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Research Article

Biodegradation of Crude Oil by Individual Bacterial Strains and a Mixed Bacterial Consortium Isolated from Hydrocarbon Contaminated Areas

A preliminary study was undertaken to determine the optimal conditions for the biodegradation of a crude oil. Among 57 oil-degrading bacterial cultures isolated from oil-contaminated soil samples, *Bacillus* sp. IOS1-7, *Corynebacterium* sp. BPS2-6, *Pseudomonas* sp. HPS2-5, and *Pseudomonas* sp. BPS1-8 were selected for the study based on the efficiency of crude oil utilization. Along with the selected individual strains, a mixed bacterial consortium prepared using the above strains was also used for degradation studies. The mixed bacterial consortium showed more growth and degradation than did individual strains. At 1% crude oil concentration, the mixed bacterial consortium degraded a maximum of 77% of the crude oil. This was followed by 69% by *Pseudomonas* sp. BPS1-8, 64% by *Bacillus* sp. IOS1-7, 45% by *Pseudomonas* sp. HPS2-5, and 41% by *Corynebacterium* sp. BPS2-6. The percentage of degradation by the mixed bacterial consortium decreased from 77 to 45% as the concentration of crude oil was increased from 1 to 12%. Temperature of 35°C and pH 7 were found to be optimum for maximum degradation.

Keywords: Bacteria; Bacterial Consortium; Crude Oil; Degradation; Hydrocarbon

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1 Introduction

In the process of fulfilling the energy requirement for today's population, various natural resources have been exploited. But the principal source of energy continues to be petroleum hydrocarbon and hence a global pollutant. During accidental spills, action will be taken to remove or remediate the contaminant immediately, whereas in the gasoline and diesel stations the spills due to leakage may be small but continuous and prolonged. Because of its persistence, the chance for groundwater contamination is high. Toxicity of crude oil includes liver necrosis, congestion of the liver, fat degeneration, and dissociation of hepatocytes. Birds and animals in oil-contaminated area are found to have black emulsion in the digestive tract with a petroleum odor. This leads to decrease in the absorption of nutrients and finally leads to death of these birds and animals due to rupture of capillaries and hemorrhage, hepatocellular dissociation, hemosiderosis, renal tubular necrosis, and anemia [1]. The aromatics in crude oils also have numerous adverse effects on the environment particularly to the local microbial flora. It was shown that α-pinene, limonene, camphene, and isobornyl acetate were inhibitory to the microorganisms. The phenolic and quinonic naphthalene derivatives inhibited the growth of the cells [2]. Calder and Lader [3] demonstrated that increasing amounts of naphthalene, 2-methylnaphthalene, pyrene, and others resulted in an increased lag phase and lowered the growth rates of two bacteria growing on these compounds. Uribe et al. [4] reported the toxic effects of cyclohexane on the energy transduction in *Saccharomyces cerevisiae*. Cyclohexane inhibited oxygen uptake in intact cells and isolated mitochondria. Studies on isolated mitochondria showed that ATP synthesis was impaired whereas ATP hydrolysis was slightly increased. Uptake of potassium ions was impaired, and dissipation of the mitochondrial membrane potential was observed [4]. These studies indicate that the permeability barrier of the inner mitochondrial membrane was disrupted by cyclohexane.

Oil contamination with petroleum and petroleum-based hydrocarbons has caused critical environmental and health defects and increasing attention has been paid for developing and implementing innovative technology for cleaning up this contaminant. Bioremediation methods are currently receiving favorable publicity as promising environmental friendly treatment technologies for the remediation of hydrocarbons. Moreover, biological methods can have an edge over the physico-chemical treatment regimes in removing spills as they offer cost effective in situ biodegradation of oil fractions by the microorganisms. Bioremediation can be described as the conversion of chemical compounds by living organisms, especially microorganisms, into energy, cell mass, and biological waste products [5]. The rates of uptake and mineralization of many organic compounds by a microbial population depend on the concentration of the compound [6]. Inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons may occur due to high concentrations of undispersed hydrocarbons in water. Extreme pH and temperature conditions are expected to have a negative influence on the ability

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of microbial populations to degrade the hydrocarbons [5]. Since the fate of hydrocarbon degradation is largely determined by the local environmental conditions, which influence the microbial growth and enzymatic activities, this research was carried out to explore the possibility of the use of selected bacterial cultures and a mixed bacterial consortium to degrade a crude oil at various pH, temperatures, and oil concentrations.

2 Materials and Methods

2.1 Bacterial Isolation

Soil samples were collected in pre-sterilized glass bottles from various gasoline and diesel spilled gas stations in Coimbatore city (India) and transported to the laboratory for analyses. Enrichment and isolation of oil-degrading bacterial cultures were done using mineral salts medium [7] with a crude oil, Bombay High (BH) crude oil as a substrate and a serial dilution-agar plating technique on nutrient agar medium [8], respectively. The isolated bacterial cultures were characterized by their morphological and biochemical characteristics [9].

2.2 Screening of Strains

Bacterial cultures (12 h old) were inoculated in mineral salts medium with 1% BH crude oil as a carbon source. They were kept in a shaker at 200 rpm at 30°C for a period of 7 days. The growth was monitored through culture densities, measuring the absorption at 620 nm, spectrophotometrically [8]. The isolates with highest rate of crude oil degradation were selected. A loopful of overnight culture was used to inoculate 100 mL sterile nutrient broth medium. The flasks were kept in a shaker at 200 rpm for 12 h at 30°C. Equal volumes (with approximately equal densities ranging between 0.81 and 1.0) of culture broth from the selected isolates were used to prepare the mixed bacterial consortium.

2.3 Degradation Studies

The individual and mixed bacterial consortium from overnight culture at the log phase of growth were adjusted with sterile distilled water to give a bacterial cell count of 1.0×10^2 CFU/g and transferred to 250 mL conical flasks containing 100 mL of sterile-defined mineral salts medium [8] with 1% BH crude oil. The flasks were then incubated in a shaker at 200 rpm at 30°C for 25 days. At every 2 days intervals, sets of flasks were used for the enumeration of the microbial population by pour plate technique on plate-count agar. The total hydrocarbons in the treatments were determined spectrophotometrically [10]. Samples (5 mL) from different treatments were mixed with equal volume of toluene to extract hydrocarbons from the sample. The extracted hydrocarbons were detected spectrophotometrically at 420 nm. A standard curve prepared using known concentrations of crude oil was used to estimate the amount of hydrocarbons in the sample. Degradation was estimated as the difference between the initial and final concentrations of total hydrocarbons.

2.4 pH and Temperature Studies

The influence of pH on the growth and degradation of 1% BH crude oil was studied at every 5 day time interval for 30 days. Mineral salts medium with BH crude oil was prepared at pH 4 to 10 using 1 N HCl and NaOH. To maintain the pH, citrate – phosphate buffer (pH 4 – 6), phosphate buffer (pH 7 and 8), and carbonate – bicarbonate buffer (pH 9 and 10) were used [11]. The flasks were inoculated with individual and mixed bacterial consortium cultures and incubated at 30°C. The populations and percentage degradations of BH crude oil at different time intervals were determined. The effect of temperature (25 – 45°C) on the growth and degradation of crude oil was studied using mineral salts medium with 1% BH crude oil at pH 7 and incubation time of 25 days. The population and percentage of degradation were determined.

2.5 Degradation Studies with Varying Crude Oil Concentrations

Biodegradation of BH crude oil with selected isolates and mixed bacterial consortium were performed with various concentrations of crude oil (1, 3, 6, 9, and 12%). For all the concentrations, the experiment was conducted at 35°C and pH 7. The inoculated flasks were incubated for 25 days and bacterial growth and crude oil degradation were estimated.

3 Results

Totally 57 pure cultures able to grow in mineral salts medium with crude oil (gases 2%, light naphtha and heavy naphtha 35%, kerosene 11%, light gas oil 20%, and heavy gas oil and residue 32%) as carbon source were identified through enrichment and isolation procedure. The isolated pure cultures were identified to belong to the genera *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Pseudomonas*, and *Vibrio* (see Tab. 1). The flora represented the normal heterotrophic bacteria present in the soil. However, the dominant strains belonged to *Bacillus*, *Corynebacterium*, and *Pseudomonas*.

Studies on the effect of the pH showed that pH 7 was favorable for all the bacterial isolates and mixed bacterial consortium (see Fig. 1). However, not much difference was seen in crude oil degradation between pH 7 and 8. Mixed bacterial consortium showed the maximum percentage of crude oil degradation with 77%, followed by *Pseudomonas* sp. BPS1-8, *Bacillus* sp. IOS1-7, *Pseudomonas* sp. HPS2-5, and *Corynebacterium* sp. BPS2-6 with 69, 64, 45, and 41%, respectively, at pH 7.

Population of the individual cultures and mixed consortium increased with time at pH 7. A population of 7.1×10^4 CFU/g in the mixed bacterial consortium and 6.5×10^4 CFU/g in *Pseudomonas* sp. HPS2-5 were recorded at 1% crude oil after 25 days of incubation. Other bacterial strains showed a population increase of 6.2×10^4 –

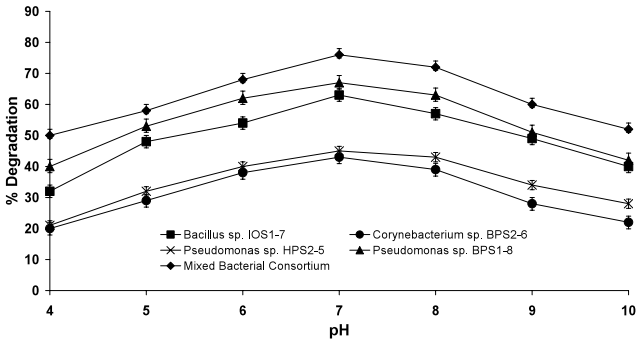

Figure 1. Effect of pH on crude oil degradation.

Table 1. Generic-wise distribution of bacteria showing different levels of growth on crude oil.

Genera	Total no.	Optical density at 620 nm [8]			
		0.21 – 0.4	0.41 – 0.6	0.61 – 0.8	0.81 – 1.0
Bacillus sp.	17	10	4	2	1
Corynebacterium sp.	16	7	5	3	1
Flavobacterium sp.	4	2	1	1	ND
Micrococcus sp.	3	2	1	ND	ND
Moraxella sp.	2	1	1	ND	ND
Pseudomonas sp.	14	6	4	2	2
Vibrio sp.	1	1	ND	ND	ND
Total	57	30	16	8	4
Percentage	100	52.63	28.07	14.04	7.02

0.21 – 0.4, Low growth; 0.41 – 0.6, moderate growth; 0.61 – 0.8, high growth; 0.81 – 1.0, excellent growth.
ND, none detected.

Table 2. Effect of various concentrations of BH crude oil biodegradation by pure and mixed bacterial cultures.

Bacteria	Oil concentration (%)	1		3		6		9		12	
	Incubation time (days)	0	25	0	25	0	25	0	25	0	25
Bacillus sp. ISO1-7	a	0	64 ± 0.7	0	58 ± 0.4	0	43 ± 1.2	0	36 ± 0.9	0	28 ± 0.7
	b	1.0×10^2	4.2×10^4	1.0×10^2	2.1×10^5	1.0×10^2	7.5×10^5	1.0×10^2	3.7×10^6	1.0×10^2	3.9×10^6
Corynebacterium sp.	a	0	43 ± 1.2	0	34 ± 1.6	0	28 ± 0.8	0	22 ± 0.5	0	19 ± 0.7
BPS2-6	b	1.0×10^2	3.3×10^4	1.0×10^2	8.1×10^4	1.0×10^2	3.6×10^5	1.0×10^2	9.5×10^5	1.0×10^2	7.7×10^5
Pseudomonas sp.	a	0	45 ± 0.9	0	39 ± 1.1	0	33 ± 1.3	0	24 ± 0.7	0	21 ± 0.5
BPS2-5	b	1.0×10^2	6.5×10^4	1.0×10^2	9.8×10^4	1.0×10^2	5.6×10^5	1.0×10^2	8.7×10^5	1.0×10^2	8.9×10^5
Pseudomonas sp.	a	0	67 ± 1.7	0	62 ± 0.8	0	52 ± 1.4	0	41 ± 1.1	0	34 ± 1.3
BPS1-8	b	1.0×10^2	6.2×10^4	1.0×10^2	1.1×10^5	1.0×10^2	3.4×10^5	1.0×10^2	7.9×10^5	1.0×10^2	7.9×10^5
Mixed consortium	a	0	76 ± 1.8	0	72 ± 1.2	0	63 ± 0.8	0	52 ± 1.5	0	41 ± 1.1
	b	1.0×10^2	7.1×10^4	1.0×10^2	2.1×10^5	1.0×10^2	5.5×10^5	1.0×10^2	8.5×10^5	1.0×10^2	2.6×10^6

a, Oil degradation (%); b, bacterial cell count (CFU/g).

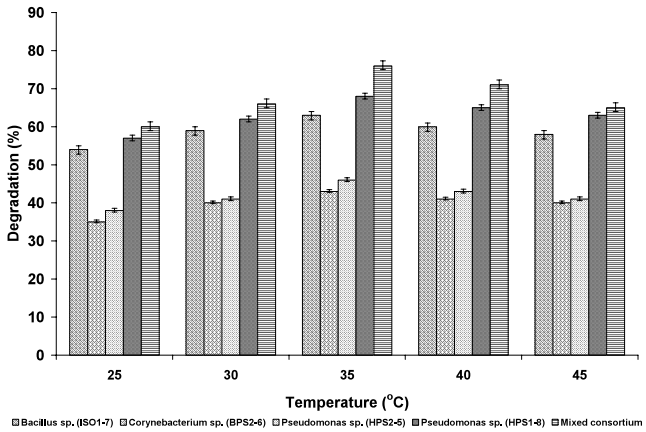

Figure 2. Effect of temperature on crude oil degradation.

3.3×10^4 CFU/g at pH 7. Hence the pH 7 was selected for further studies. All the four individual isolates and mixed bacterial consortium showed maximum crude oil degradation at 35°C and the population also corresponded (see Fig. 2). Decrease in temperature decreased the percentage of degradation and increase in temperature increased the rate of hydrocarbon metabolism to a maximum, typically in the range of 35 – 45°C.

The effects of crude oil concentrations on the growth of individual bacterial cultures and the mixed bacterial consortium, and crude oil degradation by them were tested and presented in Tab. 2. The mixed bacterial consortium showed 76% degradation at 1% BH crude oil, followed by 72% at 3%, 63% at 6%, 52% at 9%, and 41% at 12%. The individual cultures also showed the good degradation potential at 1% BH crude oil and decreased degradation at higher concentrations of the crude oil.

4 Discussions

The genera listed in Tab. 1 were also identified as hydrocarbon-degrading microorganisms by Lal and Khanna [12], Bharathi and Vasudevan [13], and Rahman et al. [5].

Similar to our results, Chhatre et al. [14], Sugiura et al. [15], Vasudevan and Rajaram [16], and Rahman et al. [5] have illustrated the ability of mixed bacterial consortia to degrade 28 to 51% of saturates and 0 to 18% of aromatics present in the crude oil or up to 78% crude oil. Crude oil contains many kinds of hydrocarbons which are fractioned into saturated and aromatic hydrocarbons, resins and asphaltenes by silica gel chromatography [17]. A number of compounds slightly degradable by microorganisms are contained in the fraction of aromatic hydrocarbons. Metagenomic analyses by many researchers have showed that only a few enzymes which degrade aromatic hydrocarbons are produced in most microorganisms in natural environments [18, 19]. Leahy and Colwell [20] reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil or diesel fuel. Such mixed cultures display metabolic versa-

tility and superiority to pure cultures [21]. Therefore, a microbial consortium containing a number of microorganisms which synthesize the degradative enzymes for different parts of the decomposition pathway is considered to be well suited to the degradation of aromatic hydrocarbons. Microorganisms not directly involved in the degradation process also probably play a role by producing micronutrients or surface-active agents for the solubilization of aromatic hydrocarbons [13, 22]. Sugiura et al. [23] reported that biodegradation caused by mixed cultures was more effective than that caused by pure cultures mainly due to the complexity of oil products. Various organisms have the capability of degrading various forms of hydrocarbons and thus when a consortium of these microbes is applied to degrade various forms of hydrocarbons in a single source like crude oil, the total degradation is more effective. Deppe et al. [24] reported that an arctic microbial consortium was able to degrade 77% of crude oil from source-I and 71% of crude oil from source-II at 20°C. This result made it obvious that the metabolic capability of the consortium was not restricted to one type of crude oil. But such type of result cannot be expected from pure cultures which are substrate specific. Gauthier et al. [25] reported that *Marinobacter hydrocarbonoclasticus* utilized linear saturated hydrocarbons (tetradecane, hexadecane, eicosane, and heneicosane) with high degradation rates and pristane, phenyldecane, and phenanthrene to a lower extent when used as carbon and energy sources. This validates the use of a microbial consortium for biodegradation of complex mixtures of hydrocarbons in crude oil.

The isolated individual oil degrades and the mixed bacterial consortium showed optimal values of pH 7 and temperature 35°C for maximum degradation. Salmon et al. [26] have reported pH 7 as the optimal range for hydrocarbon degradation. Extremes in pH were shown to have a negative influence on the ability of microbial populations to degrade hydrocarbons [5, 27, 28]. Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community [29]. At low temperatures, the viscosity of the oil is increased, volatilization of alkanes reduced, and the water solubility decreased, delaying and decreasing the onset of biodegradation [6]. Above that temperature, degradation of hydrocarbons decreased, which may be attributed to the membrane toxicity of hydrocarbons at elevated temperatures [30]. Banat [31] reported 30°C to be the optimum temperature for microbial growth and PAH degradation. Similarly, Rahman et al. [5] reported 30 to 40°C as optimum temperature for the degradation of crude oil by individual and mixed consortium of bacterial cultures. Sugiura et al. [15] reported that *Acinetobacter* sp. T_4 and 8 mS degraded 20 – 34% of Arabian light crude oil, 14 – 27% of Dubai crude oil, 14 – 25% of Shanghai crude oil, and 12 – 19% of Maya crude oil at 20°C and these were higher than the values recorded at 5°C. Hasanuzzaman et al. [32] observed 75 and 85% degradation of total crude oil by *Pseudomonas aeruginosa* strain at 20 and 30°C, respectively. Since all our isolates were mesophilic in nature, they all exhibited optimum activity at 35°C.

Increase in crude oil concentration decreased the percent degradation but an increase in the quantity of crude oil degradation was noticed. Crude oil degradation is inversely proportional to the concentration of the oil [33]. Zhang et al. [34] reported 58 and 60% degradation of crude oil with the initial concentration of 0.7 g/L in mineral salt medium by *P. aeruginosa* in the presence of 1 g/L glycerol and 0.22 g/L rhamnolipids, respectively, used as emulsifiers. Tzarkova and Groudeva [35] reported that compounds such as saturates, aromatics, and polar compounds present in different crude oil samples were degraded to different degrees by the same organisms. The degradability was not solely determined by the chemical structure but other factors as well. The bioavailability of these compounds in different crude oil samples may differ. Saturated compounds with molecular weight larger than 500 may not be degraded by the organisms, because this size corresponds to the exclusion size for passage through the outer membrane of Gram-negative bacteria [5, 33].

Generally, it is believed that microbes preferably degrade/metabolize C8 – C15 *n*-alkanes followed by C16 – C36 *n*-alkanes due to the simplicity of these hydrocarbons. Saturated, cyclic high-molecular weight compounds like hopanes are usually not attacked by the microbes due to their complexity. Most of the light aromatics like toluene, xylene, etc. are reported to be degraded either completely or partially [24]. Bacteria that degrade xylenes commonly fall into two classes: those that can degrade both *m*-xylene and *p*-xylene, and those that can degrade *o*-xylene only [24]. Thus, when a bacterial consortium is used for degradation, the possibility of degrading all three xylene isomers is very high. In the present study too it is believed that the individual microbes were selective in degrading the hydrocarbons and when the microbial consortium containing the same microbes was applied, the percent degradation of the crude oil as a whole was increased. Although it is not possible to specifically emphasize the metabolic pathway of degradation by individual microbes and microbial consortium without complete characterization of the crude oil before and after degradation, from the percent degradation of the total crude oil content we can conclude that the individual microbes and microbial consortium have the capability of degrading a wide range of hydrocarbons. Due to the highly complex nature of the crude oil, it is very difficult to understand the degradation mechanism especially for aromatics. However, some researchers have revealed the metabolic pathway for the degradation of some hydrocarbons. Wilkes et al. [36] reported that the degradation of *n*-hexane by *Azoarcus* sp. (strain HxN1) is initiated by an enzymatic radical reaction resulting in the formation of (1-methylpentyl)succinate and subsequently the product of the initial activation reaction is transformed to 4-methyloctanoyl-CoA via a complex reaction sequence involving an intramolecular rearrangement. 4-Methyloctanoyl-CoA is further degraded by β-oxidation. The pathway is active during the degradation of *n*-alkanes from crude oil by *Azoarcus* sp. (strain HxN1) which goes along with the cometabolic transformation of certain cycloalkanes such as cyclopentane by the same type of activation reaction. Gibson and Subramanian [37] proposed that the initial steps of anthracene metabolism in pseudomonads involve a dioxygenase-catalyzed oxidation to anthracene *cis*-1,2-dihydrodiol, further oxidation to 1,2-dihydroxyanthracene, and subsequent extradiol *(meta)* ring fission. Hammel [38] reported that phenanthrene oxidation to diphenic acid by *Phanerochaete chrysosporium* is a consequence of peroxidase-mediated lipid peroxidation, where the fungus oxidized phenanthrene at its 9- and 10-positions to give 10 – 15% yields of a ring-fission product, 2,2′-diphenic acid.

Since biological treatment can efficiently destroy the hydrocarbons and does not allow the contaminant to accumulate, it is considered to be a superior technology [39]. The present study revealed that the mixed bacterial consortium achieved maximum crude oil degradation at pH 7 and 35°C. Hence, it is suggested that the use of above mixed bacterial consortium under optimized conditions will be an effective and eco-friendly technology for the degradation of hydrocarbons from BH crude oil.

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