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Research Paper

Biodegradation of petroleum hydrocarbons in the presence of nickel and cobalt

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Bioremediation of environments co-contaminated with hydrocarbons and heavy metals often pose a challenge as heavy metals exert toxicity to existing communities of hydrocarbon degraders. Multi-resistant bacterial strains were studied for ability to degrade hydrocarbons in chemically defined media amended with 5.0 mM Ni^{2+}, and Co^{2+}. The bacteria, *Pseudomonas aeruginosa* CA207Ni, *Burkholderia cepacia* AL96Co, and *Corynebacterium kutscheri* FL108Hg, utilized crude oil and anthracene without lag phase at specific growth rate spanning 0.3848–0.8259 per day. The bacterial populations grew in hydrocarbon media amended with nickel (Ni) and cobalt (Co) at 0.8393–1.801 days generation time (period of exponential growth, $t = 15$ days). The bacteria degraded 96.24–98.97, and 92.94–96.24% of crude oil, and anthracene, respectively, within 30 days without any impedance due to metal toxicity (at 5.0 mM). Rather, there was reduction of Ni and Co concentrations in the axenic culture 30 days post-inoculation to 0.08–0.12 and 0.11–0.15 mM, respectively. The metabolic functions of the bacteria are active in the presence of toxic metals (Ni and Co) while utilizing petroleum hydrocarbons for increase in biomass. These findings are useful to other baseline studies on decommissioning of sites co-contaminated with hydrocarbons and toxic metals.

Abbreviations: PHs – petroleum hydrocarbons; HMs – heavy metals; PAH – polyaromatic hydrocarbon; MSM – minimum salt medium; OD – optical density; GC – gas chromatograph; FID – flame ionization detector; TCB – trichlorobenzoate; DCB – dichlorobenzoate; CB – chlorobenzoate

Keywords: Biodegradation / Nickel / Cobalt / Heavy metal / Crude oil / Anthracene / Petroleum hydrocarbon / Bacteria

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Introduction

Petroleum hydrocarbons (PHs) usually consist of 23% alkanes, 24% cycloalkanes, and 53% aromatic hydrocarbons [1]. Upon discharge, PHs spread horizontally in the groundwater surface and partition into groundwater, soil pore space, and to the surfaces of soil particles. Their presence in the environment causes serious damage to natural ecosystems and it is of public health concern [2, 3]. It has been reported to have both mutagenic and carcinogenic effect on humans [2]. The lipophilic nature of PHs makes them not only non-bioavailable for biodegradation but exert toxicity on the membranes of microorganisms. This causes increase in permeability of affected membranes to protons and ions with consequent increase in thickness of membranes and direct effect on protein–protein interactions in supramolecular protein complexes like the electron transport chain [4].

Urbanized areas, like Mexico City, are frequently characterized by concomitant presence of organic pollutants and heavy metals (HMs) [5, 6]. It was reported that 40% hazardous waste sites in industrialized nations are co-contaminated with organic and inorganic pollutants [5, 6]. There are several reports on HMs contaminations of Nigerian environments [7, 8]. The presence of HMs in the environment exerts toxicity to the biota [9, 10]. There have been contrary views on effects of

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metals on biodegradation of organic pollutants, ranging from no inhibition, and dose-dependent inhibition to nondose-dependent inhibition. Delaune *et al.* [11] demonstrated that chromium (5000 $\mu g\ g^{-1}$) and lead (2500 $\mu g\ g^{-1}$) have no effect on the biodegradation of PHs from clay sediments. Similarly, no inhibitory effects of metals were reported on reductive dechlorination of hexachlorobenzene [12] and biodegradation of polycyclic aromatic hydrocarbons (PAHs) [13], and pentachlorophenol [14]. However, HMs have been shown to inhibit biodegradation/bioremediation of organic pollutants in co-contaminated sites [15, 16], and prolong the recalcitrance of the organic pollutants in the environment. Inhibition was reportedly dose-dependence where higher metal concentrations inhibited biodegradation of toluene [15] and methyl ter-butyl ether [16]. While in other reports, pattern of nondose-dependent inhibition in which higher metal concentrations are less inhibitory than lower metal concentrations were established [17]. Despite the toxicity and consequential negative impacts of HMs to microbial communities, some bacterial strains found in soils receiving wastewaters of oil refinery have been demonstrated to show resistance to HMs [18, 19]. Various mechanisms were adopted by the resistant strains to circumvent HM stress in contaminated sites [20, 21].

The environments of industrial estates in Lagos, Nigeria are not only contaminated with HMs but rather with complex petroleum cut. The industrial plants and adjoining residences use fractions of petroleum to run heavy-duty power generators due to incessant and protracted electricity failure. Thus, accidental spills and water run-off of PHs made the industrial estates in Lagos a typical environment co-contaminated with PHs and HMs. For bioremediation to be effective in such sites where PHs and HMs coexist as pollutants, the operative microorganisms must grow, function, and out-compete other bacteria in the presence of all contaminants. Bacteria showing concomitant ability to degrade PHs and resistance to HMs have not been well established. This study, therefore, sought to investigate the ability of highly resistant bacterial strains to degrade PHs in media amended with toxic concentrations (5.0 mM) of nickel (Ni) and cobalt (Co) that are known to be associated with crude petroleum and PHs pollution. To achieve this, we simulated a model system containing PHs (crude oil and anthracene), and toxic doses of Ni and Co as co-contaminants. This will suggest the potentials of the bacterial strains for application in decommissioning of sites polluted with hydrocarbons, even when HMs coexists.

Materials and methods

Chemicals

Anthracene and naphthalene were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA), Escravos light crude oil was obtained from "Chevron Nigeria Limited," while kerosene, engine oil, and diesel were refined petroleum cuts of "Total Nigeria Limited." All other chemicals were of analytical reagent grade. Ten milligrams (10 mg) of the PAH were dissolved in hexane and vented overnight in Erlenmeyer flasks, and 100 ml minimal salt medium (MSM) [22] was added to make up a final concentration, 100 ppm, of the respective PAH before sterilization at 121 °C for 15 min. Crude oil, kerosene, and diesel were sterilized separately and added to MSM aseptically to 1% v/v final concentration. Chlorobenzoates (CB) were dissolved in MSM to make 2.0 mM, and autoclaved at 121 °C for 15 min. The MSM contained (in $g\ L^{-1}$) Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g, and $MgSO_4 \cdot 7H_2O$, 0.20 g; pH was adjusted to 7.2. Sterile trace elements solution (1.0 ml L^{-1}) was aseptically added to the medium after sterilization.

Microorganisms and culture conditions

The isolation of bacteria used in this study, and their tolerance to Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, and Cr^{6+} levels have been reported elsewhere [23]. The strains were *Pseudomonas aeruginosa* CA207Ni, *Burkholderia cepacia* AL96Co, *Rhodococcus* sp. AL03Ni, and *Corynebacterium kutscheri* FL108Hg. They were stored at −20 °C in glycerol:Luria Bertani (LB) broth (1:1). The organisms were activated by harvesting colonies on LB agar with sterile inoculating loop, pooled, and transferred to screw-capped bottles containing 5 ml of physiological saline (0.9% NaCl) solutions. The cells were pre-cultured in LB broth for 24 h at 30 °C and 175 rpm, harvested upon centrifugation at 7000*g* for 10 min, washed three times with phosphate buffer (50 mM KH_2PO_4, pH 7.2) and re-suspended in same buffer to approximately 10^6 cfu ml^{-1} cell populations.

Substrate utilization

The ability of HM resistant bacteria to grow on selected persistent organic pollutants was evaluated in MSM previously described by Kastner *et al.* [22]. The sterile medium, as earlier explained, contained the respective compound as sole source of carbon. Solid substrates were applied at a concentration of 100 ppm, while liquid hydrocarbons were applied at 1% v/v final concentration. All CB were supplied at 2.0 mM concentration. A set of the flasks were amended with filter- (0.22 μm pore size)

sterilized $NiCl_2$ (20.24 mg l^{-1} to give 5.0 mM Ni^{2+}) and $CoCl_2$ (20.19 mg l^{-1} to give 5.0 mM Co^{2+}) solution, and control set were without HM amendment. Incubation of individual bacterial strain (mono-culture) was carried out at room temperature (28 ± 2 °C) in the dark with shaking (175 rpm) for 30 days. Degradation was monitored by turbidity of culture via increase in cell population, and consequent growth-associated depletion of PHs with infra-red spectrophotometer and gas chromatography. Approximately 10^{10} cfu l^{-1} was taken as 1 optical density (OD) unit at 600 nm absorbance for *Pseudomonas*, and *Burkholderia*; while approximately 10^{10} cfu ml^{-1} was 1 OD unit at 600 nm for *Corynebacterium*, and *Rhodococcus*. MSM without carbon source was used as blank in spectrophotometry, while control consisted of MSM without the organic compounds but inoculated with the individual bacteria. Viability of the bacteria in each flask was determined by plating out appropriate dilutions of aliquot onto LB agar.

Petroleum hydrocarbons biodegradation

Triplicate 250-ml flasks containing 50 ml of MSM and supplemented anthracene (100 ppm), and crude oil (1%), as sole source of carbon and energy were prepared as explained earlier. Each flask was amended with filter-(0.22 μm pore size) sterilized $NiCl_2$ (20.24 mg l^{-1} to give 5.0 mM Ni^{2+}) and $CoCl_2$ (20.19 mg l^{-1} to give 5.0 mM Co^{2+}) solution, to simulate system co-contaminated with HMs and PHs. The metal concentrations represent higher threshold detected in the river polluted with industrial effluents as previously reported [23]. The flasks were inoculated with washed cells (1 ml) of axenic culture of *P. aeruginosa* CA207Ni, *B. cepacia* AL96Co, *Rhodococcus* sp. AL03Ni, and *C. kutscheri* FL108Hg grown overnight (final population of approximately 10^6 cfu ml^{-1}) and incubated at room temperature (29 ± 2 °C) with shaking at 175 rpm for 30 days. Degradation was accessed by determining cell biomass, as well as, GC analysis of residual substrates. Control flasks containing respective substrates were inoculated with heat-killed bacterial cells.

Gas chromatographic determination of hydrocarbon substrates

Residual hydrocarbon in the experimental and control flasks were extracted according to the method of Sarma *et al.* [24] with slight modification by using equal volume of *n*-hexane instead of toluene. After two extraction cycles, the organic phase was reconstituted to 5 ml and extracts (1.0 μl) were analyzed with Hewlett Packard 5890 Series II gas chromatograph fitted with flame ionization detector (FID) and AJ & W Scientific DB-1 fused silica 30 m long column (internal diameter, 0.32 mm; film thickness, 1.0 μm). The injector and detector temperatures were maintained at 250 and 320 °C, respectively. The column temperature was programmed to rise from 70 to 320 °C for 27 min. The GC was programmed at an initial temperature of 60 °C; this was held for 2 min, then ramped at 10 °C min^{-1} to 320 °C and held for 16 min. The mobile phase (carrier) was nitrogen. The peak areas for the residual hydrocarbons were measured, and the percentage decrease based on the areas of the control peaks were calculated. The values obtained were taken as quantitative measure of hydrocarbons.

Heavy metal analysis

Axenic cultures were centrifuged at 10,000 rpm for 10 min, supernatants were filtered through No 1 filter paper (Whatman, Inc.) and then through 0.22 μm Millipore filters (Nucleopore Corp., Pleasanton, CA, USA), and acidified with nitric acid (pH 2.0). Co and Ni were measured with their respective metal lamp by Perkin-Elmer Flame Atomic Absorption Spectrometer (Perkin-Elmer, Canada).

Results

Substrate utilization

The bacterial strains demonstrated ability to use three or more of the organic pollutants as a sole source of carbon for growth in media amended with Co^{2+} and Ni^{2+} (5.0 mM) as shown in Table 1. All the isolates grew on media supplemented with crude oil, kerosene, and engine oil as sources of carbon, except *Rhodococcus* sp. AL03Ni that failed to grow on spent oil and diesel. None of the bacteria grew on 3,4-DCB, while scanty growth of *C. kutscheri* FL108Hg was observed on biphenyl, and 1,4 DCB. About 1.25 OD unit growth on 1,4-DCB was observed with *B. cepacia* AL96Co ($1.4 \pm 0.1 \times 10^7$ cfu ml^{-1}), *C. kutscheri* FL108Hg ($1.103 \pm 0.005 \times 10^7$ cfu ml^{-1}), and *Rhodococcus* sp. AL03Ni ($1.14 \pm 0.017 \times 10^7$ cfu ml^{-1}). Moreover, more than 2.0 OD unit growth of *B. cepacia* AL96Co, and *P. aeruginosa* CA207Ni (see Table 1 for respective values) were observed on 3-chlorobenzoate (CB) and 4-CB. On 4-CB containing MSM, yellowish coloration from initial colorless medium was noticed in cultures of *P. aeruginosa* CA207Ni and *B. cepacia* AL96Co on 3 days post-inoculation. The yellowish culture of *P. aeruginosa* CA207Ni turned colorless (became clear) 2 days later unlike that of *B. cepacia* AL96Co that only faded out. At 6 days post-inoculation, cultures of *P. aeruginosa* CA207Ni and *B. cepacia* AL96Co on 3-CB appeared dark, while that of *Rhodococcus* sp. AL03Ni on 2-CB appeared

Table 1. Substrate spectra of bacteria strains.

Substrate	AL96Co ($\times 10^7$ cfu ml^{-1})	FL108Hg ($\times 10^7$ cfu ml^{-1})	AL03Ni ($\times 10^7$ cfu ml^{-1})	CA207Ni ($\times 10^7$ cfu ml^{-1})
Pyrene	0.09 ± 0.0006	1.367 ± 0.231	1.033 ± 0.058	1.633 ± 0.115
Biphenyl	0.073 ± 0.047	1.067 ± 0.058	0.09 ± 0.0006	0.099 ± 0.008
Dibenzothiophene (DBT)	0.088 ± 0.001	0.09 ± 0.0006	0.083 ± 0.003	2.61 ± 0.173
Anthracene	2.513 ± 0.289	2.493 ± 0.115	0.097 ± 0.006	2.667 ± 0.231
Naphthalene	0.09 ± 0.001	0.084 ± 0.002	0.09 ± 0.0006	2.467 ± 0.058
Phenantherene	1.947 ± 0.04	1.167 ± 0.058	0.1 ± 0.0006	0.102 ± 0.003
2-Chlorobenzoate	0.085 ± 0.001	0.089 ± 0.003	1.6 ± 0.346	0.103 ± 0.006
3-Chlorobenzoate	2.467 ± 0.231	0.097 ± 0.006	1.833 ± 0.029	2.167 ± 0.029
4-Chlorobenzoate	2.4 ± 0.1	0.102 ± 0.003	0.373 ± 0.473	2.133 ± 0.115
1,4-Dichlorobenzene	1.4 ± 0.1	1.103 ± 0.005	1.14 ± 0.017	0.101 ± 0.001
2,4-Dichlorobenzoate	1.927 ± 0.031	2.333 ± 0.115	2.533 ± 0.115	1.89 ± 0.173
2,5-Dichlorobenzoate	1.243 ± 0.058	0.095 ± 0.002	0.089 ± 0.001	0.097 ± 0.006
2,6-Dichlorobenzoate	1.787 ± 0.231	0.096 ± 0.002	2.533 ± 0.115	0.084 ± 0.002
3,4-Dichlorobenzoate	0.092 ± 0.002	0.087 ± 0.004	0.086 ± 0.002	0.084 ± 0.003
2,4,6-Trichlorobenzoate	0.07 ± 0.001	0.08 ± 0.0006	0.076 ± 0.003	1.153 ± 0.115
Crude oil	3.5 ± 0.173	3.167 ± 0.058	3.1 ± 0.173	3.567 ± 0.115
Kerosene	3.133 ± 0.115	2.867 ± 0.058	1.92 ± 0.017	3.077 ± 0.058
Spent oil	3.067 ± 0.115	3.033 ± 0.058	0.097 ± 0.006	3.367 ± 0.115
Engine oil	1.983 ± 0.012	3.067 ± 0.058	1.85 ± 0.173	3.3 ± 0.173
Diesel	2.877 ± 0.058	2.96 ± 0.052	0.102 ± 0.004	2.163 ± 0.023

Values represent mean ± SD ($n = 3$) of bacterial population. Approximately 10^{10} cfu l^{-1} = 1 OD unit at 600 nm absorbance for *Pseudomonas* and *Burkholderia*, while 10^9 cfu l^{-1} = 1 OD unit at 600 nm for *Corynebacterium* and *Rhodococcus* sp. Liquid and solid substrates were supplied at 1% v/v and 100 ppm concentration, respectively. Growth was established by turbidity through infra-red spectrophotometer, where control was MSM with appropriate hydrocarbon without viable bacterial cells.

light greenish yellow at 6 days post-inoculation. The bacteria, *C. kutscheri* FL108Hg and *Rhodococcus* sp. AL03Ni had 2.20 OD unit growth on 2,4-DCB, while 2,6-DCB allowed 2.17 OD unit growth of *Rhodococcus* sp. AL03Ni. Among the PAHs, anthracene allowed 2.35–2.60 OD units growth of *B. cepacia* AL96Co, *C. kutscheri* FL108Hg, and *P. aeruginosa* CA207Ni (Table 1). The bacterial strains exhibited varied ability to grow in pyrene, and phenantherene, but *P. aeruginosa* CA207Ni grew well in dibenzothiophene (DBT), and naphthalene (2.45 ± 0.2 OD unit). Poor performance of *Rhodococcus* sp. AL03Ni on PHs (Table 1) led to its been dropped, while strains, *P. aeruginosa* CA207Ni, *C. kutscheri* FL108Hg, and *B. cepacia* AL96Co were further investigated for their prospects to degrade PHs for growth.

Growth potentials of bacterial strains on petroleum hydrocarbons

The growth profiles of the selected isolates on hydrocarbons in the presence of Co^{2+} and Ni^{2+} with their corresponding GC-FID chromatograms were shown in Figs. 1b, 2b, and 3b. The populations of the bacteria increased by more than two order of magnitudes with concomitant drop in pH. The doubling time range of the strains were 0.8393–1.801, and 0.9597–1.362 days (t = 0–15 days) when grown on crude oil, and anthracene, respectively (Table 2). The specific growth rates of the bacteria when grown on the PHs are equally summarized in Table 2. A specific growth rate of 0.5088–0.8259 day^{-1} was observed in the culture of *P. aeruginosa* CA207Ni using PHs as carbon source. In this study, losses due to non-biological factors were eliminated since the data obtained from experimental flasks were resolved with reference to heat-inactivated controls, as well as, incubation of the flasks were in a dark room to eliminate possible photo-sequestration of metals. Degradation of PHs, as quantified by GC-FID techniques, was growth associated. With reference to growth profile revealed in Figs. 1a, 2a, and 3a, as well as, GC-FID fingerprints depicted in Figs. 1b, 2b, and 3b, *P. aeruginosa* CA207Ni, *B. cepacia* AL96Co, and *C. kutscheri* FL108Hg had depleted crude oil by 86.63, 91.45, and 85.48%, respectively, within 15 days post-inoculation when the growth phases of the bacterial strains were logarithmic. Similar trends were observed in the utilization of anthracene (Fig. 4). During this period, however, strains *P. aeruginosa* CA207Ni and *C. kutscheri* FL108Hg degraded only 46.63 and 50.81% of anthracene, respectively. Interestingly, *B. cepacia* AL96Co consumed 91.45% anthracene for growth at the same time frame. The rate at which the hydrocarbons were depleted revealed that the bacteria utilized the hydrocarbons at a faster rate (within the earlier 15 days) than in the later stage of the experiment. For example, strain *P. aeruginosa* CA207Ni utilized crude oil at average rate of 5.77 mg l^{-1} day^{-1} in the first 15 days but narrowed down to 0.82 mg l^{-1} day^{-1} in the last 15 days

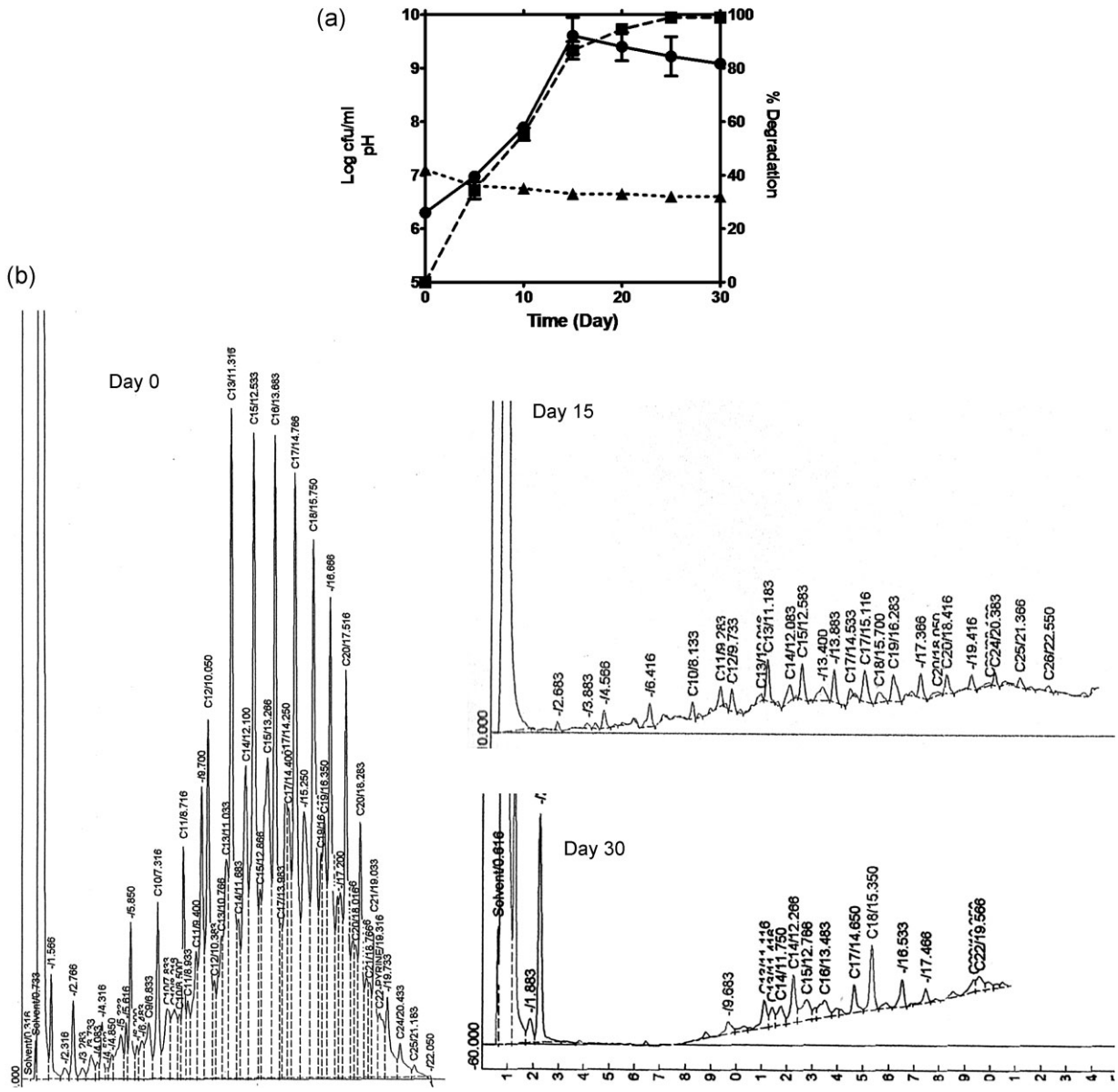

Figure 1. (a) Time course growth and degradation of crude oil for *P. aeruginosa* CA207Ni. Growth (●), % degradation (■), and pH (▲). Biodegradation medium was amended with Ni^{2+} and Co^{2+} (5.0 mM each), crude oil degradation was determined and evaluated with the net decrease in GC-FID area counts in experimental cultures, and compared with that of the heat-attenuated controls. Initial substrate concentration was 10.0 g l^{-1}. The crude oil was steadily emulsified and depleted completely with visual disappearance of the slick from the liquid surface. Data values represent averages of three replicate determinations. Bars represent standard error above and below the mean of triplicate flasks. (b) Gas chromatogram profiles of crude oil recovered from *P. aeruginosa* CA207Ni culture amended with Ni^{2+} and Co^{2+} (5.0 mM each) at Day 0, Day 15, and Day 30. There was 98.97% degradation as shown with almost complete disappearance of hydrocarbon peaks at 30 days.

of incubation. The same trend was observed in other experiments. It should be noted that cell population appeared static and slightly decreased afterwards in some cases after 15 days biodegradation studies. Despite this, metabolism of the PHs continued slowly in the last 10 days as shown in crude oil (0.05–3.21%) and anthracene (1.39–7.24%) degradation.

Overall, not <92.94% of the hydrocarbons (anthracene) were metabolized at the end of the experiment while, as much as, 98.97% of crude oil was used by *P. aeruginosa* CA207Ni during the same period. The kinetic data of other PHs and corresponding bacterial degraders are summarized in Table 2. Cumulatively, the growth increase recorded in each experiment was accompanied

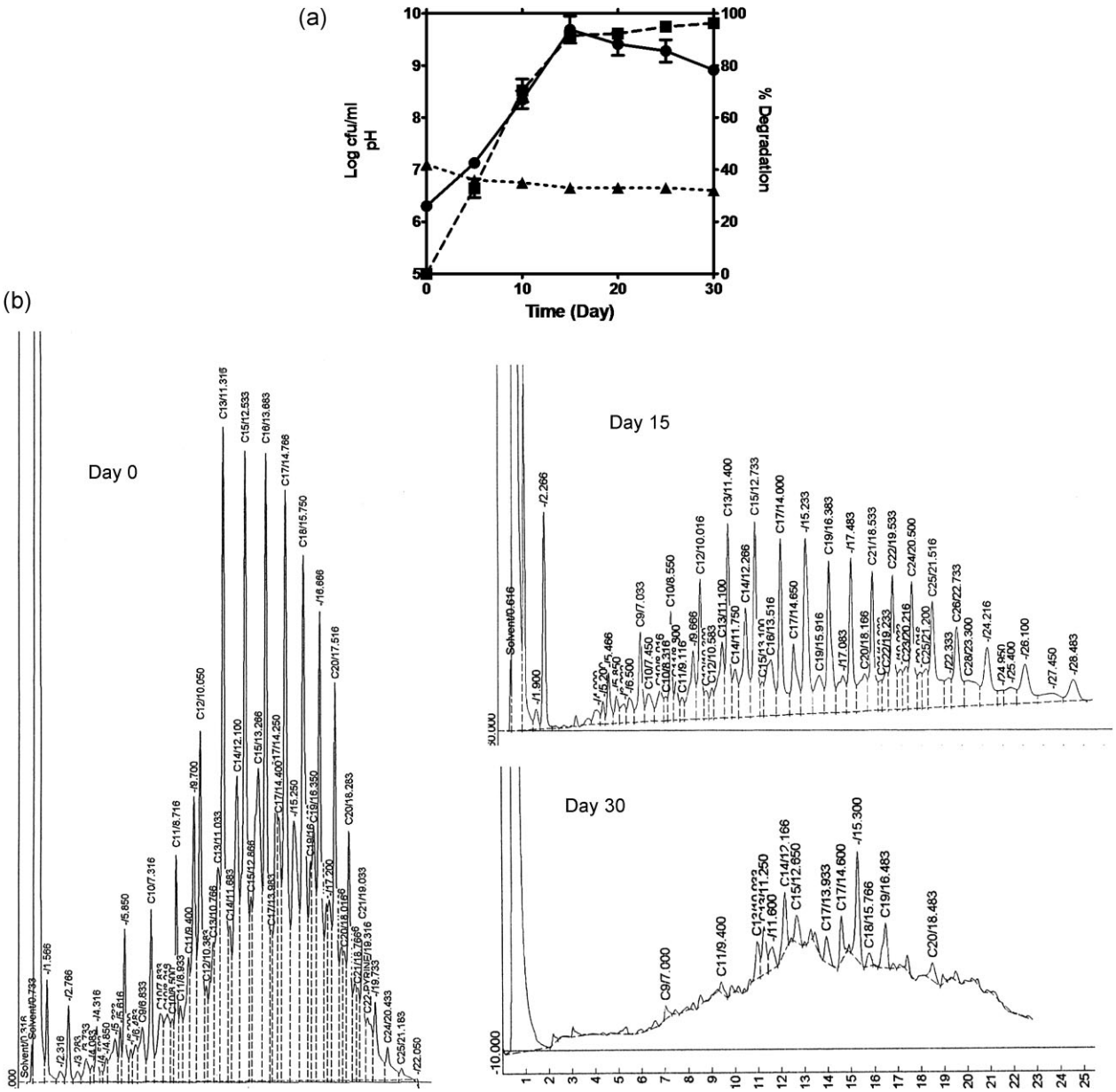

Figure 2. (a) Time course growth and degradation of crude oil for *B. cepacia* AL96Co. Growth (●), % degradation (■), and pH (▲). Biodegradation medium was amended with Ni^{2+} and Co^{2+} (5.0 mM each), crude oil degradation was determined and evaluated with the net decrease in GC-FID area counts in experimental cultures, and compared with that of the heat-attenuated controls. Initial substrate concentration was 10.0 g l^{-1}. The crude oil was steadily emulsified and depleted completely with visual disappearance of the slick from the liquid surface. Data values represent averages of three replicate determinations. Bars represent standard error above and below the mean of triplicate flasks. (b) Gas chromatogram profiles of crude oil recovered from *B. cepacia* AL96Co culture amended with Ni^{2+} and Co^{2+} (5.0 mM each) at Day 0, Day 15, and Day 30. There was 98.58% degradation as shown with almost complete disappearance of hydrocarbon peaks at 30 days.

by substrate utilization via depletion in substrate level while the experiment lasts with no effect of metal toxicity. Rather, Co concentration appreciably reduced from 5.0 to 0.12–0.13, 0.12–0.15, and 0.12–0.13 mM by *P. aeruginosa* CA207Ni, *C. kutscheri* FL108Hg, and *B. cepacia* AL96Co, respectively, during PHs biodegradation. Similarly, decrease in Ni concentration was apparent during degradation of PHs by the bacteria. It is noteworthy that there were no significant difference between biodegradation data obtained in culture amended with toxic metals (Co^{2+} and Ni^{2+}), and those of culture without metal amendment (Table 2).

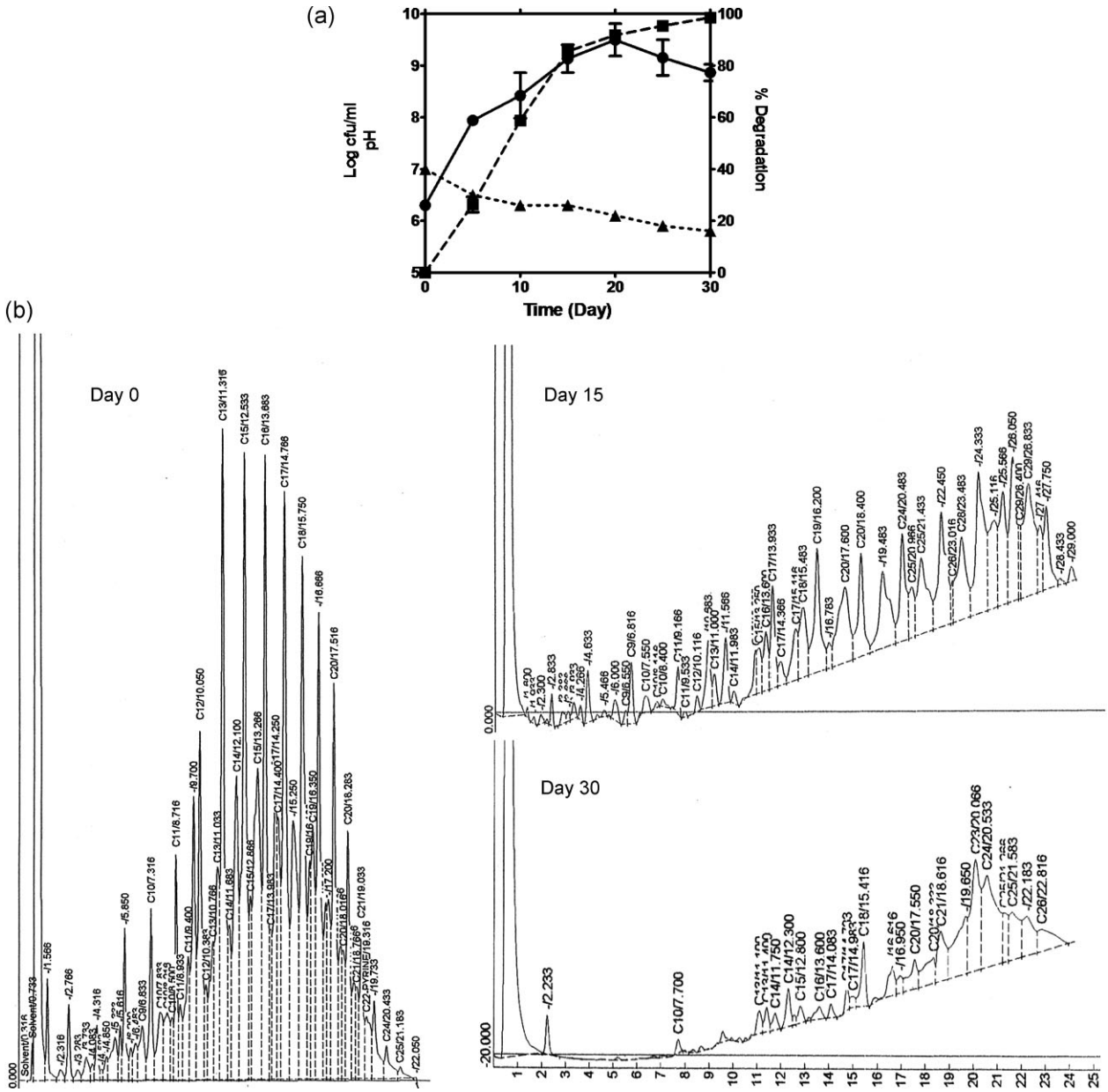

Figure 3. (a) Time course growth and degradation of crude oil for *C. kutscheri* FL108Hg. Growth (●), % degradation (■), and pH (▲). Biodegradation medium was amended with Ni^{2+} and Co^{2+} (5.0 mM each), crude oil degradation was determined and evaluated with the net decrease in GC-FID area counts in experimental cultures, and compared with that of the heat-attenuated controls. Initial substrate concentration was 10.0 g l^{-1}. The crude oil was steadily emulsified and depleted completely with visual disappearance of the slick from the liquid surface. Data values represent averages of three replicate determinations. Bars represent standard error above and below the mean of triplicate flasks. (b) Gas chromatogram profiles of crude oil recovered from *C. kutscheri* FL108Hg culture amended with Ni^{2+} and Co^{2+} (5.0 mM each) at Day 0, Day 15, and Day 30. There was 96.24% degradation as shown with almost complete disappearance of hydrocarbon peaks at 30 days.

Discussion

Discharge of effluents that contain hazardous and toxic chemicals, notably HMs and PHs, into the water body are a global threat to the aesthetic aquatic environment. Microbiological processes play a major role in the removal of contaminants and they take advantage of catabolic versatility to biodegrade. Degradation of PHs by bacteria has been extensively reported [19, 22, 25–27]. However, there is a dearth of information about bacteria that degrade hydrocarbons and reduce HMs concentration concomitantly. A few reports that are available only

Table 2. Growth and degradation kinetics of isolates on petroleum hydrocarbons.

Isolates	Crude oil							Anthracene						
	T_g (day)	μ (day^{-1})	Degr. in presence of metals (%)	Rate of degr. (mg l^{-1} day^{-1})	Res. Co^{2+} (mM)	Res. Ni^{2+} (mM)	Degr. in absence of metals (%)	T_g (day)	μ (day^{-1})	Degr. in presence of metals (%)	Rate of degr. (mg l^{-1} day^{-1})	Res. Co^{2+} (mM)	Res. Ni^{2+} (mM)	Degr. in absence of metals (%)
CA207Ni	0.8393	0.8259	98.97	3.299	0.13	0.11	98.99	1.362	0.5088	92.94	3.098	0.12	0.10	93.11
FL108Hg	1.801	0.3848	96.24	3.208	0.12	0.11	96.29	0.9597	0.7223	96.24	3.208	0.15	0.12	96.32
AL96Co	1.079	0.6424	98.58	3.286	0.13	0.08	98.61	0.9777	0.7089	93.87	3.129	0.12	0.12	93.93

Data are the average best-fit values of three replicates ($n = 3$). T_g is doubling time (day), μ is the specific growth rate (day^{-1}), degr. is degradation (%), Res. is residual metal. Doubling time was determined on Day 15, when the bacteria were actively dividing. The percent degradation values were obtained at 30 days incubation while the experiment lasts from gas chromatographic indexes (the net decrease in GC-FID area counts) in comparison with the heat-attenuated controls. The values of residual metal concentrations were detectable concentrations from experimental flasks 30 days post-inoculation from initial concentrations of 5.0 mM a piece, via atomic absorption spectrophotometer analysis.

discussed microbial catabolism of hydrocarbons in tolerance to HMs without metal sequestration [16, 28, 29], except Verma and Singh [14] that reported 78% metal reduction in simultaneous degradation of pentachlorophenol. None of such data are available with regards to Nigeria. Bioremediation of PHs in the environment where toxic metals co-exist involves selection of autochthonous strains with dual catabolic competence and tolerance to toxic metals [30].

In our previous report [23], we isolated and selected 22 autochthonous bacterial strains that exhibited dual resistances to HMs and antibiotics. The growth of the four bacterial isolates on the organic compounds in the presence of 5 mM Co and Ni is connected to their antecedent high metal-resistance as previously reported [23]. The bacterial strains were from selective enrichments of environmental samples that were further exploited for ability to tolerate unprecedented high concentrations of HMs. With the exception of *Rhodococcus* sp. AL03Ni, other isolates grew luxuriantly on spent oil and diesel along with other hydrocarbons. This shows that microorganisms, having various physiologies, are specific in the range of hydrocarbons they metabolize.

The poor performance of strain *Rhodococcus* sp. AL03Ni to utilize range of hydrocarbons suggests that it has narrow catabolic traits to degrade hydrocarbons in presence of toxic metals. Thus, as originally noted in pseudomonads, a large number of "peripheral aromatic" pathways funnel a range of natural and xenobiotic compounds into a restricted number of "central aromatic" pathways [26, 27, 29, 31]. We are yet to elucidate the catabolic pathways employed by the studied isolates. As observed in the growth of the strains on CB, the characteristic yellow coloration and subsequent disappearance within 48 h is an evidence of meta-cleavage of the compounds as previously reported [31, 32]. Inability of any of the isolates to utilize 3,4-DCB for growth is in agreement with previous reports that few bacteria are able to degrade this compound [31–33]. Lack of growth observed on 3,4-DCB, as well as, sparse growth of *P. aeruginosa* CA207Ni on 2,4,6-TCB; *C. kutscheri* FL108Hg on biphenyl and 1,4-DCB; and *B. cepacia* AL96Co on 2,5-DCB were connected to the high recalcitrant nature of the compounds due to awkward positioning of the chlorine atoms on the compounds. Similar results have also been reported for *Pseudomonas* [31, 32, 34].

The data obtained from growth kinetics and the quantitative reduction of the FID area counts of PHs biomarkers, as revealed by gas chromatographic analysis, established biodegradation potentials of the bacterial strains in the presence of Ni^{2+} and Co^{2+}. Several studies have reported that Ni and Co are essential co-factor for

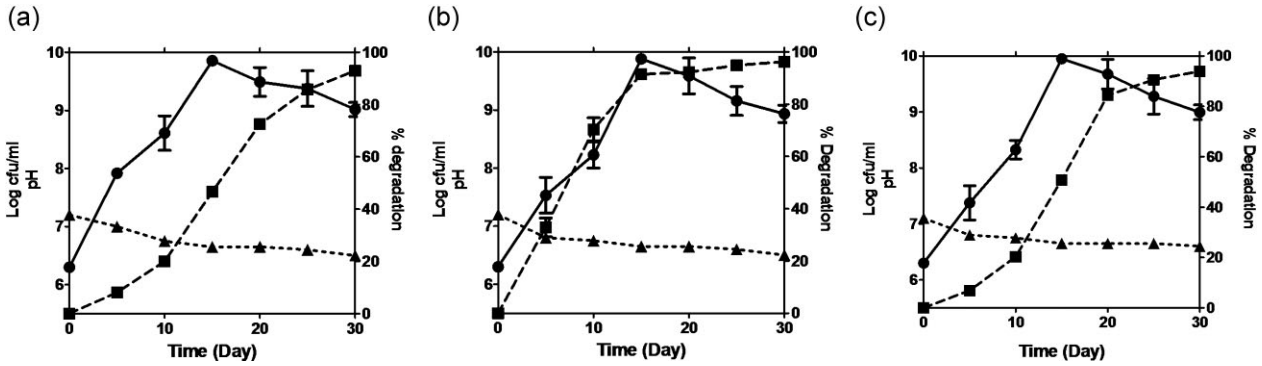

Figure 4. Time course of bacterial growth and degradation of anthracene (100 ppm). (a) *P. aeruginosa* CA207Ni; (b) *B. cepacia* AL96Co; (c) *C. kutscheri* FL108Hg. Growth (●), % degradation (■), and pH (▲). Biodegradation medium was amended with Ni^{2+} and Co^{2+} (5.0 mM each), anthracene degradation was determined and evaluated by the net decrease in GC-FID area counts in experimental cultures, and compared with that of the heat-attenuated controls. Initial substrate concentration was 100 ppm. Data values represent averages of three replicate determinations. Bars represent standard error above and below the mean of triplicate flasks.

bacterial growth and metabolism. However, high concentrations of these metals are known to exert toxicity on microorganisms [16, 35]. In this study, the bacterial strains remarkably degraded the isoprenoidal alkanes (pristine C19 and phytane C20), which are generally regarded as oil-degradation biomarkers, along with other *n*-alkanes condensed in the crude oil. Earlier on, it was reported that *P. aeruginosa* CA207Ni, *B. cepacia* AL96Co, and *C. kutscheri* FL108Hg tolerated 15 mM of Ni^{2+} (and $\geq$10 mM Co^{2+}) [24]. This high-metal-tolerance trait of the bacteria must be responsible for their triumph over Co and Ni (5.0 mM each) toxicity, while degrading PHs better than those bacteria in culture without toxic metal. For example, Chamkha *et al.* [36] reported that a strain of *Klebsiella oxytoca* degraded 75% of *n*-alkanes after 45 days without any metal toxicity, unlike in this study where 96.24–98.97% biodegradation of aliphatic alkanes were achieved in presence of Co^{2+} and Ni^{2+} within 30 days. Currently, there are few data in the literature on bacteria that circumvent inhibitory effect of toxic metals while degrading hydrocarbons [11–13]. Lin *et al.* [16] demonstrated that low concentrations of Zn^{2+} and Mn^{2+} (1–10 mg l^{-1}) stimulated biomass growth of *Ochrobactrum cytisi* on *tert*-butyl ether, unlike Ni^{2+} (1–4 mg l^{-1}) that retarded the growth. Moreover, *Brevibacterium casei* was reportedly reduced 78% Cr^{6+} along with 82% degradation of pentachlorophenol simultaneously [14]. In the contrary, Ni has been found to inhibit bacterial utilization of PHs in contaminated soils [37]. It may be concluded that lack of inhibitory effect of Ni^{2+} and Co^{2+} on the growth of the bacterial strains presently studied, was primarily due to their high tolerance to HMs. It is not farfetched, therefore, that the bacteria exhibited good cell growth in the MSM amended with 5 mM Ni^{2+} and Co^{2+}, which were less than their metal tolerance levels. Thus, enzyme synthesis towards substrate affinity and metabolism is assumed not impeded by the metal concentrations. The bacteria were observed to readily degrade the hydrocarbons in presence of the Ni and Co (at $\geq$92.94% PH degradations).

The conventional bioremediation strategy towards an environment, where HMs and hydrocarbons coexist, involves application of a consortium of metal resistant bacteria and hydrocarbon degraders [38]. Metal-resistant-bacteria are introduced to sequester the toxic metals to non-toxic level for the degraders to breakdown the hydrocarbons. In this study, the percentage degradation of PHs in presence of toxic metals has been observed to be growth associated, which compared favorably with those previously reported at lower metal concentrations [16, 28, 29, 39], and with those without toxic metal influence [26, 40]. However, concomitance metal reduction and pentachlorophenol degradation in axenic culture of *Brevibacterium casei* was reported recently [14]. Interestingly, 82% degradation of the organic compound with simultaneous Cr^{6+} reduction was reported, unlike in the present study where about 50% of the PHs were degraded along with sequestration the Ni^{2+} and Co^{2+} within the same time frame. It is noteworthy that PHs are more hydrophobic and recalcitrant than phenolic compounds, and this might be responsible for the lower degradation observed in the bacterial strains than the findings of Verma and Singh [14]. Apparently, 5.0 mM is not toxic enough to impede metabolic potential of the strains investigated, and thus incapable of retarding degradation of the PHs. It is worth mentioning that strains understudied hitherto can individually degrade PHs and simultaneously

sequester HMs. This is proven with the reduction of metal concentrations in the medium during biodegradation studies of the strains. Growth associated uptake of metals have been reported [38, 41], where *Bacillus* species were known to show rapid, pH-dependent binding of Ni and Co before bioaccumulating the HMs intracellularly in the cytoplasm [42]. The two elements needed for an efficient utilization of PHs by bacteria are the enzymes responsible for their degradation and the regulatory elements that control the expression of the catabolic operons to ensure more efficient output. The bacteria studied must have adopted one or more of the mechanisms reported by Nies [20] to sequester the HMs while harnessing some of their seemingly high numbers of homologous enzymes to metabolize the PHs.

Concluding remarks

In this study, we established that bacteria strains isolated from heavily contaminated environments individually degraded PHs and simultaneously sequester toxic metals (Ni and Co). The mineralization of the hydrocarbons in presence of the metals by the metal-resistant bacteria was assessed in batch system in chemically defined MSM. The sole sources of carbon and energy in the medium were the hydrocarbons, while the metals were supplied in ionic forms to aid bioavailability. Our results suggest that the toxic dose of the metals had no effect on the bacterial degradation of the hydrocarbons, rather, reductions in bioavailable metals were observed. The bacterial strains will be a better option in bioremediation campaign of sites co-contaminated with toxic metals and PHs. This would form part of knowledge-based approach to the selection of an inoculum to be used in bioaugmentation processes to facilitate PH degradation in PH- and HM-co-contaminated sites.

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