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Full Length Research Paper

Isolation and characterization of engine oil degrading indigenous microrganisms in Kwazulu-Natal, South Africa

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As the usage of petroleum hydrocarbon products increases, soil contamination with diesel and engine oils is becoming one of the major environmental problems. To investigate the countermeasure to remediate soils contaminated with oils, bioremediation provide an effective and efficient strategy to speed up the clean-up processes. Three bacterial isolates capable of utilizing used engine-oil as a carbon source were isolated from contaminated soils using the enrichment technique. Three isolates were identified as *Flavobacterium* sp., *Acinetobacterium calcoaceticum* and *Pseudomonas aeruginosa* based on biochemical tests and 16S rRNA sequencing. The gavimetric analysis revealed that *A. calcoaceticum* and a consortium of the isolates were capable of utilizing 80 and 90% of used engine oil, respectively, under laboratory conditions at 30°C and 160 rpm with Bushnell-Haas media in a 4 week period. An increase in oil degradation is correlated to an increase in cell number indicating that the bacterial isolates were responsible for the oil degradation. All isolates were capable of degrading the n-paraffin up to 80% in a 2 week period. The optimal temperatures at which biodegradation occurred at 30–37°C. The preference of nitrogen sources and minimal salts were different for different bacterial isolates. The results obtained demonstrate the potential for oil bioremediation of these isolates *in situ* and/or *ex situ*.

Key words: Engine oil, bioremediation, *Flavobacterium* sp., *Acinetobacterium calcoaceticum* sp. and *Pseudomonas aeruginosa*.

INTRODUCTION

Engine oil is a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents (Butler and Mason, 1997) that is used to lubricate the parts of an automobiles engine, in order to keep everything running smoothly (Hagwell et al., 1992). The most important characteristic of the lubricating oil for automotive use is its viscosity. New motor oil contains a higher percentage of fresh and lighter (often more volatile and water soluble) hydrocarbons that would be more of a concern for acute toxicity to organisms. Used motor oil contains more metals and heavy polycyclic aromatic hydrocarbons (PAHs) that would contribute to chronic hazards including mutagenicity and carcinogenicity (Keith and Telliard, 1979; Hagwell et al., 1992; Boonchan et al., 2000). Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Mishra et al., 2001; Propst et al., 1999, Lloyd and Cackette, 2001). In addition, PAHs have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the

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environment (Van Hamme et al., 2003). The illegal dumping of used motor oil is an environmental hazard with global ramifications (Blodgett, 2001). The release of oil into the environment causes environmental concern and attracts the public attention (Roling et al., 2002).

Mechanical method to reduce hydrocarbon pollution is expensive and time consuming. Hydrocarbons including PAHs have been long recognized as substrates supporting microbial growth (Bushnell and Haas, 1941; Speight, 1991; Ehrlich, 1995). Bioremediation makes use of indigenous oil–consuming microorganisms, called petrophiles, by enhancing and fertilizing them in their natural habitats. Petrophiles are very unique organisms that can naturally degrade large hydrocarbons and utilize then as a food source (Harder, 2004). Microorganisms degrade these compounds by using enzymes in their metabolism and can be useful in cleaning up contaminated sites (Alexander, 1999).

Microbial remediation of a hydrocarbon–contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil. These microorganisms can degrade a wide range of target constituents present in oily sludge (Barathi and Vasudevan, 2001; Mishra et al., 2001; Eriksson et al., 1999). A large number of *Pseudomonas* strains capable of degrading PAHs have been isolated from soil and aquifers (Johnson et al., 1996; Kiyohara et al., 1992; Fall et al., 1979). Other petroleum hydrocarbon-degraders include *Yokenella* spp., *Alcaligenes* spp., *Roseomonas* spp., *Stenotrophomonas* spp., *Acinetobacter* spp., *Flavobacter* spp., *Corynebacterium* spp., *Streptococcus* spp., *Providencia* spp., *Sphingobacterium* spp., *Capnocytophaga* spp., *Moraxella* spp., and *Bacillus* spp. (Rusansky et al., 1987; Antai, 1990; Bhattacharya et al., 2002). Other organisms such as fungi are also capable of degrading the hydrocarbons in engine oil to a certain extent. However, they take longer periods of time to grow as compared to their bacterial counterparts (Prenafeta-Boldu et al., 2001).

Bioremediation processes have been shown to be effective methods that stimulate the biodegradation in contaminated soils (McLaughlin, 2001; Swannell et al., 1996). Harder (2004) estimated that bioremediation accounts for 5 to 10 percent of all pollution treatment and has been used successfully in cleaning up the illegal dumping of used engine oil. This study reports on the isolation of indigenous engine oil-degrading bacteria isolated from contaminated soils and their degradation potentials.

MATERIALS AND METHODS

Isolation and identification of Oil degrading bacteria

Bushnell-Haas (BH) medium (Atlas, 1994) was used as the enrichment media with 10% (v/v) used engine oil, supplied by DailmerChrysler, Gateway, Durban, South Africa, as the sole carbon source to isolate engine oil–degrading bacteria. 10 g of the contaminated soil, collected from ENGEN, Amanzimtoti, South Africa, was added and incubated at 30°C at 170 rpm. After 1 week, 1 ml of enriched media was transferred into freshly prepared enrichment media and incubated at the same conditions as described above. Serial dilutions (1/10) from the third enrichment process were plated out onto BH agar plates, which were covered with 100 µl of used engine oil and incubated at 30°C. The single colonies were streaked onto nutrient agar plates, incubated at 30°C overnight, and stored at 4°C until further use. The oil-degrading isolates were identified by gram stain, biochemical tests (Balows et al., 1992) and confirmed by 16S rDNA sequencing (Marchesl et al., 1998). For long term preservation, the bacterial isolates were stored in 40% glycerol at -70°C.

Characterization of the degradation potential and its growth patterns

A single colony of the isolate was inoculated into 10 ml nutrient broth (Merck) at 30°C overnight. The overnight culture was centrifuged for 15 min at 3500 rpm. The cell pellet was washed twice and was resuspended with BH medium until OD_{600} was equivalent to 1.

One ml of bacterial inoculum (1 OD_{600} equivalent) was transferred into 100 ml BH medium with 5 ml (5%) used engine oil (or n-paraffin) and was incubated at 30°C at 160 rpm. A control devoid of the bacterial isolate was prepared for each set of experiments. All experiments were performed in duplicate. Different nitrogen sources (ammonium nitrate, sodium nitrate, sodium nitrite and urea), different pH's (5, 7 and 9) and temperatures (25, 30 and 37°C) were used to test the optimal conditions of each isolate. The growth patterns were obtained by measuring the optical density at 600 nm and total viable counts (cfu/ml) of the isolates were determined by the spread plate technique after the incubation of the nutrient agar plates at 30°C for 24 h.

Determination of used engine oil degradation

The level of used engine oil degradation was determined using the gravimetric analysis. (Chang, 1998; Marquez-Rocha et al., 2001) The percentage of engine oil remaining was calculated compared to the control.

RESULTS

Ten bacterial strains, capable of utilizing used engine oil as a carbon source were isolated from the contaminated soils. Three isolates with best oil degradation ability were identified as *Flavobacterium* spp, *A. calcoaceticum* sp. and *Pseudomonas aeruginosa,* using the biochemical tests and confirmed by the partial sequencing of 16S rDNA. *Flavobacterium* spp, isolated in this study was not viable while maintaining in the nutrient agar plates in 0°C more than 3 days and survived for 3 months while were stored in 40% glycerol at -70°C.

Figure 1 shows the percentage of engine oil remaining and the growth pattern of different isolates over a period of four weeks. An increase in cell population of each isolate was corresponding to an increase in oil degradation. The results indicate all three isolates are capable of utilize engine oil as the nutrient source. *A. calcoaceticum* was found to be the best oil degrading isolate in this stu-

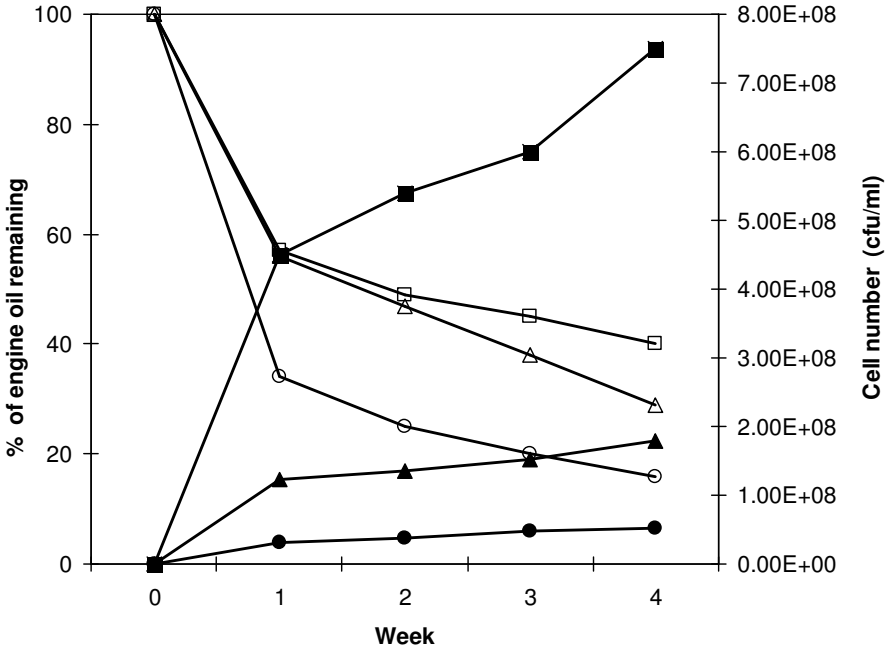

Figure 1. Percentage of engine oil remaining (□, *Flavobacterium* spp.; ○, *A. calcoaceticum*; ∆, *P. aeruginosa*) and growth patterns (■, *Flavobacterium* spp.; ●, *A. calcoaceticum*, ▲, *P. aeruginosa*) of each isolate over a period of four weeks.

dy with 84% degradation after 28 days incubation period while 60 and 71% degradation were observed using *Flavobacterium* spp, and *P. aeruginosa,* respectively, under the standard assay conditions.

All three isolates utilized *n*–paraffin as a sole carbon source with higher degradation rates comparing to utilize engine oil. *Flavobacterium* spp., *A. calcoaceticum* and *P. aeruginosa* degraded 66, 80 and 71% of n-paraffin in 2 week (Data not shown).

The results of degradations of engine oil using different consortium of three isolates showed similar patterns of all consortia as those of each individual isolate (Figure 2). The consortia proved to be a better degrader compared to individual isolate with degradation rates of 89, 82, 77 for *Flavobacterium* spp. and *A. calcoaceticum*, *A. calcoaceticum* and *P. aeruginosa*, and *Flavobacterium* spp. and *P. aeruginosa,* respectively. Ninety percent degradation in 4 weeks was obtained by the consortium of all three isolates.

Different nutrients sources and environmental conditions such as pH and temperature were substituted in the standard oil degradation assay. The results showed that *Flavobacterium* spp, isolated in this study utilized NH_4NO_3 (46%) more efficiently than utilized $NaNO_3$ in engine oil degradation metabolic mechanism. The addition of urea or nitrite reduced the cell growth and the degradation rate significantly (6-7%). *A. calcoaceticum* and *P. aeruginosa,* on another hand, were capable of utilizing different nitrogen sources including nitrite. The degradation rates by *P. aeruginosa* and *A. calcoaceticum* sp. increased as the temperature increased from 25 to 37°C while *Flavobacterium* spp, showed a better degradation rate at 30°C. *Flavobacterium* spp, and *A. calcoaceticum* preferred a pH of 7 and *P. aeruginosa* expressed a slightly higher degradation rate at pH 9.

DISCUSSION

Three bacterial isolates, namely *Flavobacterium* spp, *A. calcoaceticum* sp. and *P. aeruginosa*, were obtained from engine oil–contaminated soil in this study. An increase in oil degradation was corresponding to an increase in cell number during the degradation processes demonstrating the ability of utilizing engine oil as the energy source. All three isolates also demonstrated the ability of degrading n-paraffin with higher rates.

Pseudomonas and *Acinetobacter* species are the most common bacterial hydrocarbon–degraders reported in the literature (Rusansky et al., 1987; Kiyohara et al., 1992; Johnson et al., 1996; Barathi and Vasudevan, 2001; Bhattacharya et al., 2002; Pokethitiyook et al., 2003; Van Hamme et al., 2003). *Acinetobacter* spp. are widespread in nature and can remove or degrade a wide range of organic such as phenol (Briganti et al., 1997; Abdel-El-Haleem et al., 2002), toluene (Zilli et al., 2001) and inorganic compounds such as phosphates and metal (Auling et al., 1991; Wagner et al., 1994; Boswell et al., 2001). Species of *Acinetobacter* have been attracting increasing attention in both environmental and biotechnological applications (Abdel-El-Haleem, 2003).

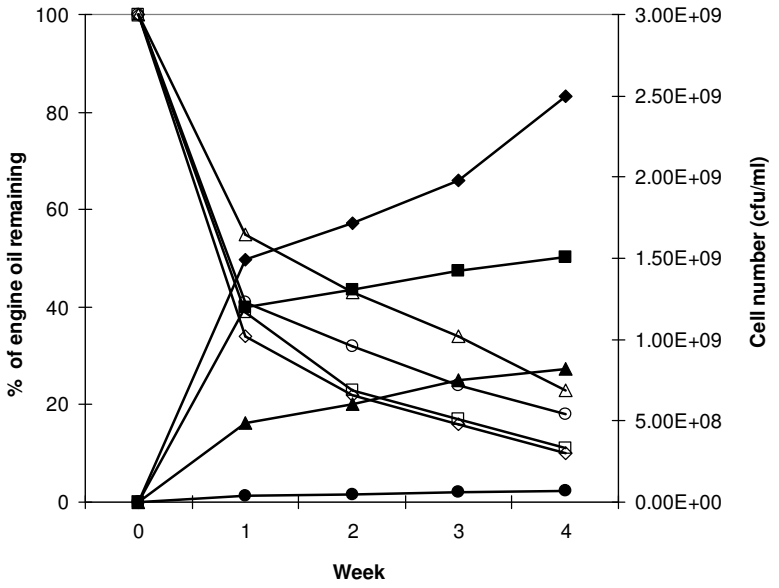

Figure 2. Percentage of used engine oil remaining (□, *Flavobacterium* spp. + *A. calcoaceticum*; ○, *A. calcoaceticum* + *P. aeruginosa*; ∆, *Flavobacterium* spp + *P. aeruginosa*; (◊, *Flavobacterium* spp. + *A. calcoaceticum*+ *P. aeruginosa*) and growth pattern (■, *Flavobacterium* spp. + *A. calcoaceticum*; ●, *A. calcoaceticum* + *P. aeruginosa;* ▲, *Flavobacterium* spp + *P. aeruginosa*; ♦, *Flavobacterium* spp. + *A. calcoaceticum*+ *P. aeruginosa*) of the bacterial consortia over a period of four weeks.

Flavobacterium spp, was also known to degrade petroleum (Atlas and Bartha, 1972). Christopher and Christopher (2004) reported that *Flavobacterium* spp. and *Pseudomonas spp*, were predominate species in the early stage of the petroleum land treatment unit. Regression analysis showed that the presence of *Flavobacterium* spp, and *Pseudomonas spp*, had a positive correlation with relative total petroleum hydrocarbon concentration. Interestingly, *Flavobacterium* was detected at very low levels in a pretreatment sample. Our results showed that *Flavobacterium* isolate could not be maintained easily indicating its liability to the environmental conditions.

The best degradation was observed by a consortium of all three isolates (*Flavobacterium* spp, *A. calcoaceticum*, and *P. aeruginosa*) with a degradation of 90%. The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been demonstrated (Alden et al., 2001). A sequential change of the composition of the oil degrading bacteria over a period of time in oil contaminated soil samples (Sorkhoh et al., 1995; Christopher and Christopher, 2004). Komukai-Nakamura and the coworkers (1996) reported the sequential degradation of Arabian light crude oil by *Acinetobacter* sp T4 and *Peudomonas putida* PB4. *Acinetobacter* sp T4 degraded alkane and other hydrocarbons in the crude oil and produced the accumulation of metabolites that were subsequently degraded by *P. putida* PB4. The use of pure cultures in the study of microbial degradation of fuels provides technical advantages by eliminating the ambiguity associated with constantly fluctuating populations (Reisfeld et al., 1972). However, individual organisms often prefer to metabolize a limited range of hydrocarbon substrates (Marin et al., 1996). Consequently, a mixed population of fungi and bacteria is usually required to provide all the metabolic capabilities for complete degradation of complex mixtures of hydrocarbons (Leahy and Colwell, 1990).

Bioremediation has been widely received by the public. However, a number of factors must be taken into consideration before *in situ* bioremediation can be applied. These include (i) type and concentration of oil contaminated; (ii) prevalent climatic conditions; (iii) type of environment that has been contaminated; and (iv) nutrient content as well as pH of the contaminated site (Rosenberg, 1992). Further research will be directed towards understanding the roles of individual members in influencing the effectiveness of a microbial association as well as the optimal degradation conditions *in situ* (Ghazali et al., 2004).

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