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Evaluation of *Alcaligenes faecalis* Degradation of Chrysene and Diesel Oil with Concomitant Production of Biosurfactant

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Abstract: *Alcaligenes faecalis* was evaluated for its potential to degrade varying concentrations of chrysene and diesel oil with concomitant biosurfactant production. Biodegradation was set up for 7 days utilizing the substrates as sole carbon and energy sources. Residual chrysene obtained after degradation of 30, 50 and 100 mg L^{-1}, respectively was 17.4 ± 1.5, 27.2 ± 1.2 and 28.7 ± 1.4 mg L^{-1} while total petroleum hydrocarbon remaining after degradation of 3, 5, 15 and 30% (v/v) diesel oil respectively was 2.58 ± 0.5, 3.09 ± 1.2, 21.65 ± 5.4 and $63.92\pm8.1\%$. Microbial cells of *A. faecalis* and sterilized cell-free extract from diesel oil media showed emulsifying activities against kerosene, diesel oil, engine oil, hexadecane, dodecane, xylene and hexane whereas no emulsifying activity was observed of microbial cells and sterilized cell-free extract from chrysene media. *Alcaligenes faecalis* cells harvested from diesel oil media also showed haemolytic activity unlike the microbial cells from chrysene media. Growth of the isolate in chrysene and diesel oil media induced secretion of protein and carbohydrate into the media which were statistically significantly ($p<0.05$) different compared to controls. This study portrays the potential of *Alcaligenes faecalis* to degrade and grow on chrysene and diesel oil and induce extracellular protein and carbohydrate with concomitant production of biosurfactant for industrial purposes and in hydrocarbon bioremediation.

Key words: Bacteria, bioremediation, pollution, petroleum products, polycyclic aromatic hydrocarbon

INTRODUCTION

Environmental pollution by crude or refined petroleum products is a global problem. Soil fertility problems arising from oil impact, leads to reduction and deformation of agricultural produce (Nwachukwu *et al.*, 2001). Domestic water supplies may become contaminated by run offs from agricultural soils polluted with crude oil. Petroleum hydrocarbon contamination is also of great concern due to the toxicity and recalcitrance of many fuel components and products such as polycyclic aromatic hydrocarbons (Saeed and Al-Mutairi, 2000; Xue and Warshawsky, 2005; Masih and Taneja, 2006). These thus warrant the need to remediate petroleum hydrocarbon polluted environment and formulate bioremediation protocols that would be safe and cost effective. Polycyclic Aromatic Hydrocarbons (PAHs) include the

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high and low molecular weight PAHss such as chrysene, pyrene, fluoranthene, benzanthracene, benzopyrene, phenanthrene, anthracene and fluorene (Kanaly and Harayama, 2000).

Bioremediation of petroleum and PAHs contaminated environment is a promising alternative remedial strategy (Gogoi *et al.*, 2003; Okoh and Trejo-Hernandez, 2006). Crude oil polluted agricultural soil has been found to be bioremediated by application of a soil bacterium and inorganic nutrient (Nwachukwu, 2001). Dean-Ross (2005) conducted slurry bioreactors bioremediation batch experiment on pristine sediment spiked with phenanthrene, anthracene, pyrene and fluoranthene and observed that bioaugmentation with *Rhodococcus* sp., resulted in enhanced bioremediation rates and yields.

One main factor that influences the extent of petroleum, aliphatic hydrocarbons and PAHs biodegradation is their bioavailability; this is a priority research objective in the bioremediation field (Makkar and Rockne, 2003). Thus, approaches to enhancing these compounds biodegradation often attempt to increase their apparent solubility by treatments such as addition of synthetic surfactants and/or release of biosurfactants (Barkay *et al.*, 1999; Makkar and Rockne, 2003; Al-Turki, 2009). Biosurfactants function to reduce surface tension and interfacial tensions between individual molecules at the surface and interface, respectively (Rahman *et al.*, 2002; Lu *et al.*, 2007). They also exhibit emulsification properties forming micelles whereby hydrocarbons become solubilized in the hydrophobic cores of the micelles thus making them available for microbial degradation (Bento *et al.*, 2005; Zhang *et al.*, 2005).

Many bacteria growing on alkanes produce biosurfactant to increase the bioavailability of these poorly available substrates (Sepahy *et al.*, 2004; Bento *et al.*, 2005). A similar strategy was suggested for PAH degrading bacteria (Déziel *et al.*, 1996). However, in other studies, no correlation was found between biosurfactant productions and PAH mineralization or dissolution rates (Volkering *et al.*, 1992; Willumsen and Karlson, 1997).

It has therefore become imperative to study the mechanisms of bioavailability of hydrocarbon pollutants to individual microorganisms. In addition, for petroleum bioremediation to be a viable alternative to non-biological remediation techniques, the search for microorganisms with degradation capabilities for petroleum products and components which are recalcitrant and poorly soluble would be a continual process. In this study, we report the potential of *A. faecalis* degradation of varying concentrations of chrysene and diesel oil with concomitant production of biosurfactant.

MATERIALS AND METHODS

This study was carried out from June 2006 to 2007 in Biochemistry Department, College of Medicine, University of Lagos, Nigeria.

Bacterial Culture
The bacterium *A. faecalis* was previously isolated in our Laboratory by chrysene enrichment from Nigerian PAHs polluted soil sites (Igwo-Ezikpe *et al.*, 2006). Chrysene enrichment is an isolation technique whereby chrysene was used as the sole carbon and energy source in Mineral Salt Medium (MSM) to isolate chrysene degraders. The isolate was maintained on nutrient agar slants and stored at 4°C when not in use.

Chemicals and Media
All chemicals were of analytical grade. Mineral Salt Media (MSM) components were supplied by (Sigma, Germany). Hydrocarbons used in this study include chrysene (Sigma,

Germany, 98% purity), ethyl acetate, dodecane, hexane, xylene and hexadecane (BDH, England). Diesel oil, kerosene and engine oil were obtained from African Petroleum (Nigeria). Nutrient agar for bacterial enumeration was purchased from Sigma (Germany) and blood agar plates for haemolytic assay were prepared using bacteriological agar (Oxoid, Germany) in our laboratory.

Preparation of Starter Culture

The MSM composed per liter (pH 7.2): NH_4NO_3, 4.0 g; Na_2HPO_4, 2.0 g; KH_2PO_4, 0.53 g; K_2SO_4, 0.17 g, $MgSO_4.7H_2O$, 0.10 g and trace elements solution (1 ml L^{-1}) as described by Ilori and Amund (2000). The MSM was sterilized by autoclaving at 121°C for 20 min. The isolate being a chrysene degrader was precultured in 3% diesel oil prior to use to ensure adaptation to diesel oil. This was achieved by growing bacterial cells in sterilized 50 mL MSM containing 3% diesel oil for 7 days at 30±0.2°C. The cells were thereafter cold harvested from the medium by centrifugation at 4,000x g, 4°C for 20 min and cultured on nutrient agar plate 48 h. Cell were harvested from surface of nutrient agar, suspended in 10 mL phosphate buffer (50 mM, pH 7.2) in a test tube, centrifuged at 10,000x g, 4°C for 10 min. The supernatant discarded while pelleted cells were resuspended in the same buffer and washed twice. The pelleted cells were finally suspended in phosphate buffer, optical density (OD_{600nm}) adjusted to 0.4.

Biodegradation Studies

The bacterial isolate was evaluated for the ability to degrade varying concentrations of chrysene (30, 50 and 100 mg L^{-1}) and diesel oil (3, 5, 15 and 30% v/v) as sole carbon and energy source. The various concentrations of chrysene were dissolved in Erlenmeyer flasks (250 mL) containing 5 mL ethyl acetate and evaporated, 20 mL MSM added and foiled to prevent photolysis. Media were sterilized by autoclaving at 121°C for 20 min. Starter cultures (10 mL) were thereafter inoculated into the media and incubated aerobically at 30±0.2°C and agitated at 150 rpm. This set up was designated Experimental (E). Two controls (C1 and C2) were also included; C1 consisted of the same materials present in E but without the carbon source while C2 contained all the materials in E with no test isolate inoculated.

Biodegradation was assayed by determining the residual chrysene or Total Petroleum Hydrocarbon (TPH) remaining and growth profile of the isolate. Residual chrysene was estimated after extracting twice with equal volume of ethyl acetate and analyzed using High Performance Liquid Chromatography (HPLC). The HPLC analyses were performed with VYDAC RP C18 reverse phase column (250×0.4 mm). Separation was achieved by gradient elution in acetonitrile: water (60, 50, 40, 30, 20, 10 and 0% water), temperature 25°C, with a flow rate of 0.8 mL min^{-1} and UV absorbance detector set at 254 nm. Estimation of diesel oil degradation was only at the end of 7 days. Diesel oil was extracted from media by mixing with equal volume of n-hexane and filtered through glasswool in a funnel. The extraction procedure was repeated three times and the extracts were pooled. The extracts were analyzed using a gas chromatography-mass spectrometry (Model: Agilent Tec 7890A, USA) and HP5-MS column (30 m×0.25 µm×0.25 µm) using helium as the carrier gas. The temperature was programmed to vary linearly from 40 to 270°C at the rate of 10°C min^{-1} and maintained for 20 min. Total Petroleum Hydrocarbon (TPH) was measured as the sum of all the peak areas on the gas chromatogram to obtain quantitative data. The degree of degradation (%) was calculated as described by Lee *et al*. (2005). The area of TPH from the experimental media was divided by the area of TPH from control C2 and multiplying by 100. Growth profile of the isolate was determined by measuring the Total Viable Counts (TVC) and population density

of the isolates. The TVC was enumerated by spread-plate technique whereby the inoculum from various media was cultured on nutrient agar for 48 h and microbial cells counted. Optical density of the media was measured spectrophometerically (OD_{600nm}) to determine the population density of the inoculum.

Emulsifying Activity Measurement

The various experimental media and controls were made cell free after 7 days of incubation by centrifugation at 10,000x g, 4°C for 10 min and heat sterilized. Cell-free extracts (2 mL) were vortex with n-hexadecane (2 mL) in a test tube and left undisturbed for 24 h. The emulsification index (E24) was calculated as the percentage of the height of emulsified layer (cm) divided by total height of liquid column (cm) (Ilori and Amund, 2001; Moraes *et al.*, 2002). Emulsifying activity was also measured against different hydrophobic sources (kerosene, diesel oil, engine oil, hexadecane, dodecane, xylene and hexane). This activity measurement was repeated using the microbial cells.

Haemolytic Activity

This was undertaken to confirm the production of biosurfactant by *A. faecalis*. The bacterial cells from MSM chrysene and diesel oil growth media were plated onto blood agar and incubated at 37°C for 48 h (Bicca *et al.*, 1999).

Determination of Total Protein and Carbohydrate Content of Cell-Free Extracts

The total protein content of the cell-free extracts was assayed by the Bradford method as described by Sepahy *et al.* (2004) using bovine serum albumin as standard. The reaction mixture contained 0.1 mL of cell-free extract to which 5.0 mL of coomassie blue dye reagent is added, incubated for 5 min and absorbance read at 595 nm. The total carbohydrate contents of cell-free extracts were estimated by anthrone method using glucose as standard (Jayaraman, 1981). A volume of 0.5 mL of cell-free extract was mixed with 2.0 mL of anthrone solution (2% anthrone reagent in conc H_2SO_4), kept in boiling water bath for 10 min. Cooled to room temperature and absorbance read at 620 nm against reagent blank. The total protein and carbohydrate content in test samples and controls were extrapolated from standard calibration curves.

Data Analysis

The experiments were set up in duplicate and analysis carried out in triplicates of each set up. Results are expressed as Mean±SEM (Standard Error of Mean). Statistically significant difference ($p < 0.05$) was determined using analysis of variance (ANOVA). All data were analyzed using Statistical Package for the Social Science15.0 for windows (SPSS 15.0).

RESULTS AND DISCUSSION

Alcaligenes faecalis used in this study is a Gram negative rod previously identified as chrysene degrading bacterium with potential to degrade petrochemical products such as diesel oil, engine oil, crude oil and kerosene of Nigerian origin (Igwo-Ezikpe *et al.*, 2006). The isolate was able to degrade varying concentrations of chrysene and diesel oil utilizing them as sole carbon and energy source. Residual chrysene obtained after 7 days degradation of 30, 50 and 100 mg L^{-1}, respectively was 17.4±1.5, 27.2±1.2 and 28.7±1.4 mg L^{-1} while THC remaining after 7 days degradation of 3, 5, 15 and 30% (v/v) diesel oil respectively were 2.58±0.5, 3.09±1.2, 21.65±5.4 and 63.92±8.1% (Fig. 1). No significant ($p < 0.05$) loss of the
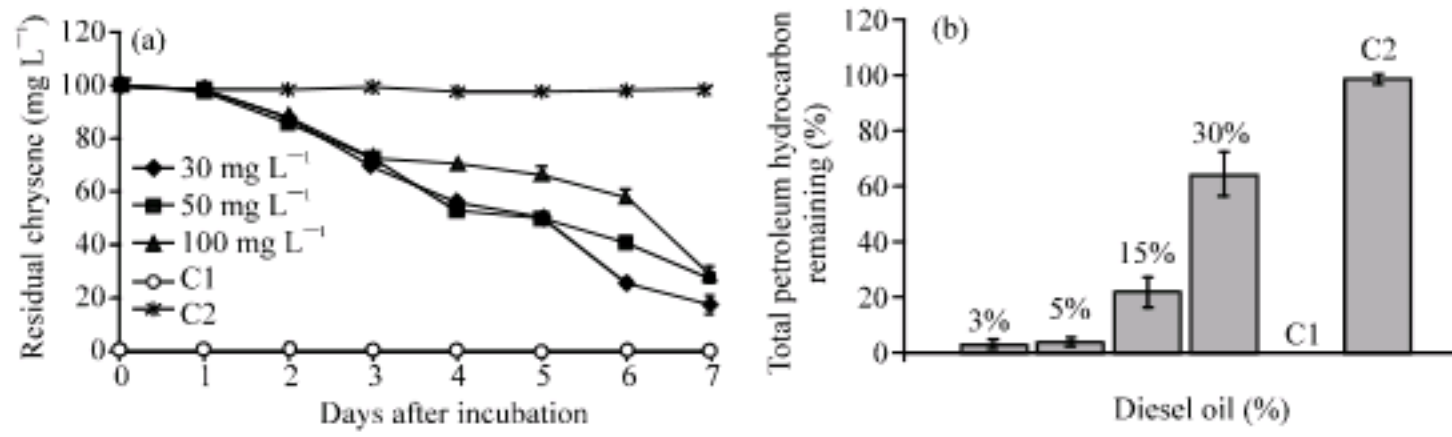
Fig. 1: (a) Residual chrysene and (b) total petroleum hydrocarbon remaining after *A. faecalis* degradation of varying concentration of chrysene and diesel oil
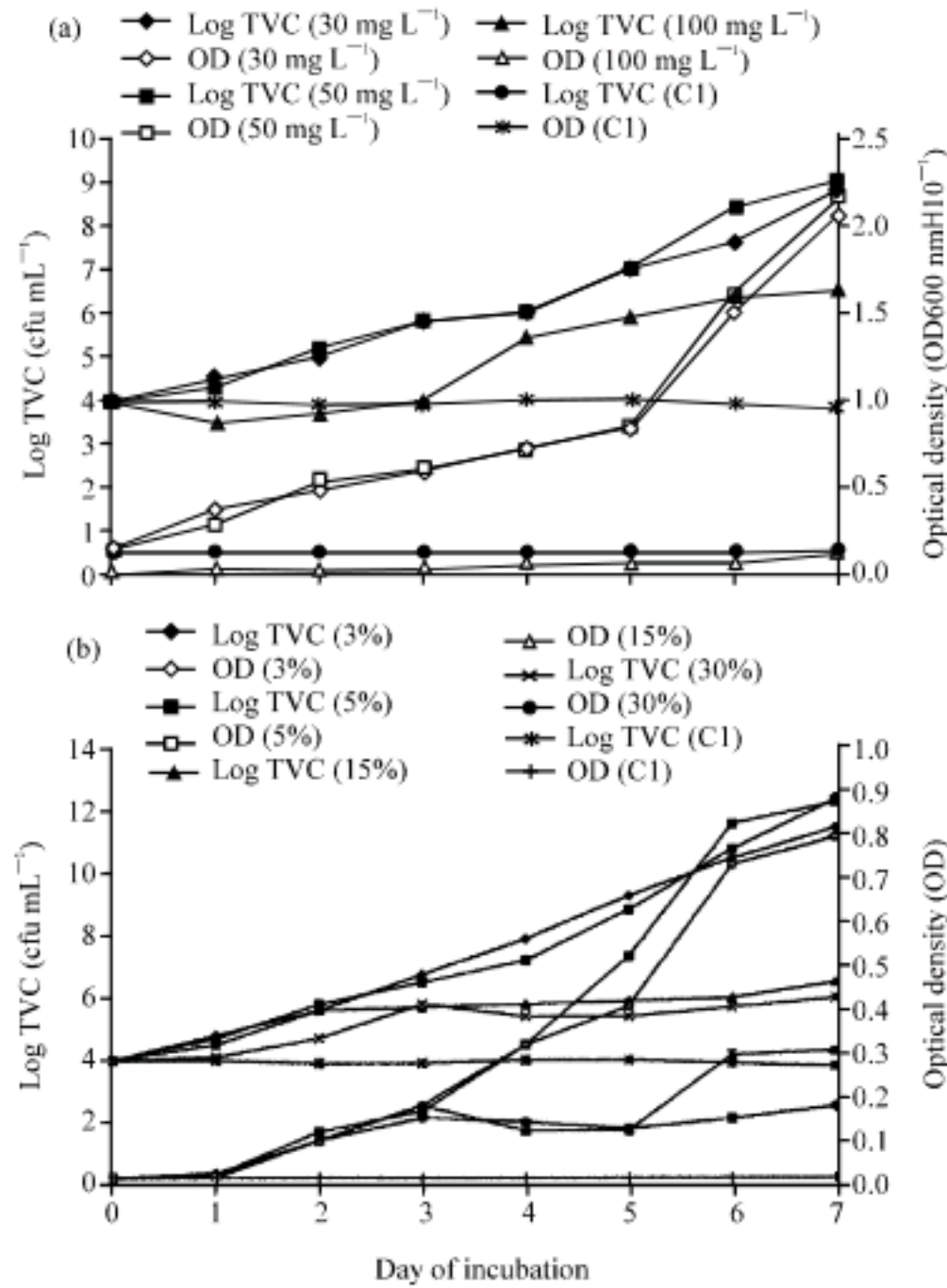

Fig. 2: Growth profile of *A. faecalis* during degradation of varying concentration (a) chrysene and (b) diesel oil

carbon sources was obtained in C2 controls indicating that the isolate mediated the degradation process. This corroborates earlier findings (Nwachukwu *et al*., 2001; Lee *et al*., 2005).

Growth profile of the isolate on the various tested concentrations of chrysene and diesel oil was found to be concentration dependent (Fig. 2). *Alcaligenes faecalis* grown on 30, 50 and 100 mg L^{-1} chrysene yielded highest cell densities of 6.3×10^8, 9.8×10^8 and
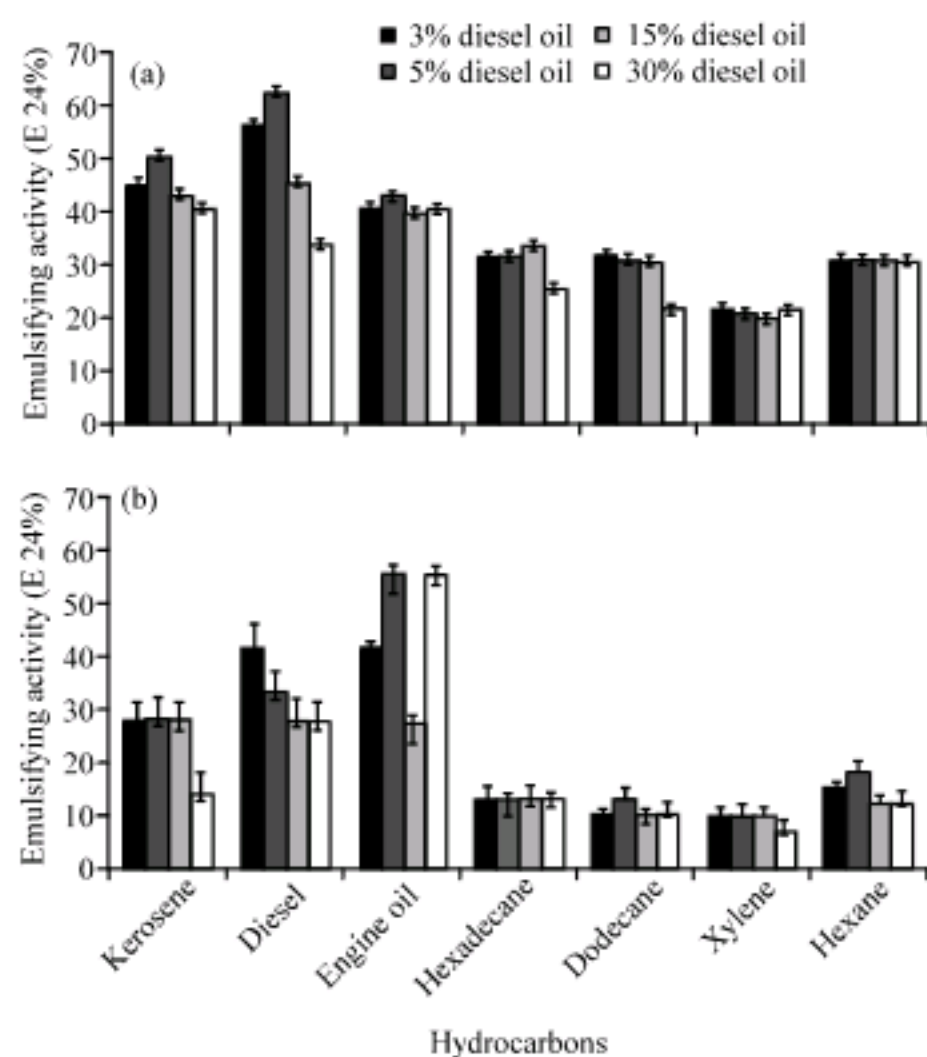
Fig. 3: (a) Emulsifying activity of microbial cells and (b) cell-free extract of *A. faecalis* grown on diesel oil

9.2×10^5 cfu mL^{-1}, respectively after 7 days of incubation. The organism was able to adapt and grew on the various concentrations of chrysene, multiplying in cell density and increasing the turbidity of the media compared to non-chrysene growth media. However, bacterial growth was best supported by 50 mg L^{-1} chrysene under the studied conditions. Previous studies have shown microbial growth and degradation of different concentrations of PAHs ranging from 5 to 100 mg L^{-1} (Yu *et al.*, 2005; Igwo-Ezikpe *et al.*, 2007). Highest cell densities of 3.2×10^{11}, 3.2×10^{14}, 2.5×10^{12} and 9.9×10^5 cfu mL^{-1} were, respectively observed for 3, 5, 15 and 30% (v/v) diesel oil media. The isolate utilized various diesel oil as the sole carbon and energy but as the concentration of diesel oil increased, microbial cell generation decreased. However, 5% diesel oil best supported cell mass generation. Possible explanation for increasing concentration of hydrocarbon-related inhibition of microbial growth include decreased bioavailability of micelle-solubilized hydrocarbon to support microbial growth or hydrocarbon interference with microbial cell integrity (Sikkema *et al.*, 1995). This may explain why crude oil and its petrochemical products cause sterility of agricultural soils and persist in the environment. The observed growth and degradation potential of the isolate on various concentrations of chrysene and diesel oil may be as a result of its previous exposure to the pollutants.

Microbial cells and cell-free extracts of the various diesel oil growth media showed emulsifying activity against various hydrocarbons such as kerosene, diesel oil, engine oil, hexadecane, dodecane and xylene (Fig. 3). This may indicate the production of biosurfactant by the isolate when grown on diesel oil. Earlier study has shown the emulsification of crude oil and other hydrocarbons by biosurfactant producing bacteria (Ilori and Amund, 2001; Rahman *et al.*, 2002; Pornsunthorntawee *et al.*, 2008). The emulsifying activities were found to be dependent on the test hydrocarbons and the source of the cell-free extract. Microbial

cells and cell-free extracts from 3 and 5% diesel oil showed better emulsifying activities than those from 15 and 30% suggesting increased constrain of microbial assess and poor solubilization of 15 and 30% diesel oil. Emulsifying activities of microbial cells from the various diesel oil media were higher than that of cell-free extracts. This may indicate that bioemulsifier of *A. faecalis* synthesized using diesel oil as sole carbon source may be cell-bound and only partially released into the liquid phase of the media. This corroborates the finding of previous research where biosurfactant activity was mediated by microbial cell mediated and/or released into growth media (Gutnick *et al.*, 2003; Bento *et al.*, 2005).

In contrast, the microbial cells and cell-free extracts from chrysene media did not show emulsifying activities against the various hydrocarbons tested. This indicated that the organism may not have produced bioemulsifier using chrysene as the sole carbon and energy source under the specified conditions of growth. This is in accordance with earlier study which reported that production of biosurfactants is largely dependent on the carbon source (Maneerat, 2005; Abouseouda *et al.*, 2008).

Haemolysis is one of the properties of biosurfactants (Tabatabaee *et al.*, 2005). Zones of clearance on blood agar plates as a result of haemolysis have been used as a rapid method for screening microorganisms for potential biosurfactant production (Lin, 1996). Cleared zones on blood agar plates were observed by microbial cells cultivated from diesel oil media (Fig. 4). This may further confirm biosurfactant production of the isolate when grown on diesel oil. However, haemolytic activity was also not observed of bacterial cells and cell-free extracts from chrysene growth media (Fig. 4).

Furthermore, growth of *A. faecalis* in the various chrysene and diesel oil growth media induced the secretion of extracellular protein and carbohydrate (Fig. 5, 6). There was statistical significant difference ($p < 0.05$) in the total protein and carbohydrate content of chrysene and diesel oil growth media compared to controls non-carbon media extracts. Results showed that an increase in chrysene and diesel oil concentrations induced relative increase in secretion of extracellular carbohydrate into the media. This may be as a result of increased toxicity of the hydrocarbons on microbial cell membrane leading to leaching of cell constituents and/or isolates response to hydrophobic environment by synthesis and release of biosurfactant components. Since, there was no carbohydrate in the media, its appearance and increase in response to hydrocarbon concentration may be due to the nature of the biosurfactant released by *A. faecalis*, this corroborates previous research (Bicca *et al.*, 1999). Previous studies have also shown hydrocarbon-degrading bacteria to produce extracellular emulsifying agents, generally consisting polysaccharides associated with proteins. Some examples included emulsan, the exopolysaccharide from *Acinetobacter*, alasan produce by

A. *faecalis* grown on diesel oil

A. *faecalis* grown on chrysne

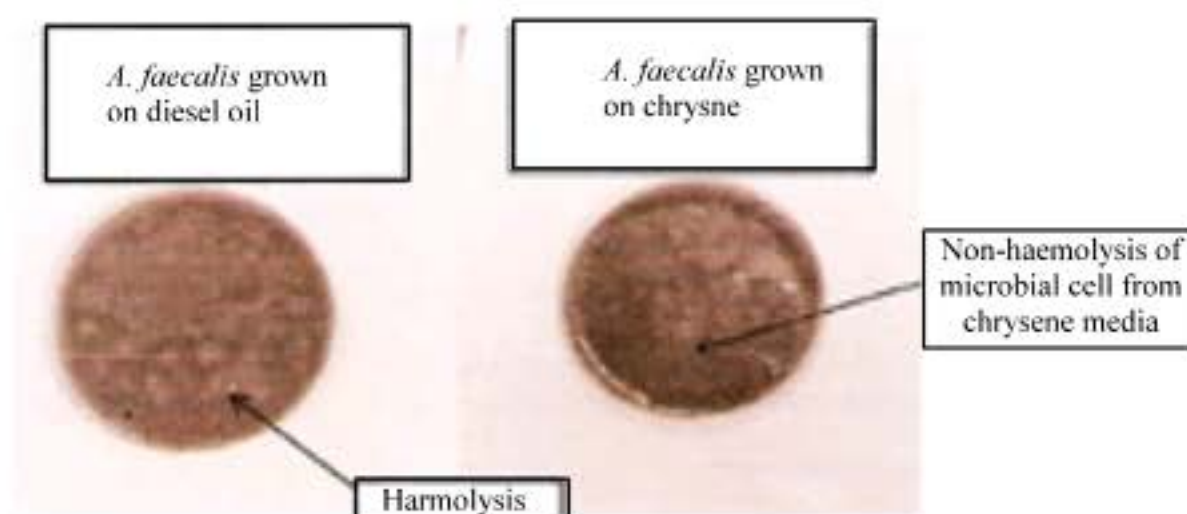Non-haemolysis of microbial cell from chrysene media

Harmolysis

Fig. 4: *Alcaligenes faecalis* harvested from chrysene and diesel oil media growth on blood agar plate
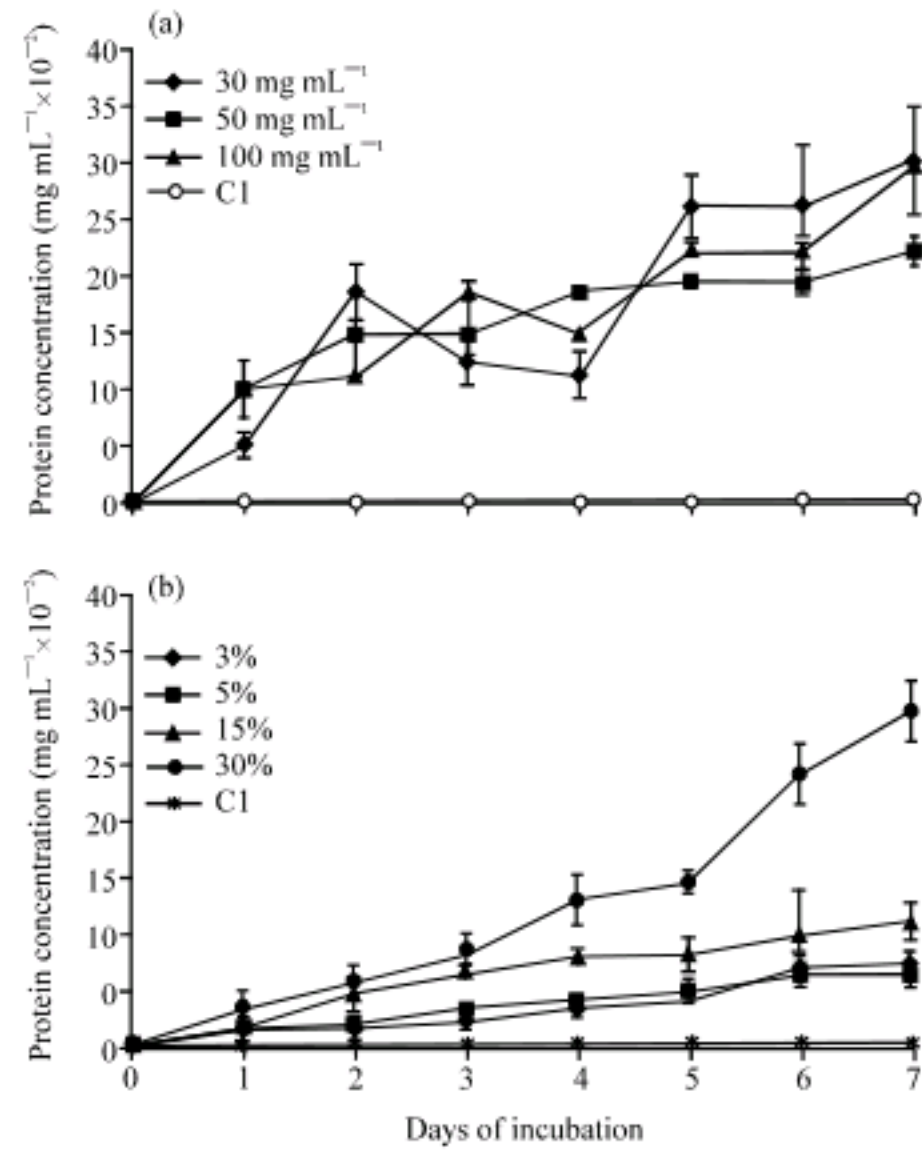
Fig. 5: Total protein of cell-free extract from (a) chrysene and (b) diesel oil media
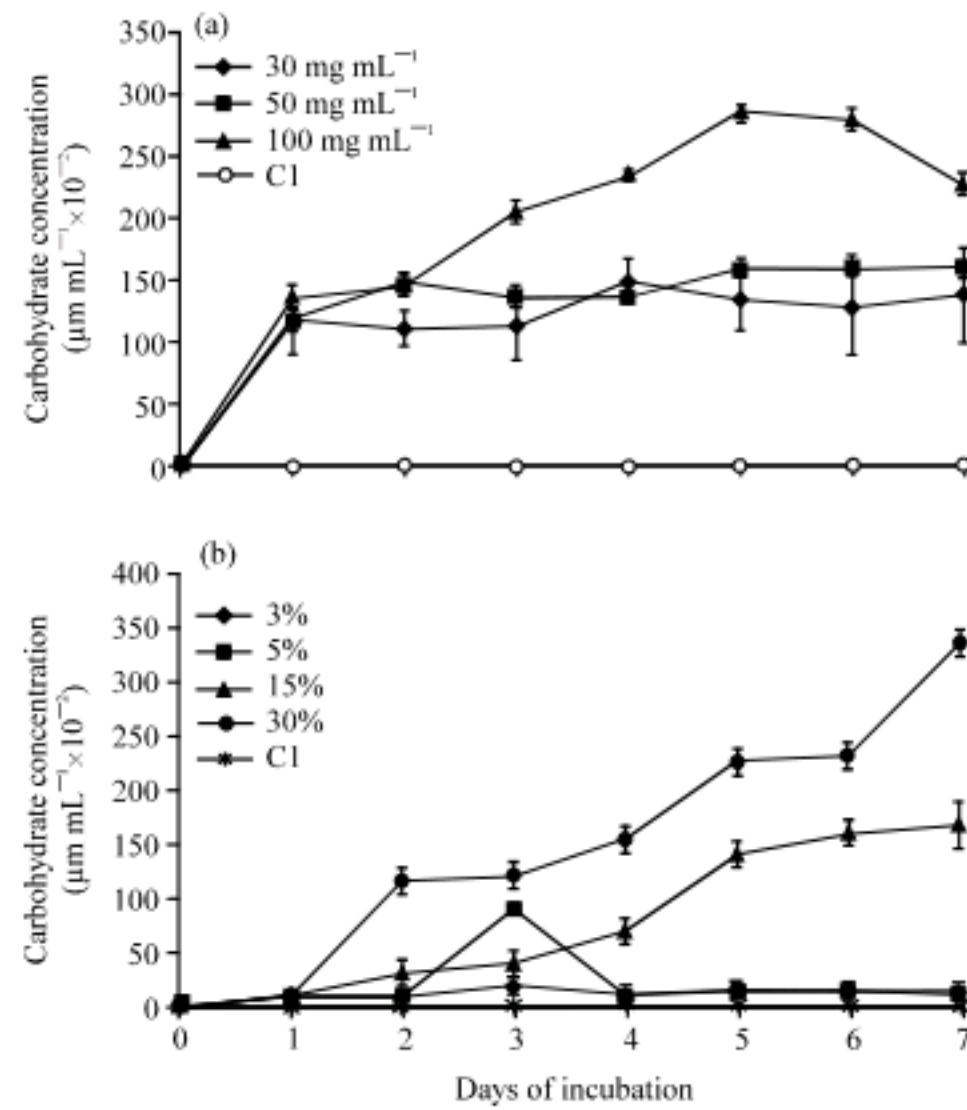

Fig. 6: Total carbohydrate of cell-free extract from (a) chrysene and (b) diesel oil media

Acinetobacter radioresistens KA53, rhamnolipid and glycolipid from *Pseudomonas* sp. (Toren *et al.*, 2001; Abdel-El-Haleem, 2003; Gutnick *et al.*, 2003). The combination of hydrophilic sugar main chain repeat units and the hydrophobic side groups of biosurfactants lead to their amphipathic behavior and therefore, their ability to form stable oil-in-water emulsions and reduce surface tension. In addition, the biosynthesis and excretion of biosurfactants into medium are considered to be cell mechanisms aimed at an adaptation of microorganism to using external lipophilic compounds as carbon and energy sources (Barkay *et al.*, 1999; Maneerat, 2005). As such, the response of the isolate in terms of possible biosurfactant production may be as a result of external factor on the microbial genetic make-up leading to the observed expression of macromolecules.

CONCLUSION

This study shows the potential of *A. faecalis* to adapt and degrade varying concentrations of chrysene and diesel oil, produce biosurfactant using diesel oil as carbon source and possible use in hydrocarbon bioremediation studies. Non-emulsification of the microbial cells and cell-free extracts from chrysene media may not totally rule out its biosurfactant production under this condition. Therefore, other parameters such as surface tension and adhesion may be investigated. We suggest that chrysene and diesel oil when used as sole carbon and energy source induced secretion of extracellular protein and carbohydrate into the growth medium.

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