J Ind Microbiol Biotechnol (2003) 30: 260–270
DOI 10.1007/s10295-003-0042-4

REVIEW PAPER

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Accelerated biodegradation of petroleum hydrocarbon waste

Received: 28 October 2002 / Accepted: 28 January 2003 / Published online: 3 April 2003

Abstract Conventional landfarming approaches to bioremediation of refinery and other petroleum sludges are not acceptable environmentally and are banned in most North American jurisdictions. While initial bioreactor-based systems for treatment of these sludges required batch-cycle process-times of 1–3 months, an accelerated process has now been developed which can be completed in 10–12 days. In this process, up to 99% of total petroleum hydrocarbons are degraded and the sludges are converted from hazardous to non-hazardous according to the United States EPA's toxicity characteristic leachate procedure criteria. Understanding and exploiting mechanisms to improve hydrocarbon accession to the degrading microorganisms was a key development component of the process. Contrasting physiological mechanisms were observed for different component organisms of the mixed culture with respect to their associations with the hydrocarbon substrate; and the beneficial effects of using surfactants were demonstrated. The mixed culture used in the process exhibited a capacity for high-rate degradation of volatile organic carbons and the potential use of the culture as a liquid biofilter was demonstrated. The culture was also effective as an inoculant for the bioaugmentation of total petroleum hydrocarbon-contaminated soil and as a de-emulsifier of oilfield emulsions and could transform some other environmental contaminants which are not predominant components of crude oil.

Keywords Mixed culture · Biodegradation · Petroleum hydrocarbons · Bioremediation · De-emulsification

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Introduction

Petroleum hydrocarbons represent high-volume global materials, most of which can be degraded or otherwise transformed by microorganisms. Crude oil production volumes surpassed 66 million barrels/day in 1998 [70]. Environmental impacts from the petroleum industry emanate through recovery, transport, refining and product usage and are modestly estimated to amount to 0.1% of the oil produced. Only 10% of this amount occurs in high-profile catastrophes [57]. These numbers certainly do not include the large quantities of petroleum-based volatile organic hydrocarbons (VOCs) emitted into the atmosphere. Because many of the constituents of crude oil have a negative impact on the environment and on human health, effective methods continue to be sought to remediate these high-volume wastes.

Microorganisms have the capacity to degrade the majority of natural hydrocarbon components, especially the dominant saturated and unsaturated alkanes, monoaromatic and low-molecular-weight polycyclic aromatic hydrocarbons (PAHs). The higher-molecular-weight PAHs, resins and asphaltenes are more recalcitrant to biodegradation. Hydrocarbon-degrading microbes must come into contact with their substrate in order for hydrocarbon uptake to occur [54, 58, 63, 75] and the insoluble nature of the majority of petroleum hydrocarbon molecules limits this contact [12]. The most widely recognized modes of hydrocarbon accession are direct microbial adherence to large oil droplets and interaction with pseudosolubilized (emulsified) oil [10]. Hence, attempts to optimize or accelerate processes for the degradation of hydrocarbons need to include strategies for improving hydrocarbon accession by microorganisms.

Since crude oils contain such a wide range of molecular structures, it was postulated that mixed cultures capable of rapidly degrading crude oil might have broader applications in the general biotransformation of hazardous hydrophobic environmental contaminants.

Landfarming bioremediation of petroleum hydrocarbons

There is ample evidence to indicate that efficient natural microbial degradation of hydrocarbon oil-contaminated beaches and soil occurs with intervention limited to ensuring that sufficient moisture, oxygen and nitrogen is available to support growth of the hydrocarbon contaminants. The comprehensive studies on the bioremediation effects related to the Exxon Valdez oil spill proved that augmentation with hydrocarbon-degrading inocula had no significant impact on the bioremediation process over and above the application of nitrogen-containing fertilizers [28]. There are also many reports where adequate hydrocarbon biodegradation has been achieved without human intervention but over longer timescales, through natural attenuation. There are many cases where short-term real estate plans are in place to develop properties on contaminated sites. In such cases, the relatively long timescales required for conventional bioremediation and natural attenuation processes eliminate these methods as remediation approaches. However, the most often reported disadvantages of bioremediation processes relate to performance unpredictability, in particular to the potential for rates of degradation to slow down as contaminant concentrations fall, likely due to unsaturating conditions, associated reductions in the viable microbial population and, in some cases, elimination of the co-substrates necessary for the degradation of other contaminants by co-metabolism. The latter disadvantages represent a major justification for the implementation of more controlled and optimized reactor-based processes, which ensure contaminants are efficiently degraded to defined criteria.

In addition to the clean-up of accidental oil spills, soil bioremediation, particularly landfarming, has also been widely used for remediating oily waste sludges. These sludges come from various sources, including storage tank bottoms, oil–water separators, dissolved air floatation units and drilling operations [46]. They vary in hydrocarbon composition with source, storage and treatment conditions, but typically contain 10–30% hydrocarbons, 5–20% solids and 50–85% water. Hydrocarbon sludges emanating from refineries are considered hazardous by the United States EPA [71]. Hence, in the United States when these wastes are disposed of in hazardous landfills, liability for the wastes still remains with the generator. In many countries, these wastes are sprayed onto land together with fertilizer and the sludge–soil mixture is tilled, to promote the activity of the soil microbial community for hydrocarbon degradation [32]. Maximum degradation rates are typically observed in the upper plough layer (10–15 cm) when hydrocarbon concentrations are maintained around 5%. The sludge–soil mix may be augmented with additives, including inocula, organic and inorganic nutrients, surfactants and bulking agents, to enhance hydrocarbon degradation [31, 48, 50, 61, 77]. Because landfarming conditions do not represent optimal and controlled conditions (temperature, pH, moisture, oxygen, mixing) for microbial activity, long treatment times are required [8, 25, 33, 40, 44].

Environmental impacts of the landfarming of hydrocarbon wastes

Landfarming bioremediation practices for the treatment of oily wastes from the petroleum industry are not considered environmentally acceptable, since in the first instance large uncontaminated areas of land are first deliberately contaminated, followed by subsequent bioremediation of the less-recalcitrant oil fractions. These landfarming operations can tie up very large tracts of land—large refineries, having capacities of 200,000–500,000 barrels/day can produce as much as 10,000 m^3 sludge/year.

Another major environmental disadvantage of landfarming processes for the bioremediation of these oily wastes relates to the large quantities of volatile organic carbons present in these wastes. Monitoring and containing these compounds is important because of their hazardous impact on health and their role in tropospheric ozone production [19, 27, 49]. In landfarming processes, the VOCs are typically transferred to the atmosphere rather than biodegraded. This is facilitated by the practice of first spraying the waste onto the land and then routinely tilling the soil to promote gas transfer. While the intended gas transfer is oxygen from the atmosphere to the soil, the process is equally effective in promoting the volatilization of VOCs. Even in the Exxon Valdez spill, 15–20% of the oil was reported to be lost to the atmosphere by volatilization [57]. Clearly, in the warmer climates in the southern United States, South America, the Middle East and Malaysia, higher rates of volatilization of VOCs is expected.

Thus, while oily sludges have traditionally been processed by landfarming bioremediation, these practices are banned in the United States and are being phased out in many other jurisdictions. Oil companies have therefore been forced to seek other disposal solutions. Treatment approaches other than biological involve capital-intensive physico-chemical methods including incineration, thermal desorption, refinery coker use, burning in cement kilns and solvent extraction, as indicated in Table 1. Incineration and thermal desorption are regarded to be among the most expensive treatment methods [60]; and the high temperatures involved requires high energy input and results in significant greenhouse gas emissions.

Benefits of bioreactor-based systems for petroleum sludge treatment

The alternative is a biodegradation process in a contained bioreactor. Liquid/solid treatment (LST) by bioremediation is recognized as a technology applicable to

Table 1 Current petroleum oily sludge treatment technologies

Treatment	Technology
Physical/thermal	Incinerator
	Thermal desorber
	Coker
	Cement kiln
Chemical	Solvent extraction
Biological	Slurry bioreactor
	Landfarming
	Biopiling
	Composting

the degradation of petroleum refinery sludge [16, 36, 67]. Application of contained bioreactor-based processes ensures no soil contamination and also, if required, provides the operator with discretion regarding disposal options for residual solids at the termination of the bioreactor process. Containment of sludge biodegradation processes in bioreactors also allows for management of off-gasses. Strategies may also be applied, for example through the use of surfactants or sorbents in the medium, to reduce volatilization. In addition, by creating culture conditions which accelerate the process of bioremediation of VOCs, the biodegradation process rather than volatilization, can become the dominant VOC-removing mechanism.

There are advantages for a biological reactor-based process over other solid-phase biodegradation approaches. The reactor-based process has the potential to be highly accelerated through proper process control/optimization and can be fully contained. Since bioreactor systems can accommodate solids concentrations across 5–50% w/v, sludge-associated solids and insoluble oil substrates can be mixed in a manner approaching homogeneity. This mixing, combined with the control of key parameters, enhances rates and extents of hydrocarbon degradation [34]. Mass transfer limitations are minimized and contaminant desorption from solids is increased, resulting in much higher hydrocarbon removal rates than are observed in landfarm and other solid-phase systems [16]. The use of mixed cultures eliminates the high cost implications of pure-culture installations, provides greater metabolic diversity and can provide a process able to degrade a variety of oily waste sludges (Table 2).

Case studies of large-scale LST processes for refinery waste treatment

A number of processes having reactor cycle-times of 1–4 months were implemented in the late 1980s and early 1990s [16, 50].

Sugar Creek, Mo.

LST combined with land treatment was used to remediate refinery sludges in three impoundments at a refinery, formerly owned by Amoco, at Sugar Creek, Mo. [18]. An unlined reactor (capacity 22.7×10^6 l) containing a float-mounted aeration and mixing system was operated to reduce oil and grease concentrations by 66%, after which the solids were land-applied to reduce residual PAHs to below 160 mg/kg (AMOCO Oil Company, Sugar Creek, Mo., unpublished data). The operating plan for closure of the sludge pond pit and wastewater treatment lagoon as a single waste management unit was submitted to the Missouri Department of Natural Resources and EPA Region VII, Chicago, Ill. Municipal activated sludge and prepared hydrocarbon cultures were used as inocula. The LST batch cycle-time ranged from <60 days to 90 days.

Gulf coast refinery

A bioreactor system (capacity 4.55×10^6 l) was used to remediate petroleum-impounded sludges at a major Gulf coast refinery [17]. Operating nominal solids contents in the reactor were about 10%. Float-mounted mixers and

Table 2 Biodegradation of petroleum hydrocarbons in oily sludges from different refineries. For shake-flask biodegradation tests, the initial total petroleum hydrocarbon (*TPH*) concentration in the sludge sample was adjusted to 5–7% and nutrient medium was added, as described by Ward and Singh [78]. Flasks were inoculated with a 10% mixed culture inoculum and incubated at 30 °C for 14 days on a rotary shaker (200 rpm)

Location of refinery	Sludge TPH (%)	Hydrocarbon fractions (% of total)				TPH degradation (%)
		Saturates	Aromatics	Resins	Asphaltenes	
Ontario (A)	18.8	49.6	32.7	10.3	7.4	93.5
Ontario (B)	15.8	42.0	42.0	6.9	9.1	95.6
Ontario (C)	13.2	44.9	40.4	7.1	7.6	94.2
Quebec	9.3	48.7	25.6	10.2	15.5	90.7
Western Canada	20.2	21.2	47.8	9.6	21.4	93.3
Eastern Canada	20.9	46.4	33.5	10.8	9.3	91.2
Western USA	17.1	45.4	37.8	3.9	12.9	95.0
Eastern USA	15.5	44.3	43.7	6.7	5.4	90.8
Latin America (A)	15.1	51.3	18.9	14.9	14.9	96.6
Latin America (B)	21.3	41.2	35.6	9.7	13.5	92.5
South-east Asia	33.7	44.7	40.8	6.5	8.0	90.3
Middle East	8.3	38.3	45.5	6.9	9.3	92.1

aerators were used and the reactor was inoculated with hydrocarbon-degrading organisms from the refinery wastewater activated-sludge system. The average temperature was 22.6 °C. The time required for a 50% reduction in oil and grease was 80–90 days; and the overall extent of removal of PAHs was 90%.

French Limited, Crosby, Tex.

Perhaps the highest profile study of a reactor-based process for the degradation of refinery and petrochemical wastes was the implementation of the slurry-phase aerated (pure oxygen) and mixed system at the French Limited Superfund site at Crosby, Tex., operated in 1992–1993 [23]. This former petrochemical waste-disposal facility contained an estimated 318.23×10^6 l of petroleum wastes. The aerated process incorporated a novel mixing/aeration system (the MixFlo system), using pure oxygen rather than air. About 0.3×10^6 t of tar-like material and associated subsoil was remediated to criteria during 11 months of treatment, with 85% of sludge contaminants being destroyed within 122 days [24]. The indigenous microflora were used to promote hydrocarbon degradation.

The Petrozyme process

A LST process, developed by Petrozyme Technologies, Ontario, Canada, was successfully operated for the treatment of sludges produced from about 75% of Venezuela's refining capacity for the past 6 years. The installation employs eight bioreactors with a total capacity of 1×10^6 l. The process was also implemented at a small number of refineries in the United States, Canada and Mexico. The process typically degrades sludges having a total petroleum hydrocarbon (TPH) content of 10% w/v. The reactor contains a sparged air-lift aeration system with no mechanical mixing; and the optimal operating temperature is 28–32 °C. The fermentation nutrient formulation is optimized to maximize hydrocarbon accession by the microorganisms, microbial growth rates and the rate and extent of hydrocarbon degradation [78]. In contrast to the LST processes described above, the Petrozyme process operates with a much shorter residence time of 12 days, with extent of degradation of TPHs up to 99% [64, 78]. The process has operated consistently over hundreds of runs at pilot and full scale.

The pattern of degradation of petroleum hydrocarbons in the Petrozyme process is illustrated in Fig. 1. Crude oil is often characterized by separation into its saturates, aromatics, resins and asphaltene (SARA) fractions [65]; and comparative laboratory tests show the ability of this culture to degrade waste sludges from different sources and containing different SARA compositions [78] (Fig. 2). Biodegradations of different oily wastes by the mixed culture are presented in Table 3 (Ward and Singh, unpublished results).

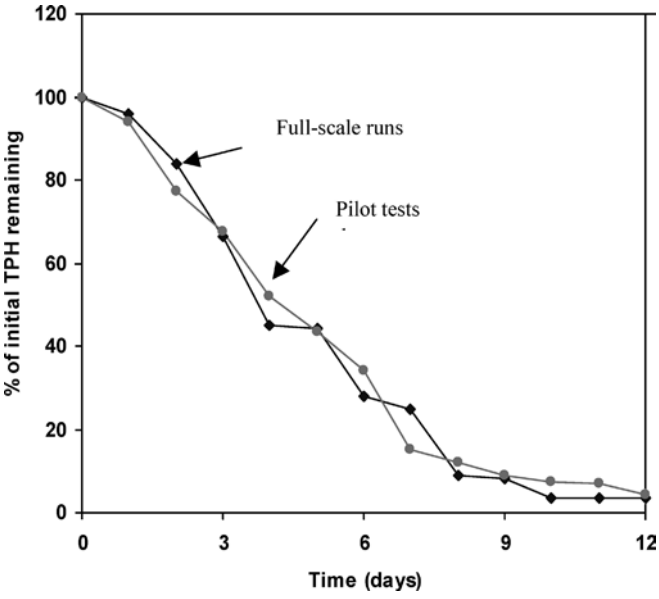

Fig. 1 Total petroleum hydrocarbon (*TPH*) degradation profile of mixed culture in refinery oily sludges. The process was based on methods described by Ward and Singh [78]

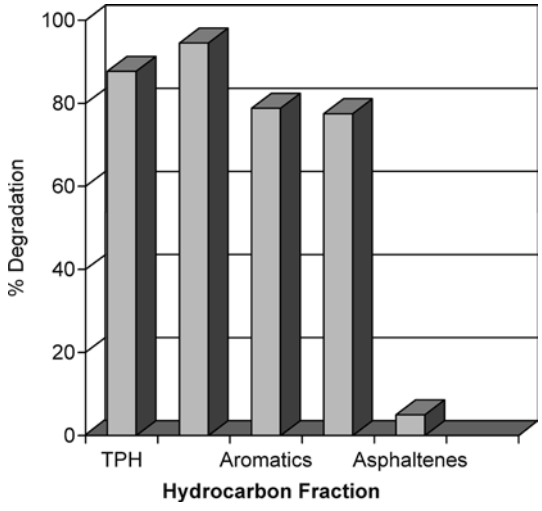

Fig. 2 Biodegradation of petroleum hydrocarbon fractions by the mixed culture. Adapted from Ward and Singh [78]

Many United States refiners currently pay in excess of U.S. $ 500/t to safely transport, treat and remove these hazardous petroleum wastes. In contrast, non-hazardous waste disposal costs are typically $< 12.5\%$ of that amount. Analysis of the residual solids from the Petrozyme process indicates that they comply with the United States Environmental Protection Agency (EPA) Toxicity characteristics leaching procedure criteria for delisting from the listed hazardous waste category [64]. Therefore, once delisted, the treated residual solids from the Petrozyme process can be sent off-site for non-hazardous disposal or reused for industrial purposes, making this process highly desirable and cost-effective.

Table 3 Application of petroleum degrading mixed culture in biodegradation of various oily sludges. For shake-flask biodegradation tests, sludge samples were mixed with nutrient medium and mixed culture (as described by Ward and Singh [78]) and incubated at 30 °C on a rotary shaker (200 rpm)

Oily waste	Initial oil concentration (ppm)	Oil degradation (%)	Time (days)
Drilling oil	50,000	99.0	7
Drilling mud	50,000	90.0	14
Steel mill scale oily sludge	41,000	80.5	24
Metal plating oily sludge	15,500	89.3	14
Paint solvent sludge	128,000	96.0	14
Lubricant oily sludge	50,000	85.0	10
Wastewater oily biosolids	26,000	92.3	10
Oily clay fines	52,000	91.8	14
Coker catcher fines	63,000	89.5	21

The mixed culture associated with the Petrozyme process can be maintained on petroleum hydrocarbons as sole carbon source with a 50% weekly medium replenishment for a period of 6 years, for use in the reactor-based process and in general research. The reactors used for maintenance are 1-l cyclone reactors (Fig. 3) having the design specification provided by Liu [42]. Some of the basic and applied properties of this mixed culture are discussed in the next section.

Hydrocarbon accession

Understanding and exploiting mechanisms to improve hydrocarbon accession by microorganisms was an important development component of the Petrozyme process. Hydrocarbon-degrading microbes produce a variety of natural biosurfactants, either as an integral part of their cell surface [11, 12, 13] or as molecules released extracellularly into the medium [3, 26, 45, 47, 62] and these biosurfactants can enhance the removal of petroleum hydrocarbons from soil or solid surfaces [1, 38, 42, 75]. Chemical surfactants have the potential to improve the accessibility of hydrophobic substrates, including hydrocarbons, to microorganisms. However, both enhancement and inhibition of biodegradation of hydrocarbons have been observed [50]. The properties of chemical surfactants which can influence their efficacy include the critical micelle concentration (cmc), the surfactant concentration at which surface tension reaches a minimum and surfactant monomers aggregate into micelles and the hydrophile–lipophile balance (HLB), a measure of surfactant lipophilicity. The cmc value is important because solubilization and biodegradation enhancement can be related both to surface tension and to the status of micelle development [4, 6, 7, 14, 21, 35, 56]. Surfactants with HLB values of 8–15 generally form oil-in-water emulsions, whereas surfactants with HLB values of 3–6 form water-in-oil emulsions [16]. Selecting surfactants to improve biodegradation is made more complex by the use of mixed cultures containing organisms having different mechanisms of access to petroleum hydrocarbons [63]. Although studies are simplified by investigating pure-culture systems, more effective degradation is generally observed with mixed cultures [68].

The effect of surfactants on the biodegradation of petroleum hydrocarbons by mixed cultures in crude oil was investigated [20, 78]. Two anionic surfactants, the alkyl phenol ethoxylate, Igepal CO-630 (HLB 13), and the alcohol ethoxylate, Biosoft EN 600, increased TPH degradation, whereas other surfactants exhibited no effect (Table 4). Igepal CO-630 increased viable counts by 4.6-fold and hydrocarbon (TPH) degradation by 57%, compared with a no-surfactant control. The effect of surfactant concentration on crude oil degradation by mixed cultures was also investigated. Enhancement of TPH degradation was observed at

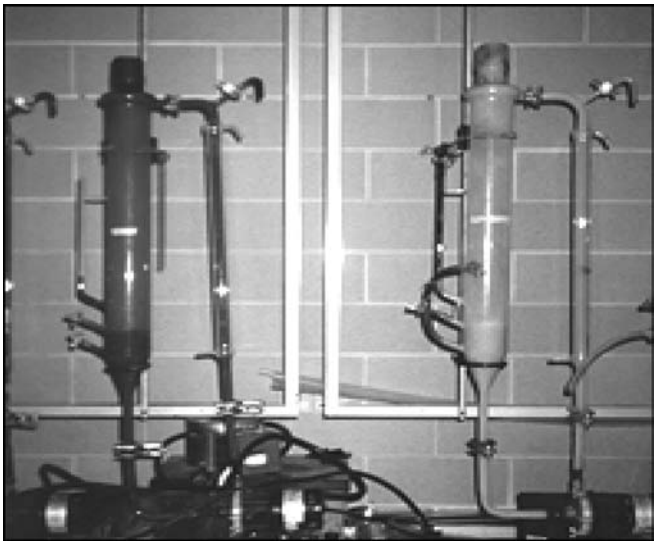

Fig. 3 Maintenance of mixed culture in cyclone fermenters at the Microbial Biotechnology Laboratory, University of Waterloo, Waterloo, Canada

Table 4 Effect of surfactant type on TPH degradation in a refinery oily sludge. Oily sludge was incubated on a rotary shaker (200 rpm) with nutrient medium and mixed culture inoculum containing 0.125% of surfactant (as described by Ward and Singh [78]) for 14 days at 30 °C. *HLB* Hydrophilic–lipophilic balance

Surfactant	Chemical class	HLB	TPH degradation (%)
None	–	–	46
Biosoft EN 600	Alcohol ethoxylate	12.2	63
Igepal CO-630	Alkyl phenol ethoxylate	13.0	66
Marlipal 013/120	Oxoalcohol polyglycol ether	14.5	45
Sorbax PMO-20	Fatty acid ethoxylate	15.0	42
Witcomul 4016	Complex alkylate	–	41

surfactant concentrations above the cmc for the surfactant, indicating that micellization was required.

The effect of the HLB value of the surfactant on TPH degradation was also explored [72]. Within the nonylphenol ethoxylate family of surfactants, the HLB value was critical for oil degradation, with a value of 13 exhibiting optimal degradation (Fig. 4). Apart from Igepal CO-630, the other nonylphenol ethoxylate surfactants in the Igepal CO class were less effective in biodegradation enhancement, although they were not inhibitory. Surfactants from chemical classes other than the nonylphenol ethoxylates either had no effect or inhibited TPH degradation.

Composition of the mixed culture

Some of the predominant organisms present in the mixed culture are shown in Table 5. They include, among others, well known hydrocarbon degraders from the *Acinetobacter*, *Pseudomonas* and *Rhodococcus* genera [76]. Community dynamics of the mixed culture growing in batch culture on petroleum hydrocarbons were monitored. Microbial counts of hydrocarbon degradation and total heterotrophic bacteria over time were nearly identical, rising from 10^6 colony-forming units (CFU)/ml to a maximum count of 10^{10} CFU/ml after 48 h. *Pseudomonas*, *Flavimonas* and *Stenotrophomonas* were the dominant genera in the early culture stages, although the addition of surfactant appeared to cause a lag in the growth of *Stenotrophomonas*. When the mixed culture was grown on the saturate fraction of crude oil, *A. calcoaceticus* was the dominant species. In the later stages of growth, a greater variety of organisms was noted in the culture, which is to be expected. As the hydrocarbon substrates are oxidized by competent strains, the resulting acids, alcohols, ketones and other metabolites, in addition to cellular materials, provide the nutrient environment to enable a wider microbial population to flourish [39]. Developing a greater understanding of the mixed culture's community dynamics has the potential to facilitate further development and optimization of processes for the treatment of heterogeneous waste substrates, such as petroleum hydrocarbons.

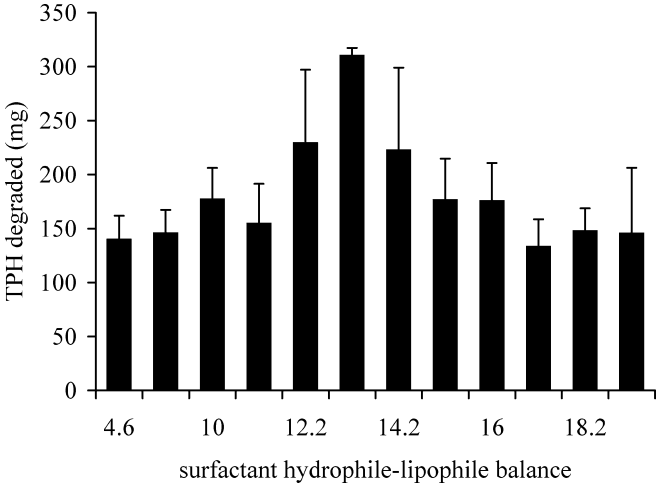

Fig. 4 Effect of hydrophilic–lipophilic (HLB) value of surfactant on biodegradation of TPH in crude oil. Adapted from Van Hamme and Ward [72]

Table 5 Predominant bacteria present in the petroleum oil-degrading mixed culture (adapted from Van Hamme Ward et al. [76])

Genus
Acinetobacter
Alcaligenes
Ochrabactrum
Pseudomonas/*Flavimonas*
Rhodococcus
Stenotrophomonas

Degradation of petroleum VOCs

Studies on crude oil degradation often fail to include the more volatile compounds, which are lost during dichloromethane or hexane extraction/GC analytical processes. Indeed, many laboratory studies on oil biodegradation purposely eliminate these compounds by investigating artificially weathered crude oil [69]. A solid-phase microextraction (SPME)/GC method was developed to monitor the degradation kinetics of individual and combined C5–C11 volatile components of crude oil [73]. The SPME method was used to determine VOC degradation kinetics by the mixed culture and selected isolates, including the *Rhodococcus* and *Pseudomonas* strains described above. The results indicate that the mixed culture had a substantially greater ability to degrade the volatile fraction, removing 45% and 55% of this component in 2 days and 4 days, respectively [73, 74]. Rates of degradation for the C8–C11 substrates are presented in Table 6 [74].

For the mixed culture, the degradation rate of individual alkanes was proportional to the initial substrate concentration and decreased from hexane to undecane. Inocula taken from early stationary phase cultures exhibited a reduced lag phase prior to hydrocarbon deg-

Table 6 Biodegradation of major volatile hydrocarbons in crude oil by mixed and pure cultures (adapted from Van Hamme and Ward [74])

Culture	Maximum degradation rate (μg/h)			
	n–C8	*n*–C9	*n*–C10	*n*–C11
Mixed culture	4.1	2.0	0.5	0.1
Pseudomonas aeruginosa	0.8	0.5	0.2	0.1
Rhodococcus globerulus	0.6	0.8	0.4	0.2
P. aeruginosa + *R. globerulus*	0.7	0.8	0.3	0.1

radation and achieved 90% removal of volatile hydrocarbons, eliminating most compounds up to C11, including methylcyclohexane. Methylcyclohexane and other branched compounds were recalcitrant to degradation by late log phase crude oil-grown inocula. The observed cyclohexane recalcitrance appears to correlate with reduced levels of hydrocarbon-degrading bacteria, the physical loss of volatile hydrocarbons from inoculum culture flasks and the presence of organic nitrogen nutrients in inoculum preparation media and it may also correlate with the loss of certain plasmids from the culture [43].

Contrasting physiological responses of bacteria to hydrocarbons and surfactants

Two of the predominant isolates from the mixed culture, a *Rhodococcus* sp. and a *Pseudomonas* sp. were examined with respect to their physiological responses to the presence of hydrocarbons [75]. The *Rhodococcus* was observed to associate strongly and grow directly on oil droplets and could be removed by the addition of exogenous surfactant, whereas the *Pseudomonas* strain remained in the aqueous phase and required surfactant-solubilized oil for growth. Surfactant altered the cellular morphology of our *Rhodococcus* isolate from rods to cocci. Rhodococci are reported to form rods or branched mycelia that fragment to irregular rods and cocci [29, 37, 79]. In co-culture, we observed a capsular mycolic acid material associated with the *Rhodococcus* species extending into the aqueous phase and the *Pseudomonas* adhering to these capsular extensions (Table 7). *Acinetobacter* sp. and *Rhodococcus* sp. have been reported to grow on hydrocarbon droplets and direct attachment to hydrocarbons is a common association mechanism for *Rhodococcus* species [2, 9, 37, 79]. The *Rhodococcus* isolate from our laboratory exhibited surface-active and emulsification properties [72, 75]. The majority of surface-active agents in *Rhodococcus* species are reported to be cell-bound glycolipids [37], while a cell-associated emulsifying activity of *R. erythropolis* ST-2 was characterized as a glycoprotein. Crude oil biodegradation by pure and co-cultures, with and without surfactant, illustrated the importance of the interactions between the strains and the substrate. Neither pure culture exhibited substantial oil degradation without surfactant; and surfactant supplementation did not promote oil degradation by the *Rhodococcus* isolate. Good degradation was observed with the *Pseudomonas* strain with surfactant, while the best degradation was observed with the co-culture plus surfactant. Apparently, the chemical surfactant was able to enhance degradation by the more metabolically active pseudomonad by disrupting the hydrophobic interactions of the rhodococci with bulk crude oil droplets, to produce more dynamic, hydrophilic hydrocarbon-surfactant micelles.

Other applications of the hydrocarbon-degrading mixed culture

Commercialization of the oil sludge process described above resulted in the production of thousands of tonnes of culture for disposal annually and generated interest in developing beneficial uses of this potentially valuable process end-product.

VOC biofiltration

The culture could be used as a liquid biofilter system for the removal of benzene, toluene and xylene components from air streams. There is significant concern in refineries and industrial plants regarding air emissions and the Clean Air Act places severe controls on the emission of VOCs. The ability of this mixed culture to reduce VOCs in air streams fed to the reactor by fine bubble-sparging was therefore tested. The results are presented in Table 8 (Ward and Singh, unpublished data). The average percentage removal rates, in a reactor height/path-length of 1.5 m, were 63%; and the rates ranged from 73.5% for ethylbenzene to 50% for *m*,*p*-xylene. Assuming that in taller reactors or in reactor series, with contaminant concentration reducing along the path length, the same percentage removal rates are achieved in a second and third 1.5-m segment, the estimated average removal rates in 3.0-m and 4.5-m path-lengths would be 86% and 93%, respectively.

Soil remediation

The culture also has potential for use as an inoculum for accelerated soil hydrocarbon bioremediation (Table 9). When diesel- and gasoline-contaminated soil was treated

Table 7 Properties of pure cultures isolated from petroleum-degrading mixed cultures. Properties are summarized from Van Hamme and Ward [75]

Property	*Pseudomonas* JA5-B45	*Rhodococcus* F9-D79
Growth	Rapid growth, in aqueous phase	Apparent slow oil-associated growth
Emulsification	Little oil emulsification	Oil emulsification, 24–48 h
Microscopy	Adheres to *Rhodococcus* extensions	Capsular mycolic acid extensions
Effect of surfactant	Facilitates degradation of alkanes and aromatics	Negative effect on alkane degradation
Co-culture	Best oil degradation	

Table 8 Use of mixed culture inoculum as a biofilter for the removal of benzene, toluene and xylene compounds (Ward and Singh, unpublished data). A reactor (4.7 l) containing oil sludge (2 l) was sparged at 0.1 vvm. The effluent gas from this reactor was fed to the biofilter inlet via an air diffuser. The biofilter dimensions were 7.5 cm (internal diameter) by 150 cm (length). The biofilter contained the nutrient medium described by Ward and Singh [78] without oil sludge and was inoculated with 10% of a culture produced by the Petrozyme process. The biofilter was operated at room temperature

Reactor location	Concentration (mg/m^3)				
	Benzene	Toluene	Ethyl benzene	*o*-Xylene	*m,p*-Xylene
Reactor inlet	10.7	14.8	15.1	11.7	10.2
Reactor outlet	4.0	4.0	4.1	4.9	5.1
Percent removal	62.6%	72.9%	73.5%	58.1%	50.0%

Table 9 Bioremediation of contaminated soils (Ward and Singh, unpublished data). Dry soil (500 g) was spiked with diesel (initial TPH = 33,500 ppm) or gasoline (initial TPH = 28,400 ppm) and placed in a box to give a soil depth of 15 cm. The contaminated soil was inoculated with 250 ml of a mixed culture and incubated for up to 28 days at room temperature. Samples were extracted with hexane and analyzed for TPH content

Time (days)	TPH degradation (%)			
	Diesel-contaminated soil		Gasoline-contaminated soil	
	Control	Inoculated	Control	Inoculated
7	19	17	9	24
14	17	31	20	46
21	19	39	27	59
28	27	55	33	69

with the mixed culture inoculum, the observed rate of removal of TPH contaminants in a 28–day timeframe was approximately doubled (Ward and Singh, unpublished data).

Microbial de-emulsification

Another interesting feature of the culture related to processing of petroleum hydrocarbons is its ability to de-emulsify oil-field emulsions. These emulsions are formed at various stages of oil production and recovery and during refining processes [5] and they represent both a serious environmental and disposal problem for the oil industry and a potential loss of valuable oil-product [46]. Conventional physicochemical processes for treating emulsions are centrifugation, heat, electrical and chemical methods [30, 41, 55]. Earlier studies with biological processes examined the de-emulsification properties of pure cultures of bacteria and yeast species, including *Nocardia* [15], *Corynebacterium* [66], *Rhodococcus* [59] and *Torulopsis* [22].

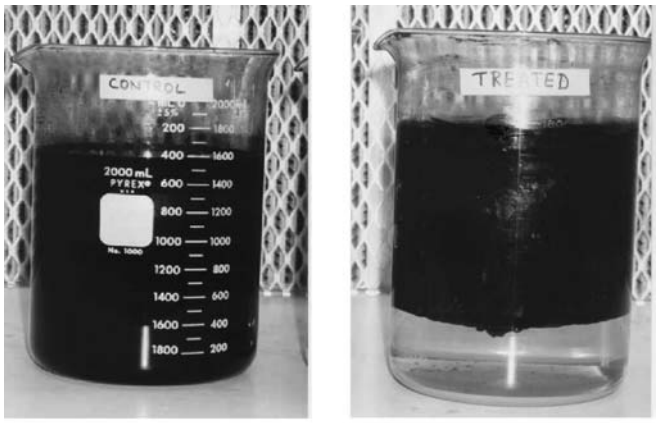

Fig. 5 De-emulsification of oilfield emulsion by the mixed culture (Ward and Singh, unpublished data). An emulsion sample (2 l) was incubated with 200 ml of a mixed bacterial culture developed by the method described by Nadarajah et al. [52, 53] and incubated at room temperature for 3 days. To an untreated control, 200 ml of distilled water was added and incubated along with the treated emulsion

The mixed culture maintained on oil exhibited a high de-emulsification ability [51]. Fig. 5 shows the de-emulsification capability of the mixed culture. Maximum activity was observed with cells grown on crude oil [52] (Table 10). De-emulsifying activity was substantially associated with the centrifuged cells rather than with the supernatant component. Emulsion-breaking ability was little affected by lyophilization or freezing and thawing but was completely destroyed by autoclaving, indicating that the de-emulsification factor was thermolabile [50]. Nine morphologically distinct pure colonies were isolated from the mixed culture, identified and characterized with respect to their de-emulsification properties [53] (Table 11). While *A. calcoaceticus* was the most effective de-emulsifying pure culture, the mixed culture exhibited the highest de-emulsification activity against oilfield water-in-oil emulsions. Little is known about the physiological or biochemical properties of cells that make them good de-emulsifiers or about other factors that affect this process. In that regard, it is curious that *A. calcoaceticus*, a species known for its biosurfactant production capabilities should also exhibit a high de-emulsification ability.

Table 10 Effect of growth substrate on de-emulsification by petroleum-degrading mixed culture (adapted from Nadarajah et al. [52])

Growth substrate	De-emulsification in 72 h (%)
Diesel	77.0
10W30 oil	80.0
Crude oil	93.5
Canola oil	65.0
Starch	59.0
Sucrose	65.0
Glucose	50.0

Table 11 De-emulsification of a model kerosene–water emulsion by pure cultures isolated from a petroleum-degrading mixed culture (adapted from Nadarajah et al. [53])

Isolate	De-emulsification in 24 h (%)
Acinetobacter calcoaceticus BV ALC	95.5
A. calcoaceticus	91.5
A. radioresistens	89.0
Alcaligenes latus	81.5
Kingella denitrificans	28.5
Pseudomonas aeruginosa	82.5
P. carboxydohybrogena	83.0
Rhodococcus globerulus	36.5
Sphingobacterium thalpophilum	30.5
Control: emulsion only	21.0

Biodegradation of other compounds

In preliminary unpublished research, we have demonstrated that the mixed culture exhibits an ability to degrade a variety of other compounds, including benzo- and dibenzothiophene (DBT), various methylthiophenes and other substituted thiophenes and DBTs. The culture can also transform many nitroaromatic compounds and other nitrogen-containing ring structures. Rapid transformation of some of these nitroaromatic compounds was observed without any prior exposure to the substrate. To date, reports on the degradation of most hazardous contaminants, involve the selection/isolation/acclimation of the degrading pure or mixed culture on the contaminant or on an analogue thereof. We are interested in reactor-based accelerated degradation of these hazardous compounds, but the use of a controlled substance to pre-acclimate cells is not attractive.

Conclusions

Currently in the United States, there is practically no application of microbial processes for the treatment of refinery sludges. Most of this waste is disposed of in hazardous landfills, which results in high costs to the producers without relieving them of the liabilities associated with these wastes. Some wastes are treated by thermal methods, especially in cement kilns or thermal desorbers. Conventional landfarming bioremediation processes for the treatment of these wastes are not environmentally acceptable and VOCs are typically volatilized to the atmosphere. Case studies describing contained LST bioreactor systems also indicate prolonged cycle-times, likely making these processes uneconomic. By optimizing fermentation process parameters and by paying particular attention to strategies for increasing hydrocarbon accession to the petroleum-degrading mixed culture, a cost-effective bioreactor-based process with a relatively short cycle-time has been developed. Process consistency has been proven over hundreds of full-scale runs and by conversion of hazardous into non-hazardous waste.

Supporting studies have illustrated the contrasting physiological mechanisms exhibited by different component organisms in the mixed culture, particularly with respect to their associations with the hydrocarbon substrate. In addition, the beneficial effects of using surfactants to improve hydrocarbon accession have been demonstrated and the effects of key surfactant properties on degradation have been shown.

From a different perspective, the process for the degradation of refinery oily wastes could be viewed as a low-cost (or negative-cost) fermentation process for the production of biomass capable of transforming other hydrophobic molecules. In this regard, preliminary evidence has been demonstrated of the utility of the culture as a liquid VOC biofilter, as a de-emulsifier of oil-field emulsions and as an inoculant for the bioaugmentation of the bioremediation of TPH-contaminated soil. What may be technically more interesting is an exploration of the ability of the mixed culture to transform other environmental contaminants, which are not predominant components of crude oil. Novel transformation applications for this culture, preferably without prior acclimation/exposure of the culture to the target substrate of interest, are being sought.

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