The use of thermophilic bacteria in accelerated hydrocarbon bioremediation

A. Perfumo, I. M. Banat & R. Marchant
Microbial Biotechnology Group, School of Biomedical Sciences, University of Ulster, Northern Ireland, UK

Abstract

Successful bioremediation of hydrocarbon contamination in terrestrial as well as aquatic ecosystems is generally limited by the low bioavailability of hydrophobic pollutants. Considerably higher mass transfer rates and hydrocarbon solubilities can be obtained at higher temperatures, but so far approaches to bioremediation at increased temperature have hardly been investigated. The biotechnological significance and the benefits of the use of thermophilic bacteria in enhancing hydrocarbon removal under different environmental conditions were investigated. Expression of the alkane mono-oxygenase *alkB* gene responsible for the initial step in the degradation of alkanes in soil geobacilli was found to be induced by the presence of such a substrate at high temperature, and this explains the increased degradation rates achieved in hexadecane-contaminated microcosms incubated at 60°C compared to mesophilic conditions. Additional benefits of increasing hydrocarbon degradation also occur when thermophilic bacteria capable of producing biosurfactants which act as solubilising agents in such systems are introduced. We conclude that thermally accelerated bioremediation may be an effective technology for hydrocarbon contaminated soil bioremediation.
Keywords: thermophiles, geobacilli, bioremediation, biosurfactants.

1 Introduction

Removal of contamination from soils, sediments, groundwater, surface water and air with hazardous and toxic compounds is a priority task for the restoration of the natural environment due to the serious threat contaminants pose to ecosystems and human health. Among the many techniques employed to

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remediate polluted sites, environmentally friendly technologies of bioremediation are gaining increasing prominence due to their obvious advantages although some disadvantages may also occur. Bioremediation is mainly carried out through the use of microorganisms or microbial processes to degrade environmental contaminants or attempts to accelerate naturally occurring biodegradation through the optimization of conditions which are otherwise limiting. A variety of bioremediation methods have been developed to support and increase the degradative activities of native microbial populations (*natural attenuation*), thus allowing a reduction in the time required and subsequent saving in costs. The two main approaches to bioremediation include environmental *biostimulation*, involving the addition of mainly oxygen and/or mineral nutrients (nitrogen, phosphorous & trace metals), and *bioaugmentation* with addition of selected degrader microorganisms to the site.

Hydrocarbon-degrading microorganisms have been widely investigated both in laboratory and field studies, but so far little attention has been paid to the role and the biotechnological significance of thermophilic bacteria in bioremediation. In this paper we will briefly present the benefits of running hydrocarbon degradation processes at elevated temperature. In particular we will discuss how to detect and best exploit the degradative capability of thermophilic geobacilli inhabiting most soil environments for high temperature bioremediation treatments. We will also investigate the aspect of production of biosurfactants as an additional property of some thermophilic degrader microorganisms for further enhancing hydrocarbon removal. Some of these aspects have been introduced in recent publications [17–19,26].

2 High temperature conditions

Biodegradation rates are influenced by several factors both physico-chemical such as environmental characteristics, nutrients, oxygen, pH value, concentration and bioavailability of the contaminants, chemical characteristics and history of the pollution, and biological aspects such as number and species of indigenous microflora, abundance of degrading microorganisms. Among these factors temperature plays a significant role in controlling bioavailability of low-solubility compounds and hence the nature and the extent of microbial metabolism and degradation [20]. The rate at which microbial cells can convert contaminants during bioremediation depends on the rate of contaminant uptake and metabolism and the rate of transfer to cells (mass transfer) [9]. The elevation of temperature is accompanied by a decrease in viscosity and an increase in the diffusion coefficient of organic compounds so that the bioavailability of recalcitrant substrates may be significantly improved allowing more efficient bioremediation [25].

3 Thermophilic degrading microorganisms

High numbers of thermophilic degraders have been isolated and characterized in the last few years, and their potential for hydrocarbon pollution remediation

investigated. A representative sample of such microorganisms with possible applications in bioremediation is shown in Table 1.

The main target of thermally enhanced bioremediation processes can be waste materials such as industrial effluents, which are often discharged at 50-130°C, and excavated contaminated soil, which could be treated in slurry reactors. The increasing number of patents indicates that there is a growing interest in the commercial application of such environmentally friendly technologies. Behmann and Tonelli [8] developed an aerated hot membrane bioreactor process for purifying any wastewater stream, and especially those containing recalcitrant compounds, suitable for treatment with thermophilic bacteria thriving in a temperature range of 45-75°C. Lugowsky et al. [16] patented a method for detoxifying liquid effluent streams contaminated with toxic hydrocarbons using a mixture of selected thermophilic degrading bacteria, mostly belonging to *Pseudomonas* genus, to be inoculated in reactors operating at 40-42°C.

Table 1: Thermophilic microorganisms potentially useful for hydrocarbon bioremediation applications.

Microorganisms	Temp	Hydrocarbons	Reference
B. thermoleovorans	70°	Long-chain alkanes	Kato et al. [15]
G. thermoleovorans T80	60°	Long-chain alkanes	Marchant et al. [19]
B. thermoleovorans DSM 10561	45-60°	Aromatic	Markl et al. [21]
Anaerobic Consortia	45-75°	Aromatic	Chen & Taylor [12]
Thermus & *Bacillus* sp.	60-70°	PAHs	Feitkenhauer et al. [14]
Consortium mainly of *Pseudomonas* sp.	40-42°	Aromatic hydrocarbons	Lugowsky et al. [16]
B. thermoleovorans Hamburg 2	60°	Naphthalene	Annweiler et al. [2]
Bacillus sp. JF8	60°	Naphthalene & PCBs	Shimura et al. [32]
Consortia of anaerobes	65°	Dibenzothiophene (DBT)	Bahrami et al. [3]
B.stearothermophilus	60°	Crude oil	Sorkhoh et al. [34]
Bacillus sp.	40-45°	Crude oil	Al-Maghrabi et al. [1]
P.aeruginosa AP02-1	45°	Crude & diesel oil	Perfumo et al. [26]

An innovative technology exploiting thermophilic degraders has recently been described by Chaalal et al. [11]. They used thermophilic bacteria growing at 80°C to inoculate a suitably designed bioreactor in which solar radiation was supplied. Solar energy allowed the increase in temperature of the reactor water to a level that promoted the growth of thermophiles while ensuring airflow through transverse perforate pipes thus creating effective mixing. Coupling these factors allowed optimum conditions (e.g. decreased viscosity and interfacial tension of

hydrocarbon contaminants, profuse emulsification and faster reaction rates) to be established and hence significantly enhanced bioremediation achieved.

Hydrocarbon-degrading thermophiles are of special significance also for accelerating bioremediation *in situ*, which is mostly related to contaminated hot-environments such as arid regions. Numerous studies on oil spill bioremediation in Kuwait after the Gulf war in 1991 reported on the different strategies for the clean up of pollution. Experimental evidence indicated a potential self-cleaning of oil-contaminated areas via the activities of indigenous hydrocarbon-degrading microorganisms [33], which could be further improved by different management practices such as irrigation and fertilization with nitrogen or organic carbons [27].

Recently, a new attractive technology that couples the degradative activities of indigenous thermophiles to primary remediation methods such as Dynamic Underground Stripping (DUS) is growing in interest as well as in applications. DUS technologies combined steam injection or electrical resistance to heat subsurface areas, vaporize volatile compounds bound to soil so as to drive contaminants to vacuum extraction wells. Although steam injection can potentially recover a large fraction of volatile contaminants, it is expected that residual amounts of the contaminants will remain, and that can be further remediated by natural attenuation or bioremediation. Taylor et al. [35] patented a process in which selected microorganisms were inoculated into the injection wells thus allowing the removal of low levels of aromatic and chlorinated hydrocarbons. Due to the heating, the subsurface environment remains at elevated temperature (50-70°C) for extended periods, which enables thermophilic bacteria to thrive and metabolize the residual contaminants. The Lawrence Livermore National Laboratory, USA, reported an unexpected benefit in encouraging the biological degradation of hydrocarbons during underground steam stripping of contaminated soils at temperatures above 100°C [24]. Such active microbial communities in underground environments maintained at temperatures between 45-75°C following heat treatments have been reported elsewhere [31]. Thus, there is a clear evidence of the usefulness of thermophilic bacteria in bioremediation.

4 Use of geobacilli in thermally enhanced bioremediation of hydrocarbons

Interest in thermophilic bacilli has been recently rekindled by the reclassification of a number of *Bacillus* species into new genus *Geobacillus* [23] coupled with the description of additional species [7]. Although many of the *Geobacillus* isolates have come from high-temperature environments, it has been pointed out that they are frequent in temperate-climate soil environments where they are apparently unable to grow due to the temperature restriction [17,18]. It has also been established that an extensive diversity of geobacilli inhabiting the soils has the capability to degrade alkanes and hydrocarbons [30]. Our current studies have been addressed to some aspects of this problem.

5 Induction of alkB gene in n-alkane degrading thermophilic geobacilli

We used molecular techniques to investigate the expression of the alkane mono-oxygenase gene (*alkB*) in thermophilic geobacilli belonging to our collection [17]. Using sequence data derived from the European Molecular Biology Laboratory (EMBL) database, we designed degenerate primers for the *alkB* gene and succeeded in amplifying a fragment of this gene in *G. thermoleovorans* T70. An RT-PCR experiment confirmed that the *alkB* gene expression was induced in the presence of *n*-hexadecane in pure cultures of *G. thermoleovorans* T70 when grown at 55°C. The same analysis was performed by extracting RNA directly from soil microcosms containing *n*-hexadecane and incubated for 1 week at 25, 30, 37 and 55°C together with a control microcosm with no alkane incubated at 55°C. The results indicated that also in soil samples *alkB* gene was expressed only in presence of *n*-hexadecane when incubated at 55°C. No positive RT-PCR signals were detected from any of the other microcosms. Thus, our data showed that the *alkB* gene in thermophilic geobacilli was induced by alkanes at high temperature.

6 Soil microcosms

Experiments were carried out to develop new strategies of thermally enhanced bioremediation technology for hydrocarbon-contaminated soils. Soil microcosms simulating the main bioremediation techniques (e.g. natural attenuation, biostimulation with inorganic nutrients, biosolubilisation with biosurfactants and bioaugmentation with a selected degrader strain together with an abiotic microcosm as control) to demonstrate the ability of thermophilic geobacilli to effectively degrade 2% (v/v) *n*-hexadecane were set up at both 60°C and at ambient temperature. Hydrocarbon removal as well as the microbiological response was monitored for a 40-day period (Table 2(a) and (b)).

7 Natural attenuation

Elevated temperature (60°C) significantly enhanced the *n*-hexadecane degradation rates in soil microcosms compared to those at ambient temperature leading to two-fold increased removal. Higher temperatures may enhance desorption of contaminants from the soil particles, mobilizing them and increasing the solubility and bioavailability. The response at 60°C of the autochthonous microbial population, which had a 10-fold-increase in number, as well as the degradation rate, which was 56.4% after 40 days, demonstrated the intrinsic capabilities of the indigenous thermophiles and encouraged the development of further bioremediation treatments.

Table 2: Soil microcosms experimental design & hexadecane degradation under various bioremediation techniques & number of Colony forming unites (CFU)/gram soil at (a) 60°C and (b) ambient temperature.

No.	Techniques	Microcosm set-up	*n*-Hexadecane % removal at 40 days	Microbiological response	
				Initial CFU	CFU at 40days
(a)					
1	Natural attenuation	No amendments	56.4	1.2×10^4	8.5×10^4
2	Biostimula-tion	NPK solution at 0.5% (v/w)	65.3	2.0×10^4	2.1×10^4
3	Biosolubili-sation	JBR-215 rhamnolipid 0.5% (v/w)	71.4	1.6×10^4	4.5×10^4
4	Bioaugme-ntation	*G.thermoleovo-rans* T80	70.3	6.5×10^4	5.0×10^4
5	All 2 +3 +4	All amendments	91.0	6.0×10^4	7.2×10^4
6	Abiotic control	Sterilized soil, no amendments	7.4	0	0
(b)					
1	Natural attenuation	No amendments	29.7	9.5×10^5	1.3×10^6
2	Biostimula-tion	NPK solution at 0.5% (v/w)	41.3	7.5×10^5	1.5×10^6
3	Biosolubili-sation	JBR-215 rhamnolipid 0.5% (v/w.	42.5	5.6×10^5	2.0×10^6
4	Bioaugme-ntation	*G.thermoleovo-rans* T80	38.0	8.2×10^5	1.8×10^6
5	All 2 +3 +4	All amendments	48.5	8.0×10^5	4.0×10^6
6	Abiotic control	Sterilized soil, no amendments	3.2	0	0

8 Biostimulation with inorganic NPK

We applied the biostimulation with inorganic nutrients (nitrogen, phosphorous, potassium), which is one of the most widely used techniques given the rapid depletion of the pool of these essential elements as a consequence of the high carbon content due to the contamination. Our results confirmed the benefit showing an increase of approximately 10% of *n*-hexadecane degradation rates both at 60°C (65.3% reduction) and at ambient temperature (41.3% reduction).

9 Solubilisation with biosurfactant

Soil microcosms treated with biosurfactants (JBR 215 rhamnolipid solution, Jeneil Biosurfactant, WI, USA) had a significant enhancement of alkane degradation, which was 71.4% and 42.5% at 60°C and 18°C respectively. These results were consistent with numerous studies demonstrating the efficacy of such molecules in remediating oil-contaminated soils [22,28,29]. It is likely that rhamnolipids, being biodegradable and containing both sugar and lipid moieties, acted as an additional carbon source thus contributing to the biodegradation enhancement.

10 Bioaugmentation

Bioaugmentation has frequently been reported unsuccessful since microorganisms able to degrade organic pollutants in laboratory conditions may fail to compete with indigenous microorganisms for many reasons. Bioaugmentation with indigenous microorganisms isolated from the contaminated soil, may help to overcome these problems and contribute to enhanced degradation rates. When we added *Geobacillus thermoleovorans* T80, an indigenous thermophilic degrading strain [18] we detected a significant increase in the *n*-hexadecane removal at high temperature (70.3%).

11 Combined treatment

When all amendments were used together up to 91% of the hydrocarbon in the soil microcosms incubated at 60°C were removed whereas 48.5% was removed in those at 18°C. Tables 2(a) and (b) show details of the experimental set-up of soil microcosms as well as the effects of treatments on soil hydrocarbon degradation and soil microbial populations. These data were subjected to a three way Analysis of Variance (ANOVA), which confirmed that the combination of the bioremediation treatments with the high temperature had a highly significant effect on hydrocarbon removal.

12 Biosurfactant-enhanced bioremediation of hydrocarbons

Bioavailability of poorly water-soluble hydrocarbons and pollutants is low. Solvents and synthetic surfactants are frequently used as additives to improve the solubility, but they are themselves a further source of contamination and mostly hazardous to the environment. Alternatively biosurfactants are microbial products that are biodegradable and non toxic and hence more suitable for environmental applications. This explains the increasing interest on these molecules, which have been extensively studied [4–6,13].

13 Thermophilic biosurfactant-producing microorganisms

Oil-degradation and biosurfactant-production investigations almost exclusively deal with mesophilic microorganisms, while a few reports refer to thermophilic conditions as reviewed by Cameotra and Makkar [10]. Thermophilic biosurfactants therefore remain an unexplored microbial resource.

A biosurfactant-producing *Pseudomonas aeruginosa* AP02-1 was isolated from a hot spring environment and grows optimally at 45°C using a variety of hydrocarbons (short and long-chain alkanes, aromatic and polycyclic aromatic hydrocarbons, heterocyclic sulphorate hydrocarbons) as carbon source [26]. The strain is able to degrade crude oil and diesel oil with high efficiency: gas chromatographic analyses for cultures grown on both compounds showed 99% degradation of the total petroleum hydrocarbons (TPHs) after only 7 days of incubation [26].

The involvement and role of rhamnolipids in promoting the uptake of hydrocarbons is well known but under such thermophilic conditions the solubilisation process was accelerated. The fast dispersion of the diesel layer into oil droplets and the subsequent formation of a stable emulsion observed during the bacterial growth was attributed to biosurfactant production which led to the lowering of the surface tension coupled to an increase in the emulsification activity. The Bacterial Adhesion To Hydrocarbons (BATH) test showed 71% of cells adhered directly to hydrocarbon droplets. Clear evidence came from Scanning Electron Microscopy, which showed diesel oil droplets breaking down to smaller sizes, and after 96 h of incubation, bacterial cells tightly adhered to the surface of microdroplets of approximately 30-μm diameter. Thus, we concluded that such a multiphase, thermally enhanced and biosurfactant-mediated process allowed *P. aeruginosa* AP02-1 to degrade almost completely diesel as well as crude oil in less than 1 week of incubation.

14 Conclusions

High temperature (60°C) significantly enhanced *n*-hexadecane degradation in soil leading to approximately two-fold increased overall removal. The supplementation with limiting nutrients, augmentation with thermophilic geobacilli or addition of biosurfactants all contributed to additional degradation and combining all resulted in up to 90% removal of *n*-hexadecane in microcosms within 40 days. Such high degradation rates of hydrocarbons were also observed when a thermophilic biosurfactant-producing oil degrading *P. aeruginosa* AP02-1 was used and which efficiently utilised crude oil and diesel within a short period (<7 days) at 45°C. It is concluded therefore that the combination of thermal treatment for pollution removal and bioremediation has potential to be used as an effective environmental biotechnology.

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