lett.*, 1984, **22**, 289-95.

29. Singh, M. & Desai, J.D. Uptake of water-insluble substtrates by microorganisms. *J. Sci. Ind. Res.*, 1986, **45**, 413-17.

30. Neu, T.R. & Pralla, K. Emusifying agent from bacteria isoalted during screening for cells with hydrophobic surfaces. *Appl. Microbiol. Technol.*, 1996, **60**, 151-66.

31. Miura, Y. Mechanism of liquid hydrocarbon uptake by microorgasnism and growth kinetics. *In* Advances in biochemical engineering, edited by T.K. Ghosh, A. Fiechter, and N. Blakebrough. Springer Verlag, New York, 1978. pp. 31-56.

32. Mihelcic, J.R.; Pritschow, A. & Leuking, D.R. Uptake of dissolved and oil phase organic chemicals by bacteria. *Ground Water Monit. Rev.*, 1995, **15**, 100-06.

33. Bruheim, P.; Bredholdt, H. & Eimhjellem, K. Bacterial degradation of emulsified crude oil and the effect of various surfactants. *Can. J. Microbiol.*, 1997, **43**, 17-22.

34. Zajic, J.E. & Smith, S.W. Oil separation relating to hydrophobicity and microbes. *In* Biosurfactants and biotechnology, edited by N. Kosaric, W.L. Cairns, and N.C.C. Gray. Marcel Dekker Inc, New York, 1987. pp. 121-42.

35 Ratledge, C. *In* Hydrocarbons in biotechnology, edited by D.E.F. Harrison, I.J. Higgins and R. Watkinson. Heyden and Son, London, 1980. 133p.

36. Jarvis, F.G. & Johnson, M.J. A glycolipid produced by *Pseudomonas aeruginosa*. *J. Am. Chem. Soc.*, 1949, **71**, 4124-126.

37. Edward, J.R., & Hayashi, J.A. Structure of a rhamnolipid from *Pseudomonas aeruginosa*. *Arch. Biochem. Biophys.*, 1965, **111**, 415-21.

38. Histasuka, K.; Nakahara, T.; Sano, N. & Yamada, K. Formation of rhamnolipid by *Pseudomonas*

aeruginosa: its function in hydrocarbon fermantation. *Agric. Biol. Chem.*, 1971, **35**, 686-92.

39. Itoh, S.; Honda, H.; Tomita, F. & Suzuki, T. Rhamnolipids by *Pseudomonas aeruginosa* growth on n-paraffin. *Journal of Antibiotics*, 1971, **24**, 855-59.

40 Itoh, S. & Suzuki, T. Effect of rhamnolipid on the growth of *Pseudomonas aeruginosa* mutant deficient in n-paraffin-utilizing ability. *Agric. Biol. Chem.*, 1972, **36**, 2233-235.

41. Rapp, P.; Bock, H.; Wray, V. & Wagner, F. Formation, isolation and characterisation of trehalose dimycolates from *Rhodococcus erythropolis* grown on *n*-alkanes. *J. Gen. Microbiol.*, 1979, **115**, 491-03.

42. Li, Z.Y.; Lang, S.; Wagner, F.; Witte, L.; & Wray, V. *Appl. Environ. Microbiol.*, 1984, **48**, 610-17.

43. Lang, S. & Wagner, F. Structure and properties of biosurfactants. *In* Biosurfactants and biotechnology, edited by N. Kosaric, W.L. Cairns, and N.C.C. Gray. Marcel Dekker Inc, New York, 1987. pp. 21-47.

44. Cooper, D.G. & Paddock, D.A. *Appl. Environ. Microbiol.*, 1983, **46**, 1426-429.

45. Cooper, D.G. & Paddock, D.A. Production of a biosurfactant from *Torulopsis bombicola*. *Appl. Environ. Microbiol.*, 1984, **47**, 173-76.

46. Culter, A.J. & Light, R.J. Regulation of hydroxydocosanoic and sophoroside production in *Candida bogoriensis* by the level of glucose and yeast extract in the growth medium. *J. Biol. Chem.*, 1979, **254**, 1944-950.

47. Inoue, S. & Itoh, S. Sopholipids from Torulopsis bombicola as microbial surfactants in alkane fermentation. *Biotechnology Letters*, 1984, **4**, 3-8.

48. Arima, K.; Kakinuma, A. & Tamura, G., a crystalline peptidolipid surfactant produced by Bacillus subtilis: isilation, characterisation and its inhibition of clot formation. *Biochem. Biophys. Res. Commun.*, 1968, **31**, 488-94.

49. Asselineau, C. & Asselineau, J. Trehalose containing glycolipids. *Prog. Chem. Fats Lipids*, 1978, **16**, 59-99.

50 Cirigliano, M.C. & Carman, G.M. Purification and characterisation of liposan, a bioemulsifier from *Candila lipolytica*. *Appl. Environ. Microbiol.*, 1985, **50**, 846-50.

51. Robert, M.; Mercade, M.E.; Bosch, M.P.; Parra, J.L.; Espuny, M.J.; Manresa, M.A. & Guinea, J. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa*. *Biotechnology Letters*, 1989, **11**, 871-74.

52. Gutnick, D.L. & Shabti, Y. Exopolysacchraide bioemulsifiers. *In* Biosurfactants and biotechnology, edited by N. Kosaric, W.L. Cairns, and N.C.C. Gray. Marcel Dekker Inc, New York, 1987. pp. 211-46.

53. Grund, A.; Shapiro, J.; Fennewald, M.; Bacha, P.; Leahy, J.; Markbreiter, K.; Nieder, M. & Toepfer, M. Regulation of alkane oxidation in *Pseudomonas putida*. *Journal Bacteriology*, 1975, **123**, 546-56.

54. Van Beilen, J.B.; Wubbolts, M.G. & Witholt, B. Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegradation*, 1994, **5**, 161-74.

55. Kostal, J.; Suchanek, M.; Klierova, H.; Kralova, B.; Demnerova, K. & Mcbeth, D. *Int. Biodeterio. Biodegrad.*, 1998, **42**, 221-28.

56. Bouchetz, M.; Blanchet, D. & Vandecasteele, J.P. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomene and co-metabolism. *Appl. Microbiol. Biotechnol.*, 1995, **43**, 156-64.

57. Rosenberg, M.; Rosenberg, E. & Gutnick, D. *In* Microbial adhesion to surfaces, edited by R.C.W. Berkeley, J.M. Lynch, J. Melling, R.R.

Rutter & B. Vincent. Ellis Harwood Publisher, UK. pp. 514-41.

58. Zajic, J.E. & Panchal, C.J. *Crit. Rev. Microbiol.*, 1976, **5**, 39-66.

59. Reddy, P.G., Singh, H.D., Roy, P.K. and Baruah, J.N. Predominant role of hydrocarbon solubilisation in the microbial uptake of hydrocarbons. *Biotechnology Bioengineering*, 1982, **24**, 1241-269.

60. Aronstein B.N.; Calvillo, Y.M. & Alexander, M. Effect of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soil. *Environ. Sci. Technol.*, 1991, **25**, 127-33.

61. Oberbremer, A.; Muller-Hurtig, R. & Wagner, F. Effect of the additiion of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. *Appl. Microbiol. Biotechnol.*, 1990, **32**, 485-89.

62. Laha, S. & Luthy, R.J. Effect of non-ionic surfactants on the solubilisation and mineralisation of phenanthrene in soil-water systems. *Biotechnology Bioengineering*, 1992, **40**, 1367-380.

63. Volkering, F.; Breure, A.M., Sterkenburg, A. & Andel, J.G. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. *Appl. Microbiol. Biotechnol.*, 1993, **36**, 548-52.

64. Shaw, A. *Soap Cosmet. Chem. Specilities*, 1994, **70**, 24-34.

65. Zajic, J.E.; Gignard, H. & Gerson, D.F.Properties and biodegradation of a biomulsifier from *Corynebacterium hydrocarblastus. Biotechnology Bioengineering*, 1977, **19**, 1303-320.

66. Georgiou, G.; Lin, S.C. & Sharma, M.M. Surface active compounds from microorganisms. *Biology/ Technology*, 1990, **10**, pp. 60-65.

67. Desai, J.D.; & Banat, I.M. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.*, 1997, **61**, 47-64.

68. Razafindralambo, H.; Paquot, M.; Baniel, A.; Popineau, Y.; Hbid, C.; Jacques, P. & Thonart, P. Foaming properties of surfactin, a lipopeptide biosurfactants from *Bacilluc subtilis*. *J. Am. Oil Chem. Soc.*, 1996, **73**, 149-51.

69. Kretschmer, A.; Bock, H. & Wagner, F. Chemical and physical characterisation of interfacial active lipids from *Rhodococcus erythropolis* grown on *n*-alkane. *Appl. Environ. Microbiol.*, 1982, **44**, 864-70.

70. Chakrabarty, A.M. Genetically manipulated microorganisms and their products in the oil service industries. *Trends Biotechnology*, 1985, **3**, 32-38.

71. Harris, P.M.; Rice, S.D.; Babcock, M.M. & Brodersen, C.C. Within-bed distribution of Exxon Valdez crude oil in Prince William Sound blue mussels and underlying sediments. *In* Proceedings of the Exxon Valdez oil Spill Symposium, edited by Rice, S.D., R.B. Spies, D.A. Wolfe, and B.A. Wright. American Fisheries Society Symposium 18, American Fisheries Society, Bethesda, Maryland. pp. 298-308.

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