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ORIGINAL PAPER

Degradation of hydrocarbons and biosurfactant production by *Pseudomonas* sp. strain LP1

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Abstract *Pseudomonas* sp. strain LP1, an organism isolated on the basis of its ability to grow on pyrene, was assayed for its degradative and biosurfactant production potentials when growing on crude, diesel and engine oils. The isolate exhibited specific growth rate and doubling time of 0.304 days^{-1} and 2.28 days, respectively on crude oil (Escravos Light). The corresponding values on diesel were 0.233 days^{-1} and 2.97 days, while on engine oil, were 0.122 days^{-1} and 5.71 days. The organism did not show significant biosurfactant production towards crude oil and diesel, but readily produced biosurfactant on engine oil. The highest Emulsification index (E_{24}) value for the biosurfactant produced by LP1 on engine oil was 80.33 ± 1.20, on day 8 of incubation. Biosurfactant production was growth-associated. The surface-active compound which exhibited zero saline tolerance had its optimal activity at 50°C and pH 2.0.

Keywords Biodegradation · Biosurfactants · Engine oil · Hydrocarbons · *Pseudomonas*

Introduction

Petroleum exploitation, exploration, transportation, consumption, attendant spills and disposal often lead to release of hydrocarbon (HC) pollutants into the environment with serious ecological problems (Okonkwo 1984; Rhodes and Hendricks 1990; Oluwole et al. 2005; Okoh 2006). Petroleum pollutants are not only toxic to biological components of the environment, some are indeed carcinogenic. Mechanical and chemical methods to reduce hydrocarbon pollution are often expensive, time consuming and not environment friendly (Office of Technological Assesment 1990; Mandri and Lin 2007). Thus bioremediation remains the method of choice for total removal of HC pollutants in the environment.

It is usually difficult to get isolates with degradative abilities for all the components of petroleum. Total degradation of oil component often results from the activities of consortium consisting of mixture of organisms with degradative potentials for the diverse fractions of which the oil is composed (Lal and Khanna 1996; Marin et al. 1996a; Ko and Lebeault 1999). Individual organisms are able to metabolise a limited range of hydrocarbon substrates (Marin et al. 1996a). Most of the bacteria frequently isolated from hydrocarbon-polluted sites belong to the genera *Pseudomonas, Sphingomonas, Acinetobacter, Alcaligenes, Micrococcus, Bacillus, Flavobacterium, Arthrobacter, Alcanivorax Mycobacterium, Rhodococcus* and *Actinobacter* (Atlas 1992; Okoh and Trejo-Hernandez 2006).

Detailed studies of organisms with pyrene degradative ability as well as light aromatic and n-alkane components of petroleum have not been well-reported in the literature as focus has been on mono-substrates and co-substrates (exploring the cometabolism of polycyclic aromatic hydrocarbons [PAHs]). Kastner et al. (1994) and Churchill et al. (1999) limited their studies to substrate specificities on a number of hydrocarbons including pure alkanes and aromatics. However, Ilori (1998) reported in detail the isolation and characterisation of anthracene degraders with remarkable potentials on crude oil and hexadecane from a crude oil polluted site.

The low solubility and high hydrophobicity of many hydrocarbon compounds make them highly unavailable to

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microorganisms. Release of biosurfactants is one of the strategies used by microorganisms to influence the uptake of PAHs and hydrophobic compounds in general (Marin et al. 1996b; Johnsen et al. 2005). Many hydrocarbon utilising bacteria and fungi possess emulsifying activities, due to whole cell or to extracellular surface active compounds (Ilori and Amund 2001). Microorganisms synthesise a wide variety of high and low molecular mass bio-emulsifiers (Rosenberg and Ron 1997). The commonest genera include *Corynebacterium*, *Rhodococcus*, *Pseudomonas*, *Serratia* and *Bacillus*. Conflicting results have been reported concerning the effect of surfactants/emulsifiers on biodegradation of hydrocarbons (Foght and Westlake 1988; Zhang and Miller 1992; Rosenberg and Ron 1997; Johnsen et al. 2005). A study of biosurfactant production properties of hydrocarbon degraders is crucial to understanding the physiology of the organisms and maximizing their industrial and commercial potentials. The isolation, characterisation and degradative potentials of *Pseudomonas* sp. LP1 on pyrene has been reported in a previous paper (Obayori et al. 2008). In this study, we investigated the biodegradative and biosurfactant production properties of this organism on crude oil and petroleum cuts known to be common contaminants in the Nigerian environment.

Materials and methods

Microorganism and inoculum development

The organism used in this study was maintained in glycerol: nutrient broth (1:1) at −20°C. Colonies growing on Luria Bertani agar with very low percentage of PAH (0.005%) were harvested with sterile inoculating loop, pooled and transferred to screw-capped bottles containing 5 ml of physiological saline (0.9% NaCl). Enough cells were transferred to achieve an OD_{600} of approximately 1.5.

Medium and culture conditions

The mineral salt medium (MSM) described by Kastner et al. (1994) was used. The pH of the medium was adjusted to 7.2 and further fortified with trace elements solution (1 ml/l) previously described by Bauchop and Elsden (1960). The trace elements solution was sterilised separately and added aseptically to the medium. Unless otherwise stated, all incubations were performed at room temperature (29 ± 2.0°C).

Growth of isolates on hydrocarbons

Replicate flasks containing 50 ml of MSM with 0.5 ml of crude oil, diesel or engine oil were prepared. The flasks were inoculated to achieve an initial concentration of the total viable count (TVC) between 2.0×10^6 and 2.0 x 10^7 cfu/ml and incubated for a period of 21 days. Flasks inoculated with heat-inactivated cells served as controls Total viable counts were determined at 3 days interval by plating out appropriate dilutions of the cultures onto nutrient agar. Residual oil concentration and pH were also determined at the same interval. Mean generation times and specific growth rates were calculated using nonlinear regression of growth curves for the period when growth rates were maximal. Regression and variance analyses were performed using Prism version 5.0 (Graphpad software, San Diego, CA, USA).

Extraction of residual oil

Residual oil was extracted by adding 20 ml of hexane to broth culture in flask and shaking thoroughly as recently described by Adebusoye et al. (2007). After removing the aqueous phase with separating funnel, the residual oil concentration was determined by gas chromatography. Control flasks were also extracted similarly. Results were expressed as percentages of respective controls.

Analytical method

Hexane extracts (1.0 μl) were analyzed with Hewlett Packard 5890 Series II gas chromatograph equipped with flame ionization detector (FID) and 30 m long HP-5 column (internal diameter, 0.25 mm; film thickness, 0.25 μm). The carrier gas was nitrogen. The injector and detector temperatures were maintained at 250°C and 350°C, respectively. The column was programmed at an initial temperature of 70°C; this was held for 2 min, then ramped at 10°C/min to 320°C and held for 10 min.

Production and partial purification of biosurfactant

Mineral medium (350 ml) for biosurfactant production contained 1% liquid hydrocarbon in an Erlenmeyer flask (1,000 ml). Bacterial growth was initiated by introducing 0.35 ml inoculum. Incubation was carried out with shaking at room temperature. Cell-free extract was obtained by centrifugation at 5,000 *g* for 10 min. The OD_{600}, pH, biomass and emulsification index (E_{24}) were determined at 48 h interval.

Emulsification measurement

The emulsification index (E_{24}) was measured using the method described by Ilori et al. (2005). Biosurfactant activity was measured by adding 2 ml of crude oil to 2 ml of cell-free extract and vortexing at high speed for 2 min. Measurement was taken 24 h later. The E_{24} is the height of

the emulsion divided by the total height multiplied by 100. Kerosene, diesel, crude oil, cyclohexane and hexane were also assayed for their ability to serve as substrate for emulsification.

Effects of pH, heat and salinity on the activity of biosurfactant

Stability studies were carried out using the cell-free broth (crude biosurfactant) obtained by centrifuging the cultures at 5,000 *g* for 20 min. The pH of the biosurfactant (4 ml) was adjusted to 2, 4, 6, 8, 10 and 12 using NaOH or HCl after which E_{24} was determined. To test the heat stability of the biosurfactant, the broth was heated at 50, 70 and 100°C for 15 min, cooled to room temperature and emulsification index determined. Emulsification was also determined after exposure to refrigeration (4°C) for 30 min.The effect of NaCl on the biosurfactant was also assayed at concentrations of 2, 4, 6, 8, 10%) of NaCl.

Results

Growth dynamics of isolate on petroleum hydrocarbons

The growth potentials of the isolate on crude oil, diesel oil and engine oil are shown in Fig. 1 a–c. After an initial slow growth, the population increased exponentially from 2.0×10^6 cfu/ml to 1.50×10^9 cfu/ml in 12 days (Fig. 1a). Thereafter, it declined rapidly. Oil degradation was accompanied by lowering of pH from 7.0 to 5.8 in 15 days followed by a rise to 6.2 on day 21. The isolate (Table 1) exhibited specific growth rate and mean generation time of 0.304 days^{-1} and 2.28 days, respectively.

On diesel oil, the highest population density of 6.6×10^{10} cfu/ml was on day 12 from an initial value of 1.7×10^7 (Fig. 1b) after a lag phase of about 3 days. The pH dropped from 7.14 to 6.50 at the end of the experiment. On engine oil the bacterium also displayed brief lag phase of about 3 days from an initial population of 8.0×10^7 cfu/ml. The population reached stationary phase on day 12 with population density of 5.7×10^{10} cfu/ml, followed by a steady decline to 3.3×10^{10} cfu/ml on day 21 (Fig. 1c). Lower specific growth rates and higher mean generation times were observed compared to diesel, with values of 0.122 days^{-1} and generation time of 5.71 days.

The results displayed in Figs. 2 and 3 confirmed the ability of strain LP1 to metabolise the HC components of crude and diesel oils and that the growth recorded was as a result of consumption of these components. The comparison of the GC fingerprints of crude oil recovered from heat-inactivated controls and experimental flasks showed a near

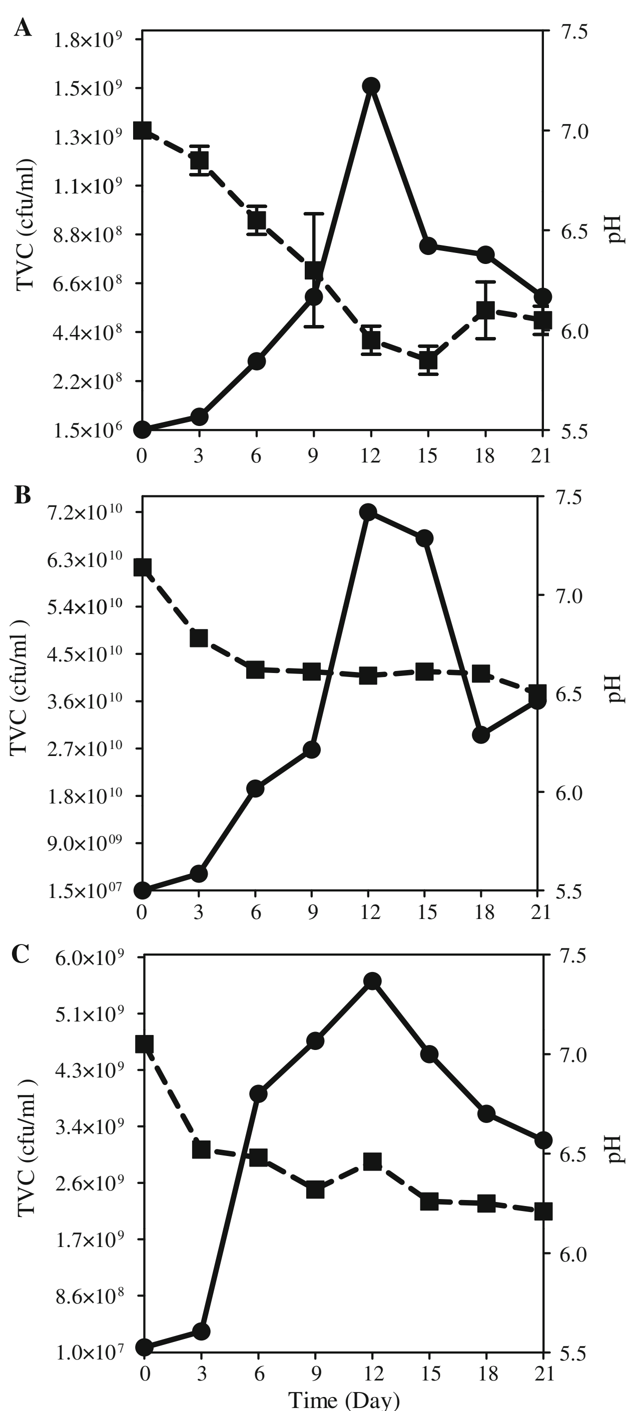

Fig. 1 Population (●) and pH (■) changes during growth of *Pseudomonas* sp. LP1 on crude oil **a**, diesel **b** and engine oil **c**. The hydrocarbon substrates were supplied at the concentration of 1% (v/v). Data points represent the mean of three replicate flasks, while *error bars* represent standard deviation. In the case of population counts, *error bars* were removed for clarity

total disappearance of the major alkane peaks including the isoprenoid molecules. Degradation of the oil fractions did not follow a particular fashion, as nearly all the component HCs were depleted at relatively equal rates. Similar trend

Table 1 Growth kinetics of *Pseudomonas* sp. LP1 on crude oil and petroleum cuts

Substrate	Specfic growth rate (day^{-1})	Mean generation time (day)	% degradation[a]	Emlusification index (%)[b]
Crude oil	0.304	2.28	92.34	3.33 ± 0.83
Diesel	0.233	2.97	95.29	0
Engine oil	0.122	5.71	ND	80.25 ± 1.20

[a] Percent degradation values represent the net decrease (FID area counts) calculated with reference to the amount recovered from heat attenuated control tubes

[b] Emulsification index was determined as the percentage of emulsion divided total liquid column as described in the materials and methods

was observed when the organism was propagated on diesel oil with some exceptions. While C21, C23, C24 and C25 hydrocarbons were completely degraded, traces of lighter fractions of C9, C11, C12 and C15 were still noticeable in the recovered oil (Fig. 3). Therefore, on the basis of this observation coupled with the fact that 95.29% of the oil was metabolised compared to 92.34% obtained for crude oil, it would appear that diesel was much more amenable to microbial degradation compared to the latter.

Production of biosurfactant

Time course production of biosurfactant by *Pseudomonas* sp. strain LP1 on engine oil is shown in Fig. 4. The highest E_{24} value of 80.25 ± 1.20 was recorded on the 8th day of incubation when the optical density was 0.53. Even though there was no increase in biosurfactant production between days 8 and 10, there was a sharp increase in the population density of the organism as indicated by increase in OD_{600}–1.04. When grown on crude oil, LP1 showed marginal biosurfactant production with E_{24} value of 3.33 ± 0.83. It equally failed to produce biosurfactant on diesel and pyrene. Furthermore, the biosurfactant produced on engine oil showed poor emulsification activity towards crude oil, diesel and hexadecane.

Effects of salinity, pH and temperature on emulsification activity of biosurfactant

The effects of salinity, pH and temperature on emulsification activity of the biosurfactant produced by LP1 is summarised in Fig. 5. Biosurfactant production was negatively affected by salinity (Fig. 5a). The lowest value (12.86) was recorded at 10% NaCl concentration, while the highest value (82.82) was obtained in the tube without NaCl (0%). Surprisingly, the activity at 8% (38.0) was higher than at 6% (24.27). As shown in Fig. 5b, no activity was recorded at pH 10, while the highest value of 85.0 was recorded at pH 2. Temperature also had a remarkable impact on the E_{24} with the highest value of 92.43 obtained at 50°C and no loss of activity even at temperature of 100°C ($E_{24} = 60.0$; Fig. 5c).

Discussion

The biodegradation of hydrocarbons in polluted environment is mainly through the activities of bacteria and fungi. Typically, individual organisms degrade only a limited range of hydrocarbons. *Pseudomonas* sp. represents one of the most versatile groups of organisms involved in the degradation of hydrocarbons (Wackett and Hershberger 2001). *Pseudomonas* sp. LP1, the organism used in this study, has been reported to have specificity for a range of hydrocarbon compounds including biphenyl, PAHs and petroleum products commonly used in the Nigerian environment (Obayori et al. 2008).

Pseudomonas sp. LP1 grew well on crude oil, diesel and engine oil and in all cases stationary phase was attained within 12 d, with considerable disappearance of oil and biomass accumulation as revealed by turbidity and increase in viable counts. The growth rate and generation time of 0.304 $days^{-1}$ and 2.28 days, respectively exhibited by LP1 on crude oil were similar to the results previously obtained for a strain of *Pseudomonas aeruginosa* isolated from a polluted stream by Adebusoye et al. (2007). It is however remarkable that, while the article reported percentage oil degradation of 65.8% by the axenic culture and only achieved 100% removal in microbial consortia, LP1 removed 92.34% of oil in 21 days. Furthermore, this organism showed an almost complete removal of the isoprenoid molecules.

The ability of LP1 to remarkably degrade diesel is of interest because diesel is an excellent model for studying hydrocarbon biodegradation since it consists of a variety of molecules such as paraffin, olefins, naphtha and aromatic compounds (Ilori et al. 2008). The strain also exhibited remarkable growth rate on engine oil. The lower growth rate observed on engine oil compared to diesel may be attributed to the constituent of the oil which may be sparingly soluble (Amund and Adebiyi 1991). Another factor frequently fingered for this is the fact that substrates such as diesel contain more variety of hydrocarbons making it more likely to support microbial growth than the more refined engine oil (Adebusoye et al. 2007). It is also noteworthy that whereas diesel oil contains n-alkanes with

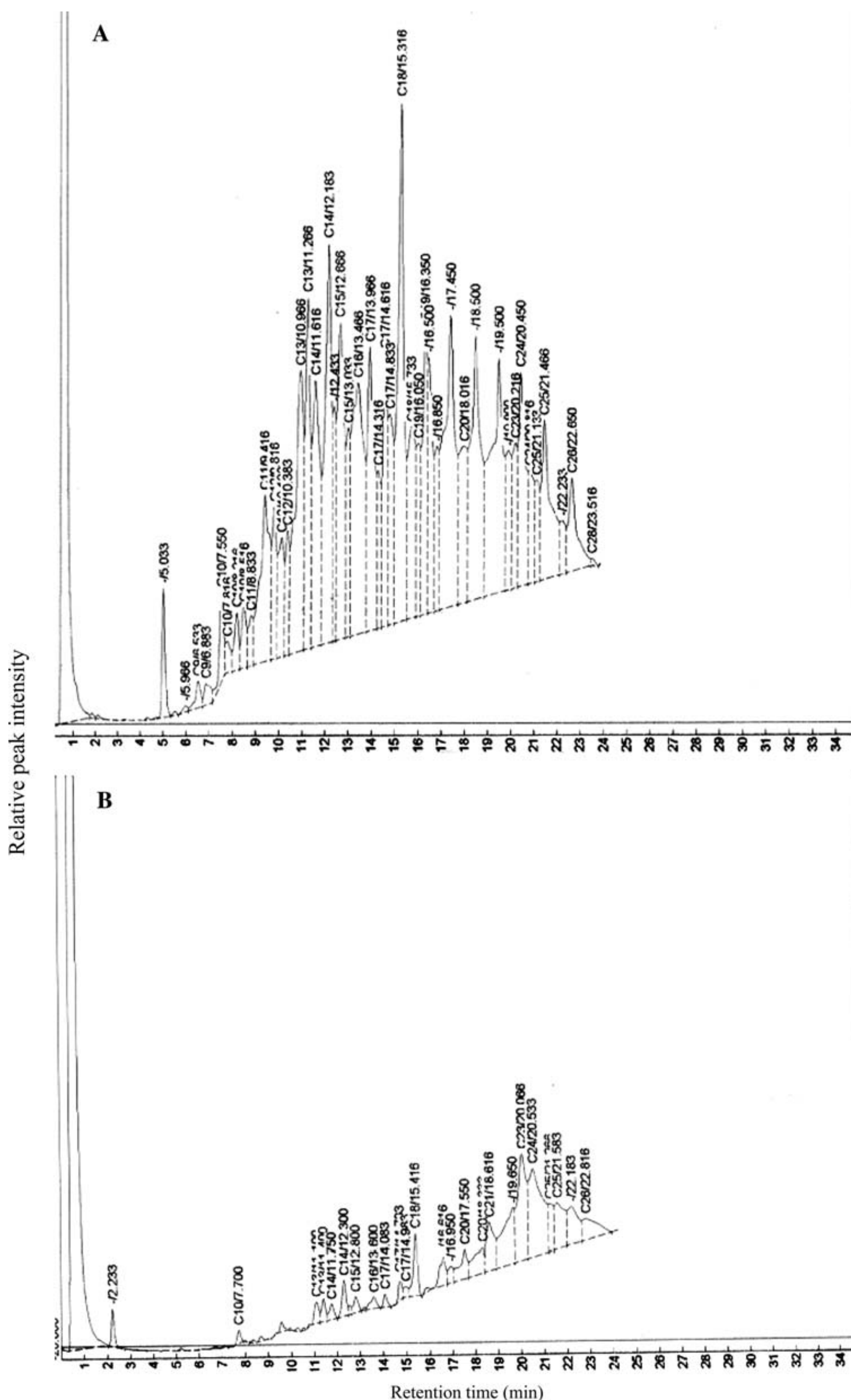

Fig. 2 Gas chromatographic traces of n-hexane extract of recovered crude oil from culture fluids of heat-killed cells *Pseudomonas* sp. LP1 (**a**) and experimental flasks (**b**) after incubation at room temperature for 21 days. The aliphatic fractions were nearly completely utilised in panel (b). The oil components were separated on a 30 m long HP-5 capillary column

medium range chain length (n-C_9–n-C_{25}), longer chain lengths (up to n-C_{50}) which may proof more recalcitrant to biodegradation as a result of higher molecular weights are found in engine oil. The good diesel degradation ability of LP1 is underscored by the 94% degradation recorded and near total removal of n-C_{17}/pristane and n-C_{18}/phytane biomarkers. This report is similar to previous findings. For example, Kucerova (2006) reported 87% removal of NEL

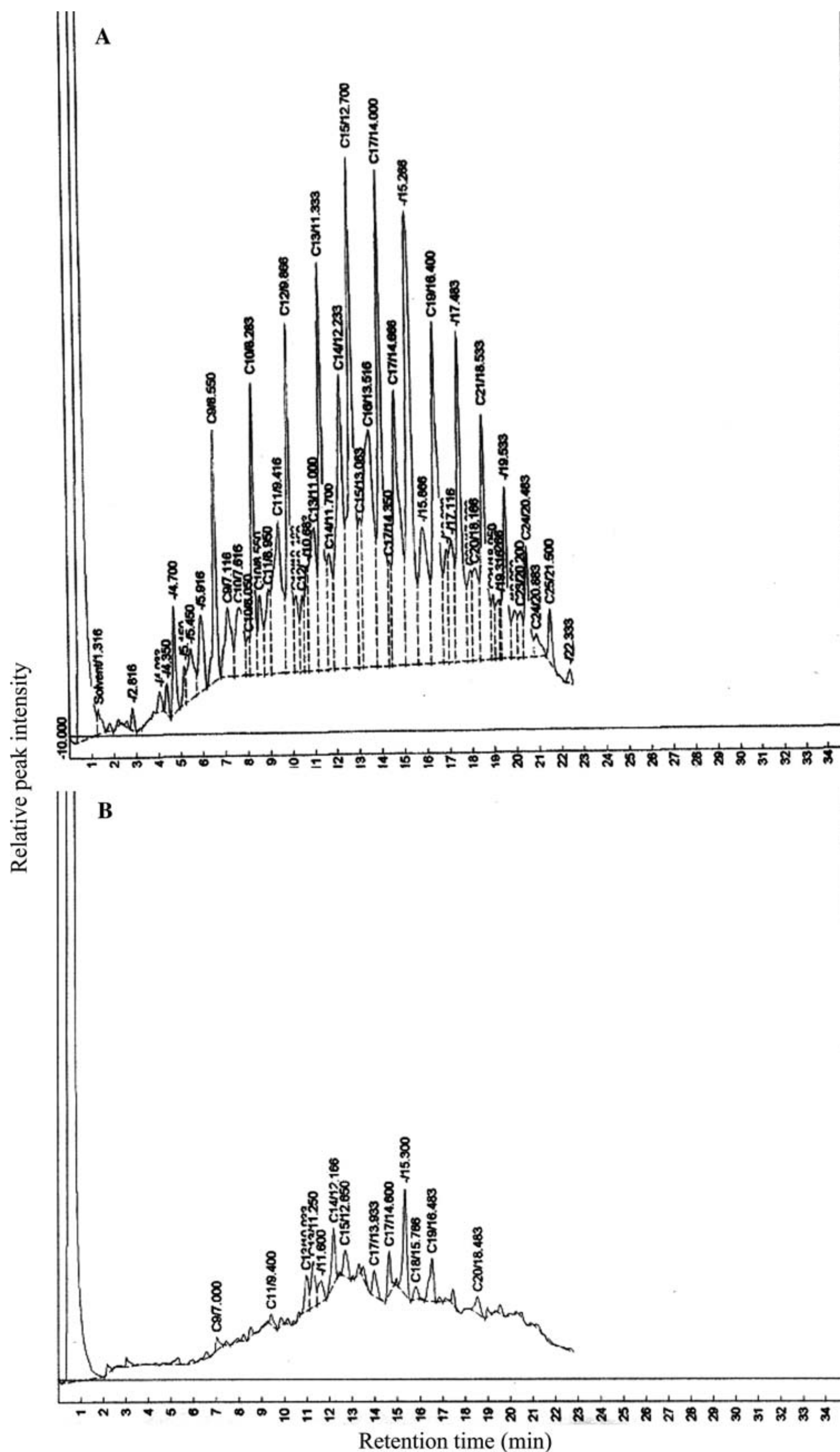

Fig. 3 Gas chromatographic traces of n-hexane extract of recovered diesel oil from heat-inactivated control (**a**) and experimental flasks (**b**) after incubation at room temperature for 21 days. The aliphatic fractions were nearly completely utilised in panel (b). The oil components were separated on a 30 m long HP-5 capillary column

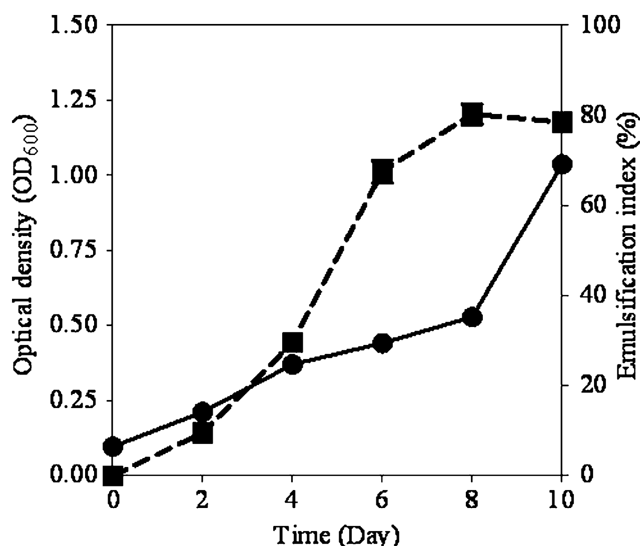

Fig. 4 Growth (λ_{600}, ●) and biosurfactant production (■) dynamics of *Pseudomonas* LP1 during degradation of engine oil as the sole source of carbon and energy. Data points represent the mean of three replicate flasks. Emulsification index was determined as percentage of emulsion divide the total liquid column as described in the materials and methods

(gasoline range organic diesel) by a strain of *Pseudomonas putida*, while Singh and Lin (2008) reported 86.94% degradation of diesel oil by *Bacillus pumilus* strain JL_B in 2 weeks. The lowering of pH observed during the growth of LP1 on the different substrates (crude oil, diesel oil and engine oil) is as a result of production of organic acids (Amund and Adebiyi 1991).

Members of the genus *Pseudomonas* are among the best known biosurfactant producers but rate of emulsifier production and degree of emulsification is usually a function of growth substrate and physico-chemical conditions among other factors (Tuleva et al. 2002; Rashedi et al. 2006). The biosurfactant produced by *Pseudomonas* sp. LP1 on engine oil was growth-associated, similar to those reported in *Bacillus stearothermophillus* VR-8 (Gurjar et al. 1995), *Pseudomonas aeruginosa* (Ilori and Amund 2001) and *Aeromonas* sp (Ilori et al. 2005). Tabatabaee et al. (2005) also documented that a biosurfactant synthesised by a strain of *Bacillus* sp. was a primary metabolite produced during cellular biomass formation. This is at variance with some other reports which showed that microorganisms also produce biosurfactants optimally at stationary or death phase (Deziel et al. 1996; Rodrigues et al. 2006; Ilori et al. 2008). Most biosurfactants are specific and emulsify different substrates differently (Ilori et al. 2005). The biosurfactant produced by LP1 is highly specific, showing emulsification activity for only engine oil, crude oil and diesel. Formation of emulsion usually results from the dispersion of a liquid phase as microscopic droplets in another liquid continuous phase (Desai and

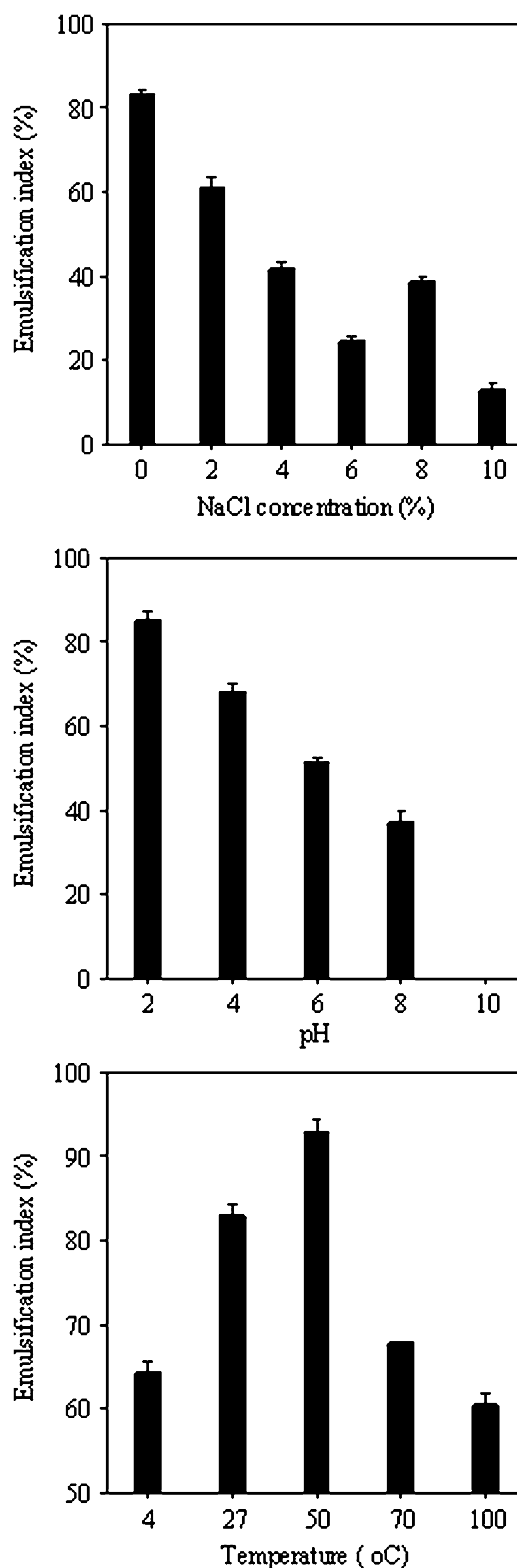

Fig. 5 Effects of salinity, pH and temperature on the emulsification activity of biosurfactant produced by *Pseudomonas* sp. LP1. Data points represent the mean of three replicate flasks, while *error bars* represent standard deviation

Banat 1997). Lack of emulsification of most of the substrates tested might be due to the inability of this highly specific biosurfactant to stabilise the microscopic droplets. As pointed out by Amund and Adebiyi (1991), the physical state of petroleum hydrocarbons exerts a marked effect on their biodegradation. Hydrocarbon degrading microorganisms act mainly at the oil-water interface and availability of increased surface area is known to accelerate biodegradation. Increase in vicousity decreases the dispersion potential of oils in aqueous medium. From the point of view of microbial degradation, dissolution and emulsification of hydrocarbons appear to have a positive effect on degradation rate.

The production and emulsification activities of biosurfactants are affected by environmental factors such as salinity, pH and temperature. The emulsification index of the biosurfactant produced by LP1 on engine oil was found to be inversely proportional to NaCl concentration, but still retained its activity even at concentration of 10% and exhibited an emulsification increase at 8%. The slight enhancement of activity suggests that this biosurfactant might be useful in marine environments and other systems where salt concentration is above physiological level. Previous reports have also shown stability of some biosurfactants in the presence of high salt concentration (Ilori et al. 2005; Sarubbo et al. 2007).

The biosurfactant had its highest activity at pH of 2 and no activity at all at pH of 10. Increasing activity with lowering of pH may have resulted from the transformation of less active species into more active emulsifier by denaturation of proteinaceous component or by increased ionisation (Sarubbo et al. 2007). This suggests that it may be very useful in environment or system where acidic condition obtains or where system progression is accompanied with increasing acidity. The retention of over 60% activity at 4°C and 100 suggests that this biosurfactant might be useful in extreme environments such as temperate marine compartments and industrial systems where extremes of temperature are integral elements.

Conclusively, this study has shown that *Pseudomonas* sp. LP1, an organism isolated from contaminated Nigerian soil on the basis of its ability to grow on pyrene also has degradative potential for crude oil and its cuts such as diesel and engine oil. In the case of latter degradation was accompanied by production of a highly specific biosurfactant which was stable under extreme temperature and pH conditions. Because of the substrate diversity of this organism it is adjudged as a good candidate for bioremediation of polluted sites and a potential resource for surface-active molecules of industrial importance.

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