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Full Length Research Paper

Diesel degradation and biosurfactant production by Gram-positive isolates

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Accidental leakages during hydrocarbon fuels transportation and other activities are inevitable, making these hydrocarbons the most common global environmental pollutants. Current understanding in diesel degradation involves the study of Gram-negative microorganisms. Seven Gram-positive and one Gram-negative diesel degrading bacteria isolated from contaminated soil were used in this study. The overnight bacterial cultures were standardized and transferred into Bushnell-Haas medium supplemented with glucose and incubated at 30°C at 160 rpm for 48 h. The level of diesel degradation was determined using gravimetric analysis. Cell numbers were calculated using total heterotrophic plate count. All isolates were capable of degrading 70 - 80% of n-paraffin whilst isolates D2, D9, D10 and DJLB possessed better abilities of diesel degradation at 65.4 - 83.12% under the standard conditions. Diesel degradation rates and microbial cell number, increased with an increase in glucose composition. The addition of glucose to the liquid medium had a positive effect, with an increase in growth of the isolates thus leading to significantly ($p < 0.05$) higher percentages of diesel degradation and greater emulsification activity. The ability of Gram-positive bacteria to degrade diesel increased in a comparable trend as its biosurfactant production increased. The E_{24} index was highest at 87.6% for isolate D9. Isolates D2, D9 and D10, were identified as *Paenibacillus* sp. whilst isolate DJLB was found to belong to *Stenotrophomonas* sp. This study clearly demonstrates that Gram-positive biosurfactant producing bacteria are effective in diesel degradation.

Key words: Diesel, biodegradation, *Paenibacillus* sp., *Stenotrophomonas* sp., Gram-positive bacteria, biosurfactant.

INTRODUCTION

Hydrocarbons such as diesel fuel, crude oil and petroleum distillates are some of the world's most widely used primary energy and fuel resources, due to the energy they produce when combusted (Watanabe, 2001). Huge quantities of fuel are required to power industry, automobiles and heat homes and with the number of times each gallon of petroleum is stored, transported, or transferred, accidents and leakages are inevitable (Surridge, 2007), making these hydrocarbons the most common global environmental pollutants. This contamination are hazardous to the health of plants and are also carcinogenic, mutagenic and potent immuno-toxicants, posing a serious threat to human and animal health (Atlas, 1981; Zhou and Crawford, 1995; Liebeg and Cutright, 1999; Ting and HuTan, 1999; Vasudevan and Rajaram, 2001).

Hydrocarbons are considered to be of biological origin, since short and long chain hydrocarbons (alkanes: C_{10} - C_{20}; C_{20}-C_{40}) appear to be exclusively the origin of biological processes (Surridge, 2007). Petroleum and coal contain a class of molecules known as hopanoids, which are commonly found in bacterial cell walls (Gold, 1985). Gold (1985) indicated that these fuels at some point originated at least in part from microorganisms and that biodegradation of these fuels has always been occurring

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to some extent. The 'biological evidence' within these hydrocarbons could be the reason for the adaptation of microorganisms to degrade them so readily upon technological industrialisation of the earth and why biodegradation is such an applicable method for polluted soil reclamation (Surridge, 2007). Biodegradation of hydrocarbons by natural populations of microorganisms allows for the conversion of hazardous substances into forms that are less or non-toxic and represents one of the primary mechanisms by which petroleum and diesel products are removed from the environment inexpensively (Atlas, 1981; Floodgate, 1984; Leahy and Colwell, 1990; Lidderdale, 1993).

One of the most important characteristics of hydrocarbon-degrading bacteria is the ability of emulsifying hydrocarbons in solution by producing surface-active agents such as biosurfactants (Hommel, 1990; Neu, 1996; Desai and Banat, 1997; Bredholt et al., 1998; Das et al., 1998). Biosurfactants are directly involved in the process of hydrocarbon removal from the environment through increased bioavailability and subsequent biodegradation of the hydrocarbons by direct cell contact (Hommel, 1990; Leahy and Colwell, 1990; Deleu et al., 1999; Banat et al., 2000).

Bacterial communities from several petroleum-contaminated sites have been effectively characterized by culture-independent molecular techniques using 16S rRNA gene sequences (Giovannoni et al., 1988; Hugenholtz et al., 1998; Amann et al., 1995; Clement et al., 1998; Dojka et al., 1998; Ficker et al., 1999; Alfreider et al., 2002; Ayala-del-Río et al., 2004; Philip et al., 2005; Philip and Atlas, 2005). Gram-negative bacteria (*Acinetobacter , Pseudomonas, Alkanivorax* and related genera) are often dominant in microcosms after oil spill simulations (Cho et al., 1997; MacNaughton et al., 1999; Margesin et al., 2003; Kasai et al., 2005; Brakstad and Bonaunet, 2006) and in hydrocarbon contaminated environments after biostimulation (Kaplan and Kitts, 2004; Röling et al., 2004). These groups of bacteria are associated with a fast petroleum-degradation phase and their large quantity is positively correlated to total petroleum hydrocarbons (TPH) (Kaplan and Kitts, 2004; Röling et al., 2004; Yakimov et al., 2005).

According to Kaplan and Kitts (2004), Gram-positive bacteria, when detected in environment, are never prevailing. The role of Gram-positive bacteria is largely unknown and it can be hypothesized that they belong to minor communities having a role in the degradation of more specific hydrocarbon classes. Very few Gram-positive bacteria, mainly *Rhodococci,* have been described as having the capability of degrading alkanes. Gram-positive bacteria have been shown as a great potential for the biotransformation and biodegradation of organic compounds (Larkin et al., 2005). Gram-positive bacteria particularly *Bacillus* sp. has been attracting interest in both environmental bioremediation strategies and biotechnological applications. This study therefore aimed to demonstrate the diesel degradation ability of Gram-positive bacterial isolates which coincided with biosurfactant production.

METHODS AND MATERIALS

The diesel fuel used in this experiment was purchased from a local garage and stored in the dark at ambient temperature (± 20°C) throughout the study. Prior to use the diesel and n-paraffin were sterilized using 0.2 µm membrane filter (Merck). Diesel-contaminated soil was collected from a transport company in Durban, South Africa.

Isolation and identification of bacterial diesel degraders

Bushnell Haas (BH) liquid medium (Bushnell and Haas, 1941; Atlas and Bartha, 1992) was used as the enrichment medium with 1% (v/v) diesel as the sole carbon source to isolate diesel degrading bacteria. 1 g of the diesel contaminated soil sample was added to 100 ml of the enrichment medium and incubated at 30°C at 160 rpm. After two weeks, 1 ml of enriched medium was transferred into freshly prepared enrichment media and incubated at the same conditions as described in previous studies (Singh and Lin, 2008; Mandri and Lin, 2007). Serial dilutions (1/10) from the third enrichment process were plated out into BH agar plates, which were covered with 100 µl of diesel oil and incubated at 30°C for approximately one week. The single colonies were streaked into nutrient agar plates, incubated at 30°C overnight and stored at 4°C until further use. For long-term preservation, the bacterial isolates were stored in 30% glycerol at -70°C.

Several basic morphological and biochemical tests (Balows et al., 1992) were performed including colony morphology, cell micromorphology and Gram-reaction to identify the bacterial diesel degraders. This was confirmed by API 50CHB and 16S rRNA sequencing (Marchesi et al., 1998), followed by NCBI Blast comparison software to reveal the identities of the isolates (Altschul et al., 1990). Thereafter the sequences were submitted to Bankit (GenBank) to obtain valid accession numbers.

Diesel/n-paraffin degradation assay

The bacterial isolates were tested for their ability to grow on diesel, using a liquid mineral medium, Bushnell-Haas (BH) medium (Bushnell and Haas, 1941). A single colony of the isolate was inoculated into 10 ml nutrient broth (Merck) and incubated at 30°C overnight at 160 rpm. The overnight culture was centrifuged for 10 min at 10000 rpm. The cell pellet was washed twice in phosphate buffer saline (pH 7.6) and re-suspended in BH medium until $OD_{600\ nm}$ was equivalent to 1. 1 ml of bacterial inoculum (1 $OD_{600\ nm}$ equivalent) was added to 250 ml Erlenmeyer flasks containing 100 ml of liquid BH medium supplemented with 1% carbon source (diesel or n-paraffin). Uninoculated control flasks with 1% (v/v) diesel were incubated in parallel to monitor abiotic losses of the substrate. The culture flasks were incubated at 30°C at 160 rpm for 20 days. The effect of glucose supplement in diesel degradation was studied by adding glucose (0.1 and 1%) in the above media. The growth patterns were obtained by measuring total viable counts (cfu/ml) of the isolates, which were enumerated by the spread plate technique using the nutrient agar plates at 30°C for 36 h. All experiments were performed in triplicate.

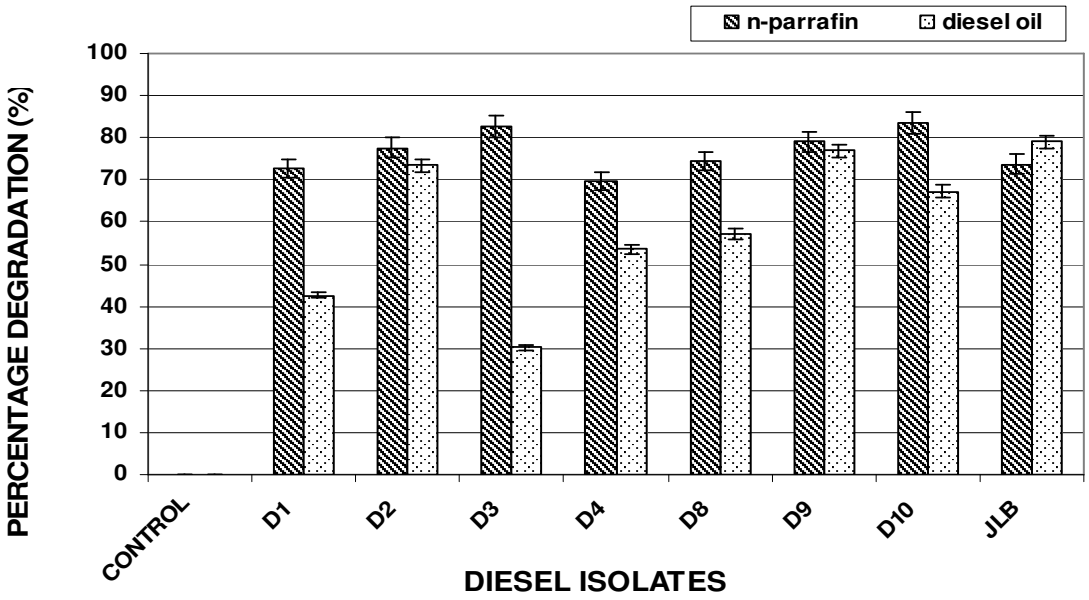

Figure 1. The percentage of n-paraffin and diesel degradation by the bacterial isolates after a 20-day incubation at 30°C, 160 rpm^{-1}.

Determination of diesel degradation

The level of diesel degradation was determined using the gravimetric analysis (Chang, 1998; Marquez-Rocha et al., 2001). In brief, 100 ml of media was transferred into a separation funnel and the aqueous phase containing bacterial cells was separated with the remaining diesel. The aqueous phase was used for the emulsification test. The remaining diesel was extracted using dichloromethane as described previously (Mandri and Lin, 2007). The percentage of diesel oil degraded was calculated as follows: % diesel oil degraded = (weight of diesel oil degraded/original weight of diesel introduced) x 100, where the weight of diesel oil degraded was determined as the weight of diesel oil plus flask minus the original weight of the flask.

Emulsification index (E_{24})

The emulsification index (E_{24}) of culture samples was determined by adding 2 ml of diesel to the same amount of culture media obtained above, mixing with a vortex for 2 min and leaving to stand for 24 h. The E_{24} index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (Desai and Banat, 1997).

Student t-tests were performed for statistical significance

All values were presented as the mean ± the standard deviation. Student t-tests were used to examine the statistical significance (SPSS version 13) between different diesel degradation with various glucose concentrations. Probability was set at 0.05.

RESULTS

A total of twelve hydrocarbon-utilizing microorganisms were isolated from the contaminated soil after the enrichment process. Seven isolates were discovered to be Gram-positive spore forming rods with the remaining five being Gram-negative rods. These seven Gram-positive isolates, including one Gram-negative isolate (DJLB) were tested for n-paraffin and diesel degradation abilities. Results from these assays (Figure 1) showed that all isolates have a high degradative potential of n-paraffin from 69 - 84%. Isolates DJLB , D9, D2 and D10 had a high percentage degradation of 79.1, 76.85, 73.5 and 67.31% for diesel respectively as compared to isolate D3 having least degradative ability at 30%. Four isolates namely (D2, D9, D10 and DJLB) were shown to possess good degradation ability of diesel and were selected to be studied further.

Additional degradation assays were performed in liquid media using glucose as a nutrient supplement. Figure 2 illustrates that an increase in glucose concentration in the media resulted in an increase in diesel degradation under the standard degradation assay conditions. The diesel degradation in the presence of 0.1% glucose was 76.02, 74.36, 65.32 and 80.22% for isolates D2, D9, D10 and DJLB respectively. The percentage of diesel degradation went up to 80, 82.7, 65.4 and 83.12% for isolates D2, D9, D10 and DJLB respectively with the presence of 1% glucose, as compared to 62, 61, 56 and 73.1% without glucose. These results indicate that the addition of increased glucose concentrations to the liquid medium increased the degradation rate of diesel substantially.

The diesel degradation level by various Gram-positive isolates was coincided with the amount of biosurfactant produced. Biosurfactant production of these Gram-

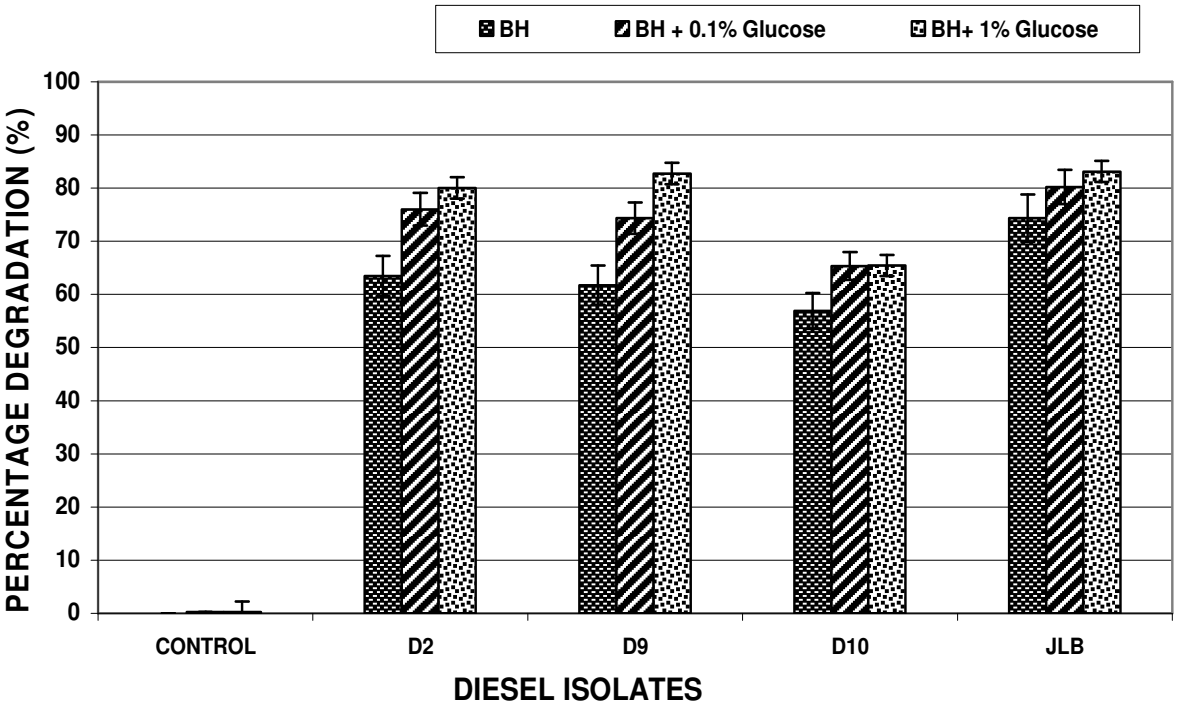

Figure 2. The percentage of diesel degradation of the bacterial isolates in BH medium with different glucose concentrations after a 20-day incubation.

Table 1. Emulsification evaluation of bacterial isolates (aqueous phase) after 3 days of growth in glucose and BH medium spiked with diesel oil.

Isolate	E_{24} (B/A)*100	
	Cell (%)	Cell-free (%)
D2	24	NE
D2*	76.8	13.6
D9	32	NE
D9*	87.6	32.8
D10	16	NE
D10*	74	28
JLB	24	NE
JLB*	79	22

NE = Not emulsified; * = 0.1% glucose added; B= height of emulsion; A= total height.

positive isolates, according to the emulsification capacity, was enhanced significantly ($P < 0.05$) when different concentrations of glucose enriched medium were used. The E_{24} emulsification index was highest at 76.8%, 79% and 87.6% in the cultures of isolates D2, DJLB and D9 respectively (Table 1), when glucose concentration of 0.1% was added to the degradation assay. This study furthermore demonstrated (data not shown), that as glucose concentrations increased (1%) the amount of biosurfactant production increased concomitantly, resulting in enhanced diesel degradation. There was a significant decrease in the emulsifying capacity when the bacterial cell was removed from the culture media.

The patterns of microbial growth differed from each isolate as shown in Figure 3. The diesel isolates were grown in BH media for approximately 36 - 48 h, after which half of the culture isolates were spiked with 1% diesel. Figure 3a illustrates that the addition of diesel to the degradation assays after day 3 increased the growth of the microorganisms considerably. The isolates thrived on the diesel substrate when supplied as the sole source of carbon and energy. The utilization of the diesel resulted in an increase in the number of colony forming units per ml with a concomitant visual gradual reduction in the diesel layer and substantial disappearance of the diesel with prolonged incubation. The addition of glucose (Figure 3b) to the liquid medium had a positive effect, with an increase in the growth of the isolates thus leading to significantly higher percentages of diesel degradation ($P < 0.05$) and greater emulsification activity.

The identities of the diesel degrading isolates after analysis of the 16S rRNA gene sequences and results of biochemical tests and the API tests were isolates belong to the genus *Paenibacillus* (accession number GQ 384459, GQ368737, GQ384458 for isolate D2, D9 and D10 respectively) while isolate DJLB was found to belong to *Stenotrophomonas* sp. (accession number GQ 368736).

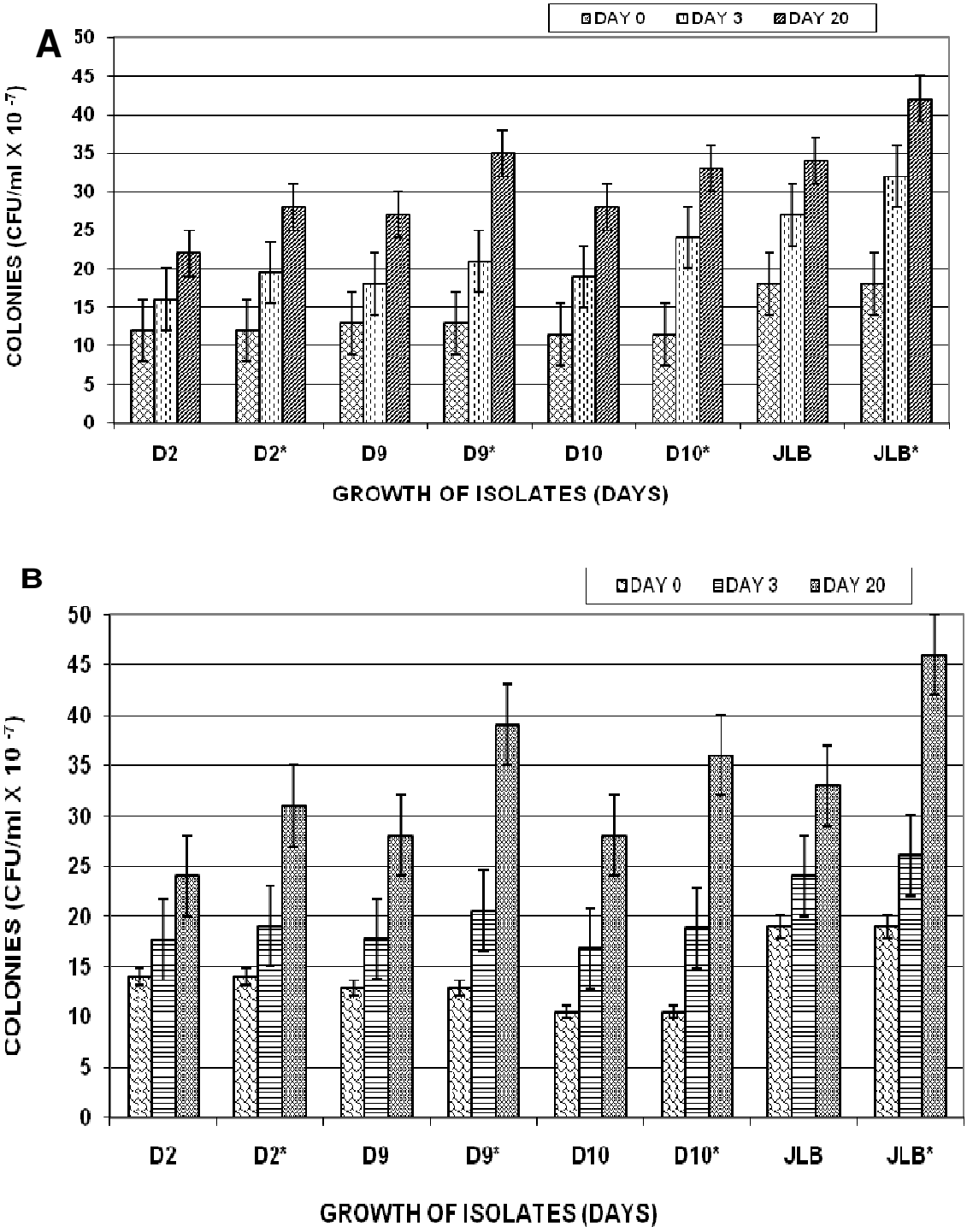

Figure 3. Growth of bacterial isolates in BH medium spiked with 1% diesel (*) over 20 days under diesel degradation assay conditions. A) No glucose added. B) Addition of 0.1% glucose.

DISCUSSION

Research performed in the early 20th century has provided us with an extensive knowledge on the microbiology of alkane degradation. Leahy and Cowell (1990) demonstrated that microorganisms are the main degraders of petroleum hydrocarbons in contaminated ecosystems. Enrichments to isolate oil-degraders using diesel as sole carbon and energy source provides various microorganisms from contaminated soil (Jirasripongpun,

2002). In this study, screening for diesel degrading bacteria from contaminated soil resulted in the recovery of 12 candidate isolates. Seven of these isolates, constituting more than 58% of the total isolates were Gram-positive bacteria capable of degrading n-paraffin and diesel in the enrichment culture. An increase in diesel degradation corresponded to an increase in cell number during the degradation processes demonstrating the isolates ability of utilizing diesel as the energy source. The dominance of Gram-positive bacteria in this study is not surprising, since these bacteria have a stronger cell envelope and are more tolerant to high levels of hydrocarbons due to their resistant endospores, than Gram-negative bacteria which allow them to thrive in the highly variable diesel contaminated environment (Zhuang et al., 2002). The use of pure cultures in this study, in addition, provides practical advantages by eliminating the ambiguity associated with constantly fluctuating microbial populations. There is growing evidence that isolates belonging to the *Bacillus* sp. are effective in clearing oil spills (Ghazali et al., 2004).

Microorganisms capable of surviving on these highly reduced organic compounds have been identified (Marchant et al., 2006). More recently, *Bacillus, Geobacillus* (phylum *Firmicutes*) and *Thermus* (phylum *Deinococcus-Thermus*) isolates were found to degrade alkanes (Marchant et al., 2006; Meintanis et al., 2006). The best diesel degrading Gram-positive microorganisms (D2, D9, D10) isolated in this study were identified as *Paenibacillus* sp. and one (DJLB) as *Stenotrophomonas* sp. *Bacillus pumilus* has also been isolated in our previous study's as one of the potential diesel degraders (Singh and Lin, 2008). Isolates found in this study are not known common hydrocarbon degraders. Recently, Obuekwe et al. (2009) identified *Bacillus* sp. and *Paenibacillus* sp. as two of the most prominent crude-oil degraders in the Kuwait desert environment. The dominant isolation of these spore-forming Gram-positive bacteria was predicated on their ability to survive the prevalent high soil temperature (40 - 50°C) and tailings from oil-recovery operations (e.g. water flooding). *Stenotrophomonas* sp. has not been reported in literature as having diesel degradation ability. However, *Stenotrophomonas* does possess phenanthrene degradation ability as reported by Vacca et al. (2005). Our results showed that both *Paenibacillus* sp. and *Stenotrophomnas* sp. are effective in degrading diesel up to 83% making them prominent hydrocarbon degraders.

In the present study, isolates D2, D9, D10 and DJLB proved to be better hydrocarbon degraders than the other isolates. This effect was consistent with increased cell growth. The addition of glucose caused a significant difference in the ability of the diesel degraders to break down diesel of up to 84%. The results of this study indicated that an increase in glucose concentration (0.1 and 1%) increased the diesel degradation by the isolates significantly and increased the microbial cell numbers after the addition of diesel (Figure 3).

Fava et al. (1995) and Loh and Wang (1998) demonstrated that minerals or supplementary carbon substrates increase the rate of biodegradation. The addition of nutrients stimulates the degradative capabilities of the indigenous microorganisms thus allowing the microorganisms to break down the organic pollutants at a faster rate (Ausma et al., 2002). Okerentugba and Ezeronye (2003) stated that microbiological communities exposed to hydrocarbons will adapt to this exposure through selective enrichment and genetic changes, resulting in an increase in hydrocarbon-degradation. This pre-exposure of microorganisms makes them better suited to degrade diesel through higher growth and reproduction and more efficient metabolism thus maximizing the rate of diesel removal from the culture media.

According to Rehm and Reiff (1981) and Finnerty (1992), a well-known property of Gram-positive bacteria, *Rhodococci*, is the capability of degrading alkanes, which is often accompanied, by the ability to produce biosurfactants. Our results support this observation. An approach for screening potential biosurfactant-producing microorganisms is the estimation of the emulsification index (E_{24}). The possession of cell-surface hydrophobicity and biosurfactant production by microorganisms creates chemical and physical compatibility between the organisms and the hydrophobic substrates, thus resulting in enhanced interaction between them (Stelmack et al., 1999). Many bacteria are known to degrade liquid hydrocarbons only after adherence to the substrate (Rosenberg and Rosenberg, 1981; Marcoux et al., 2000; MacLeod and Daugulis, 2005).

Isolates D2, D9, D10 and DJLB showed substantial biosurfactant production when stressed with diesel after being grown in glucose enriched medium. Emulsification activity was observed to be highest at 87.6% by isolate D9. This increased emulsification activity coincided with isolate D9's ability to utilize diesel more efficiently and initiate degradation of up to 83%. Published data on E_{24} in literature reported emulsification activity in the region of 65% (Menezes Bento et al., 2005). However, a *B. subtilis* strain demonstrated E_{24} values close to 90% in a study by Banat et al. (2000). Findings from the investigation by Obuekwe et al. (2009) have shown that the ability of Gram-positive bacteria especially *Bacillus* sp. and *Paenibacillus* sp. to degrade crude oil increased in a similar trend as their biosurfactant production increased. This relationship between biosurfactant production and crude oil-degrading ability was revealed to be highly significant ($P < 0.01$) (Obuekwe et al., 2009). Our study agrees with these findings, since diesel degradation increased significantly when biosurfactants were produced. In addition this study established that no considerable emulsification activity was attained with the cell-free extracts as compared

to cellular material, implying that the emulsifying activity by these isolates might be cell (membrane)-bound, not extracellular. Thus it can be suggested that *Paenibacillus* sp. and *Stenotrophomonas* sp. are now becoming promising microbial candidates for bio-surfactant production.

Hydrophobic surfaces increase the interaction between the microbial cells and the hydrocarbon substrates, thus resulting in increased diffusion rate, substrate utilization and growth in hydrocarbon in two phase systems (Rosenberg and Rosenberg, 1985; Stenstrom, 1989; van der Mei et al., 1993). The widespread occurrence of hydrophobic cell-surface character among members of hydrocarbon-degrading population of microorganisms will be a favourable feature for hydrocarbon pollution bioremediation activity in any environment (Obuekwe et al., 2009).

Microbial surface active agents (biosurfacants) are significant biotechnological products, with a wide variety of applications in many industries. Their properties of interest include: (i) changing surface active phenomena, such as lowering of surface and interfacial tensions, (ii) wetting and penetrating actions, (iii) spreading, (iv) hydrophylicity and hydrophobicity actions, (v) microbial growth enhancement, (vi) metal sequestration and (vii) antimicrobial action (Kosaric, 2001). Among these biosurfactants, glycolipids are the most important group as they show a high surface or interfacial activity as well as pH and temperature stability, low toxicity and good biodegradability (Rapp and Gabriel-Jürgens, 2003). Amid the main types of biosurfactants produced by microorganisms, surfactin is one of the most well recognized products with commercial application (Menezes Bento et al., 2005). Only *B. subtilis* and *B. pumilus* have been confirmed as surfactin producers (Banat et al., 2000).

Conclusion

This study clearly demonstrates that Gram-positive biosurfactant producing bacteria are effective in diesel degradation. The ability of Gram-positive bacteria to degrade diesel increased in a comparable trend as its biosurfactant production increased. Further studies are under way to scale up growth conditions and quantify biosurfactant production and to identify genes involved in diesel degradation by Gram-positive microorganisms. Ultimately, an understanding of the Gram-positive microbial community gene organization, structure and function could provide a mechanistic model for evaluating the effectiveness and sustainability of Gram-positive bacteria in the bioremediation of diesel-contaminated sites.

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