



# International Journal of **Biological Chemistry**

ISSN 1819-155X



Academic  
Journals Inc.

[www.academicjournals.com](http://www.academicjournals.com)

## **Influence of pH on Cadmium Toxicity To *Bacillus* Species (02 and 12) During Biodegradation of Crude Oil**

M.G. Ekpenyong and S.P. Antai  
Department of Microbiology, University of Calabar  
P.M.B. 1115, Calabar, Nigeria

---

**Abstract:** *Bacillus* sp. -02 and *Bacillus* sp. -12 produced Optical Density (OD) readings of 1.228 and 0.540, respectively at 540 nm wavelength during screen tests in crude oil/mineral salts broth medium. In addition, both organisms caused extensive shredding (breakdown) of the overlaid oil in tubes indicating oil utilization. Gravimetric measurement of hydrocarbonoclastic potentials of the organisms revealed that such potentials were similar in both species (49.8 and 48.6% weight losses, respectively). Carbon dioxide evolution studies under the influences of cadmium toxicity at concentrations of 1, 10, 100 and 1000 mg L<sup>-1</sup> and pH changes from 5.5 through 7.0 to 8.5, revealed that *Bacillus* sp. -12 produced higher amounts of CO<sub>2</sub> and was less sensitive to cadmium toxicity and pH changes during the process than *Bacillus* sp.-02. This shows that *Bacillus* sp.-12 would be useful in the bioremediation of petroleum-polluted environments co-contaminated with cadmium in the Niger Delta region of Nigeria.

**Key words:** *Bacillus* species, crude oil biodegradation, pH, cadmium

---

### **INTRODUCTION**

Biodegradation of petroleum hydrocarbons, thought to be mainly (if not entirely) aerobic (Ward *et al.*, 1980) produces as end products, innocuous substances including microbial biomass, carbon dioxide and water, which are all environment-friendly (National Research Council, 1994). These products have often been used to monitor the rate and extent of biodegradation and determine the overall success or failure of a bioremediation exercise (Malakul *et al.*, 1998; Nweke and Okpokwasili, 2003; Ijah and Antai, 2003; Gonzalez- Gil *et al.*, 1999).

The major drawback of a bioremediation exercise is the relatively slow rates at which the process occurs. Heavy metals and pH, among other implicated factors, are on the frontline of this limitation (Malakul *et al.*, 1998; Sandrin and Maier, 2002; Benka-Coker and Ekundayo, 1998). The manipulation of pH (which curiously has not been well studied) has been suggested as a possible approach to reducing heavy metal toxicity to hydrocarbon-degrading microorganisms (Sandrin and Maier, 2003).

Microorganisms play a role in producing complex products (like the formation of tarballs) from hydrocarbon metabolism, which may persist in the environment (Atlas, 1981). The synthesis of complex high molecular weight hydrocarbons, like asphaltenes, whose content Esin and Antai (2002) found to increase as biodegradation of Nigerian light crude oil progressed, would suggest that microorganisms can play a role in prolonging the impact of such environmental contaminants through biodegradative processes. Carbon dioxide evolution provides the much-needed information on the overall success or failure of a bioremediation process, since it is one of the end products of a successful biodegradation process.

---

**Corresponding Author:** M.G. Ekpenyong, Department of Microbiology,  
University of Calabar P.M.B. 1115, Calabar, Nigeria

The search for competent oil degrading bacterial populations for use in seeding petroleum-polluted environments especially in the Niger Delta region of Nigeria where oil drilling and refining activities are popular is on the increase. The selection of such populations with additional proven abilities to degrade crude oil even when concomitantly challenged with hazardous levels of toxic metals at their optimal metabolic pH is the main focus of this study.

## MATERIALS AND METHODS

The Nigerian light crude oil used for the study was obtained from Mobil Producing Nigeria Unlimited, Ibeno, Akwa Ibom State, Nigeria. The oil was filter-sterilized using Millipore 0.45 µm membrane filter and left at room temperature until required.

The bacterial cultures were isolated from water and sediment samples obtained from Qua Iboe Estuary; on mineral salts agar medium (MSM) of Zajic and Supplisson (1972), using the vapour phase transfer method of Thijsee and van der Linden (1961). The MSM contained in g L<sup>-1</sup>: K<sub>2</sub>HPO<sub>4</sub>, 1.8; KH<sub>2</sub>PO<sub>4</sub>, 1.2; NH<sub>4</sub>Cl, 4.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; NaCl, 0.1; Fe SO<sub>4</sub>·7H<sub>2</sub>O, 0.01; Agar, 15; adjusted to pH 7.2. The bacterial cultures were identified following the schemes of Cowan and Steel (1985). Concentrated stock solution of cadmium was prepared according to the method described by Asuquo *et al.* (2004). Working concentrations of 1, 10, 100 and 1000 mg L<sup>-1</sup> were prepared by serial dilutions of the concentrated stock solution in sterile deionised distilled water (Zhang and Crow, 2001).

### Screen Tests for Crude Oil Utilization by Bacterial Isolates

The mineral salts broth screen test followed the method of Okpokwasili and Okorie (1988). Screen tubes were incubated at 30°C for 16 days. Growth in tubes was scored as high (+++), moderate (++) , low (+) and no growth (-). Extents of shredding (breakdown) of overlaid oil were recorded as fine (F), moderate (M), low (B) and no shredding (NS). Optical density readings of growth in tubes were taken at the end of 16 days incubation period at a wavelength of 540 nm (Spectro 22RS: Digital Spectrophotometer; Lab. Med. Inc. England).

### Determination of Crude Oil Biodegradation Potentials of Bacteria

The rate and extent of biodegradation of Nigerian light crude oil by four bacterial isolates namely *Bacillus* sp. -02, *Pseudomonas* sp. -04, *Bacillus* sp. -10 and *Bacillus* sp. -12 were assessed using the method of Okpokwasili and Okorie (1988) and the amounts of crude oil degraded calculated by the gravimetric method of Odu (1972). Degradation study flasks were incubated at room temperature (28±2°C) for 24 days. The amounts of the crude oil left after every 6 days were determined by extracting the residual crude oil with n-hexane and noting their absorbance readings at 450 nm using HACH DR 300 Spectrophotometer and expressed as percentage weight loss of crude oil.

### Measurement of Cadmium Toxicity to *Bacillus* sp.-02 and *Bacillus* sp. -12 During Biodegradation of Crude Oil at Varying pH Levels by the Carbon Dioxide Evolution Method

The toxicities of varying concentrations of cadmium to *Bacillus* sp.-02 and *Bacillus* sp.-12 during biodegradation of Nigerian light crude oil at pH 5.5, 7.0 and 8.5 were determined by the Carbon dioxide evolution method of Cornfield (1961). Flasks containing crude oil as carbon source and different concentrations of cadmium which included 1, 10, 100 and 1000 mg L<sup>-1</sup> were incubated in triplicates at room temperature (28±2°C) after they have been connected via glass tubings to vials containing 10% w/v barium peroxide in distilled water. The barium peroxide vials served as traps for CO<sub>2</sub> evolved during the biodegradation process (Skoog *et al.*, 2004). Barium peroxide vials were also set up to trap CO<sub>2</sub> in the air of the vials and served as control 1. Other controls included; Control 2

(un-inoculated); MSM + crude oil without inoculum and heavy metal. Control 3 (inoculated): MSM + crude oil + inoculum without heavy metal.

Each bubble tower formed due to the absorption of CO<sub>2</sub> was rinsed with 20 mL of distilled water and transferred into a conical flask for titration with 1N hydrochloric acid using phenolphthalein as indicator (Skoog *et al.*, 2004). Amounts of CO<sub>2</sub> evolved during biodegradation were calculated using the formula of Stotzky (1965).

The residual hydrocarbon remaining in the flasks after 4, 8, 12 and 16 days was also determined to validate the results of the CO<sub>2</sub> evolution technique (Odu, 1972). Mean rates of CO<sub>2</sub> evolved as well as inhibition efficiencies of the different concentrations of cadmium at the different pH levels were determined from the amounts of CO<sub>2</sub> evolved during the study.

### Statistical Analysis

Data obtained from the study was subjected to analysis of variance (ANOVA) to determine whether cadmium and/or pH significantly influenced the CO<sub>2</sub> evolution process at 95% confidence limit. The amount of carbon dioxide evolved during biodegradation was also correlated with percentage weight loss of crude oil at the end of the incubation period.

## RESULTS

A total of twelve hydrocarbon-utilizing bacteria were isolated from the study samples. The bacterial genera identified were *Bacillus*, *Nocardia*, *Pseudomonas*, *Proteus*, *Micrococcus* and *Vibrio*.

*Bacillus* sp.-02 showed the highest turbidity (+++), with an optical density reading of 1.228 at a wavelength of 540 nm. The organism was also able to cause extensive shredding (breakdown) of the overlaid oil (F). Despite the low level of turbidity (+) and comparatively low OD reading of 0.540 in the tubes, *Bacillus* sp.-12 was still able to cause similar level of shredding (breakdown) of overlaid oil (F) in the tubes (Table 1).

**Table 1: Screen tests for utilization of Nigerian light crude oil by microbial isolates**

Isolate code	Turbidity	OD	Extent of oil Shredding
<i>Nocardia</i> sp.-01	+	0.297	M
<i>Bacillus</i> sp.-02	+++	1.228	F
<i>Micrococcus varians</i> -03	+	0.242	B
<i>Pseudomonas</i> sp.-04	+++	0.944	B
<i>Pseudomonas aeruginosa</i> -05	+	0.421	M
<i>Vibrio</i> sp.-06	+	0.268	NS
<i>Bacillus</i> sp.-07	+	0.495	M
<i>Nocardia</i> sp.-08	++	0.590	B
<i>Proteus mirabilis</i> -09	++	0.550	NS
<i>Bacillus subtilis</i> -10	+++	0.855	M
<i>Proteus</i> sp.-11	++	0.640	M
<i>Bacillus</i> sp.-12	+	0.540	F

+ Little growth, ++ Moderate growth, +++ Heavy growth, - No growth., OD (Optical density at 540 nm), M Moderate shredding of overlaid oil., F Extensive shredding of overlaid oil., B Low shredding of overlaid oil, NS No Shredding

**Table 2: Hydrocarbonoclastic potentials of some bacterial isolates.**

Isolate code	Weight loss (%) <sup>a</sup> of crude oil over a 24-day period			
	6	12	18	24
<i>Bacillus</i> sp.-02	19.4±0.6	35.6±0.2	42.6±0.1	49.8±0.6
<i>Pseudomonas</i> sp.-04	12.6±0.5	28.3±0.6	37.3±0.1	40.6±0.2
<i>Bacillus subtilis</i> -10	10.3±0.2	26.4±0.3	35.2±0.2	39.1±0.4
<i>Bacillus</i> sp.-12	8.4±0.5	31.2±0.3	42.7±0.2	48.6±0.3

<sup>a</sup>mean of three determinations

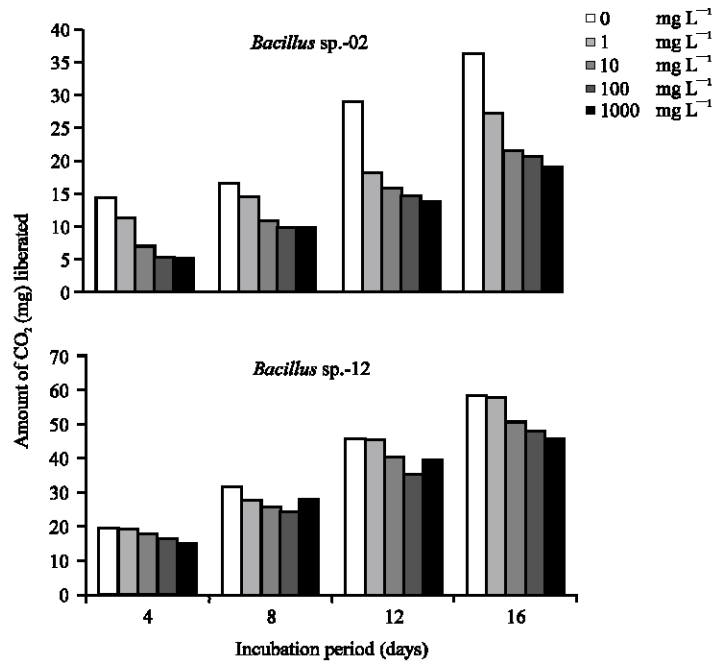


Fig. 1: Influence of cadmium on CO<sub>2</sub> evolution during biodegradation of Nigerian light crude oil at pH 5.5

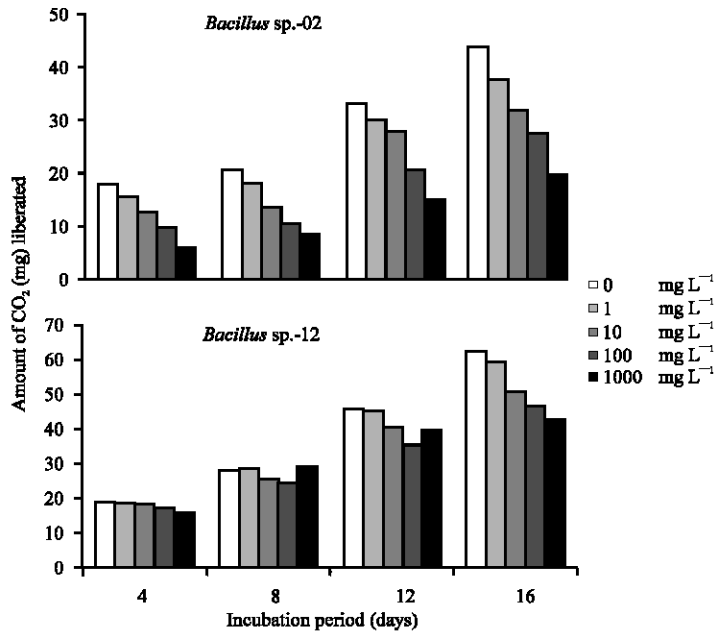


Fig. 2: Influence of cadmium on CO<sub>2</sub> evolution during biodegradation of Nigerian light crude oil at pH 7.0

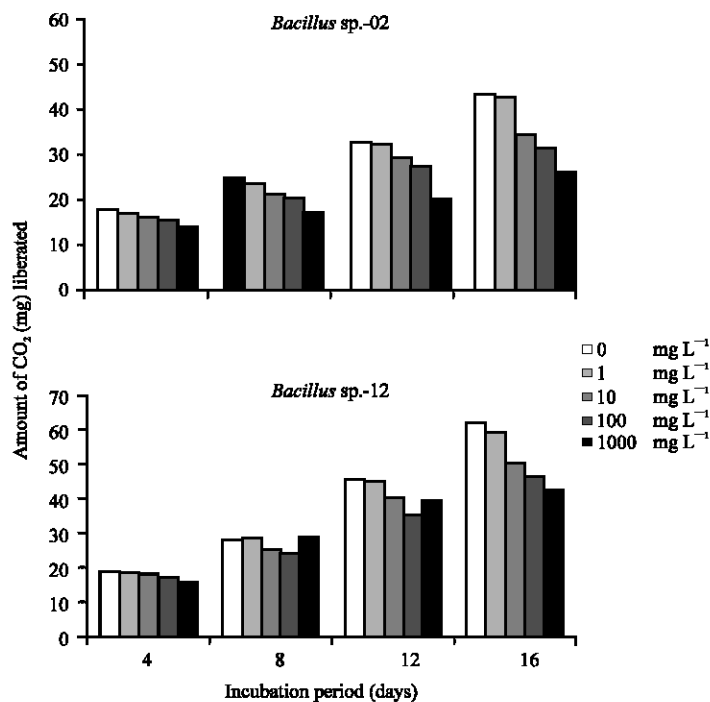


Fig. 3: Influence of cadmium on CO<sub>2</sub> evolution during biodegradation of Nigerian light crude oil at pH 8.5

The Table 2 results reveal that *Bacillus sp. -02* caused a 49.8% weight loss of crude oil in 24 days. This was closely followed by the weight loss of 48.6% caused by *Bacillus sp. -12*. *Pseudomonas sp. -04* and *Bacillus subtilis-10* recorded 40.6 and 39.1% weight losses, respectively.

From the Fig. 1-3 cumulative amounts of CO<sub>2</sub> evolved increased throughout the sampling period for both organisms. The cumulative amount of CO<sub>2</sub> evolved by *Bacillus sp. -12* at pH 5.5, 7.0 and 8.5 in the controls (without cadmium) were 58.2, 59.9 and 60.8%, respectively while those for *Bacillus sp. -02* were 36.6, 44.4 and 48.1% at pH 5.5, 7.0 and 8.5, respectively (Fig. 1-3) Cadmium toxicity to the CO<sub>2</sub> evolution process by these organisms increased with increasing concentrations of the metal at all the pH levels tested.

Figure 4 reveals that the mean rate of CO<sub>2</sub> evolution by *Bacillus sp. -02* decreased with decreasing pH (8.5>7.0>5.5) in the presence or absence of cadmium. Highest mean rate (3.55 mg day<sup>-1</sup>) of CO<sub>2</sub> evolved by this organism was observed at pH 8.5. No definite pattern was observed for *Bacillus sp. -12*, although pH 7.0 was the least favourable for CO<sub>2</sub> evolution by the organism. Highest rate of CO<sub>2</sub> evolution by this organism was 3.99 mg day<sup>-1</sup> also observed at pH 8.5. In Fig. 5, inhibition efficiencies (IE) of the different concentrations of cadmium are shown. Only 1000 mg L<sup>-1</sup> cadmium was able to cause up to 50% inhibition of CO<sub>2</sub> evolution by *Bacillus sp. -02* as observed at pH 7.0. The highest IE value observed for *Bacillus sp. -12* was 31% caused by 1000mg L<sup>-1</sup> cadmium at pH 7.0.

The Table 3 reveals that cumulative amounts of crude oil lost as a result of biodegradation by *Bacillus sp. -02* were higher in the control and at cadmium concentrations of 1, 10 and 100 mg L<sup>-1</sup> cadmium (51.3, 40, 34.3 and 30.1%, respectively) than those by *Bacillus sp. -12* (43.3, 40.1, 31 and 26.9%).

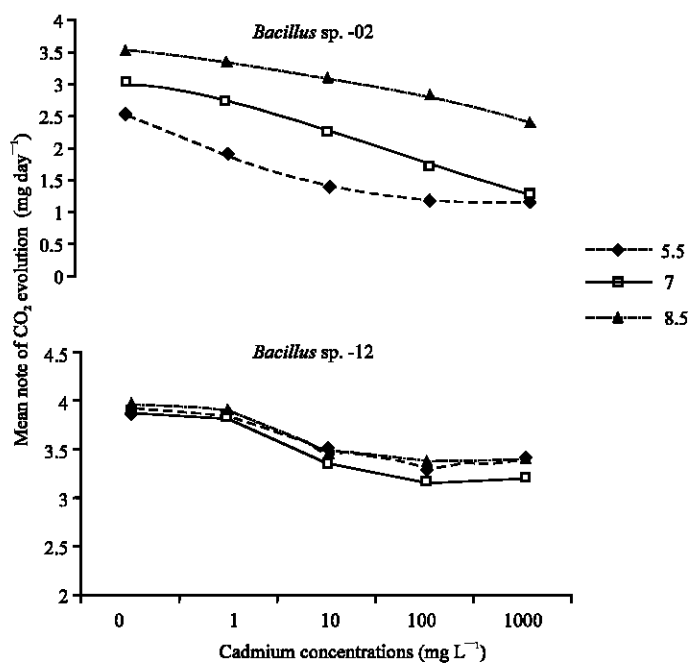


Fig. 4: Influence of cadmium on mean rate of CO<sub>2</sub> evolution during biodegradation of Nigerian light crude oil at varying pH levels

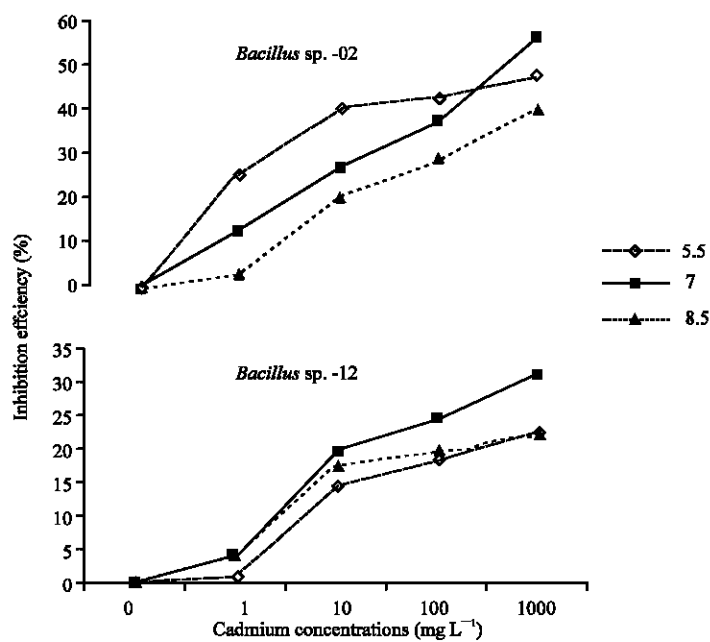


Fig. 5: Cadmium inhibition efficiency of CO<sub>2</sub> evolution during biodegradation of Nigerian light crude oil at varying pH levels

Table 3: Influence of cadmium on weight loss of crude oil during biodegradation by species of *Bacillus*

Incubation period (days)	Percentage weight loss of crude oil at varying cadmium concentrations (mg L <sup>-1</sup> ) at pH 8.5									
	0.0	0.0	1.0	1.0	10.0	10.0	100.0	100.0	1000.0	1000.0
	A	B	A	B	A	B	A	B	A	B
4	12.4	5.7	10.3	5.4	10.9	4.9	9.3	4.6	5.0	4.8
8	22.3	10.3	19.3	9.3	17.7	8.5	13.2	8.0	6.7	9.6
12	36.8	31.8	33.4	26.7	30.1	16.2	27.4	15.2	10.2	18.2
16	51.3	43.3	40.0	40.1	34.2	31.0	30.1	26.9	14.3	25.4

Key: A. *Bacillus* sp. -02 B. *Bacillus* sp. -12

## DISCUSSION

*Bacillus* species constituted about 33% of the total hydrocarbon-utilizing bacteria (HUB) isolated from water and sediment samples obtained from the Qua Iboe River estuary in the Niger Delta region of Nigeria. Other genera isolated were *Micrococcus* and *Vibrio* (8.3% each), *Pseudomonas*, *Proteus* and *Nocardia* (16.7% each). This shows that *Bacillus* species are actively involved in the natural purification of oil-polluted ecosystems and so differs from the reports of Bossert and Bartha (1984), who observed that spore-forming bacteria in general have negligible role in oil biodegradation. The result is however supported by the work of Atlas (1992), Antai, (1990), Esin and Antai, (2002) and Itah and Essien, (2005), who observed that species of *Bacillus* show promise as good bioremediating microorganisms

It was interesting to observe that *Bacillus* sp.-12, which produced low level of turbidity (+) in screen test tubes and low OD reading of 0.540 could cause the same level of shredding (F) of the overlaid oil as *Bacillus* sp. -02 with high turbidity (+++) and high optical density reading of 1.228 in the screen test tubes. This prompted the use of the gravimetric method to assess properly the actual biodegradation potentials of the two species of *Bacillus*. Despite its impressive turbidity during screen tests, *Bacillus* sp. -02 could cause 49.8% weight loss of crude oil after 24 days as against 48.6% by *Bacillus* sp. -12. This observation was earlier made by Itah and Essien (2005), for which they argued that the ability of microorganisms to produce turbidity in culture media does not necessarily indicate efficient hydrocarbon-degrading potential. They reasoned that such capability is determined by the ability of the organism to elaborate the vital enzymes required for decomposition of the recalcitrant components of the hydrocarbons, rather than being nutritionally fastidious. The results of the influences of varying concentrations (1, 10, 100 and 1000 mg L<sup>-1</sup>) of cadmium at varying pH (5.5, 7.0 and 8.5) levels on the amount of carbon dioxide evolved during biodegradation of crude oil by *Bacillus* sp. -02 and 12 reveal that cumulative amounts of carbon dioxide evolved increased throughout the sampling period, at all pH levels tested, for both isolates, whether in the presence or absence of cadmium. The results obtained show that the amount of CO<sub>2</sub> evolved depended on pH during biodegradation by *Bacillus* sp. -02. However, analysis of variance (ANOVA) of data revealed that there was no significant (p>0.05) difference in the amounts of carbon dioxide evolved during the same process by *Bacillus* sp. -12 among the pH levels tested. *Bacillus* sp. -02 was found to cause the liberation of the highest amount of CO<sub>2</sub> at pH 8.5 (alkaline) against the least amount at pH 5.5 (acidic). This indicates the preference for alkaline pH by this particular species of *Bacillus* during oil biodegradation. Generally, cumulative amounts of CO<sub>2</sub> evolved decreased with increasing cadmium concentrations at all pH levels.

In order to validate the results of the carbon dioxide evolution technique, the amounts of crude oil lost during oil biodegradation were determined (Table 3) and results were found to be strongly correlated (r = 0.96) with the amounts of CO<sub>2</sub> evolved during oil biodegradation. However, on careful examination of the data obtained, it was observed that, although *Bacillus* sp. -02 was able to cause



higher percentage weight losses of crude oil than *Bacillus* sp. -12, generally at all the pH levels tested without cadmium and particularly at pH 8.5 even in the presence of cadmium, it produced comparatively lower amounts of CO<sub>2</sub> during the degradation process. This may indicate that the enzyme systems for oil degradation are quite distinct from those for carbon dioxide production.

*Bacillus* sp. -02 was found to be more sensitive to cadmium than *Bacillus* sp. -12. More than fifty-percent inhibition of CO<sub>2</sub> evolution process was produced at a concentration of 100 mg L<sup>-1</sup> cadmium at pH 7.0, as against a 31.0% inhibition of CO<sub>2</sub> evolution at the same pH, in the presence of 1000 mg L<sup>-1</sup> in *Bacillus* sp.-12. The later organism would therefore be useful in the bioremediation of petroleum-polluted environments co-contaminated with cadmium in the Niger Delta region of Nigeria.

## REFERENCES

- Antai, S.P., 1990. Biodegradation of Bonny light crude oil by *Bacillus* sp. and *Pseudomonas* sp. *Waste Manage.*, 10: 61-64.
- Asuquo, F.E., I. Ewa-obo, E.F. Asuquo and P.J. Udo, 2004. Fish species used as biomarker for heavy metal and hydrocarbon contamination for cross river, Nigeria. *The Environmentalist*, 24: 29 -37.
- Atlas, R.M., 1981. Microbial degradation of petroleum hydrocarbons: An environmental perspective. *Microbiol Rev.*, 45: 180-209.
- Atlas, R.M., 1992. Petroleum Microbiology, *Encyclopedia of Microbiology*. Academic Press Inc, New York, pp: 362-369.
- Benka-Coker, M.O. and J.A. Ekundayo, 1998. Effects of heavy metals on growth of species of *Micrococcus* and *Pseudomonas* in a crude oil/mineral salts medium. *Biores. Technol.*, 66: 241-245.
- Bossert, I. and R. Bartha, 1984. The Fate of Petroleum in Soil Ecosystems, In: *Petroleum Microbiology*. Atlas, R.M. (Ed.), Macmillan Publishing Company New York, pp: 435-437.
- Cornfield, A.H., 1961. A simple Technique Determining mineralization of carbon during incubation of soils treated with organic material. *Plant and Soil*, 9: 10-93.
- Cowan, S.T. and K.J. Steel, 1985. *Manual for the Identification of Medical Bacteria*. 2nd Edn., Cambridge University Press.
- Esin, A.A. and S.P. Antai, 2002. Biodegradability of the major components of Bonny light crude oil by *Bacillus subtilis*. *Global J. Pure Applied Sci.*, 8: 215-222.
- Gonzalez, Gil, G.K. Kleerebezem and G. Lettinga, 1999. Effects of Nickel and Cobalt on kinetics of methanol conversion by methanogenic sludge as Assessed by on-line CH<sub>4</sub> Monitoring. *Applied Environ. Microbiol.*, 65: 1789-1793.
- Ijah, U.J.J. and S.P. Antai, 2003. The potential use of Chicken-drop Microorganisms for oil spill remediation. *The Environmentalist*, 23: 89-95.
- Itah, A.Y. and J.P. Essien, 2005. Growth profile and hydrocarbonoclastic potential of microorganisms isolated from tarballs in the Bight of Bonny, Nigeria. *World J. Microbiol. Biotechnol.*, 21: 1317-1322.
- Malakul, P., K.R. Srinivasan and H.Y. Wang, 1998. Metal toxicity reduction in naphthalene biodegradation by use of metal chelating adsorbents. *Applied Environ. Microbiol.*, 64: 4610-4613.
- National Research Council, 1994. *Alternatives for Groundwater Cleanup*. Washington, DC: National Academy Press.
- Nweke, C.O. and G.C. Okpokwasili, 2003. Drilling fluid base oil biodegradation potential of a soil *Staphylococcus* species. *Afr. J. Bacteriol.*, 2: 293-295.

- Odu, C.T.I., 1972. Microbiology of soils contaminated with petroleum hydrocarbons, III. Natural rehabilitation and reclamation of soils affected. Institute of Petroleum Technol. Publication, pp: 77- 102.
- Okpokwasili, G.C. and B.B. Okorie, 1988. Biodeterioration potentials of organisms isolated from car-engine lubricating oil. *Tribol.I.*, 21: 215-220.
- Sandrin, T.R. and R.M. Maier, 2002. Effect of pH on cadmium toxicity, speciation and accumulation during naphthalene biodegradation. *Environ. Toxicol. Chem.* 21: 2075-2079.
- Sandrin, T.R. and R.M. Maier, 2003. Impact of metals on the Biodegradation of Organic Pollutants. *Environ. Health Perspect*, 111: 1093-1101.
- Skoog, D.A., D.M. West, F.J. Holler and S.R. Crouch, 2004. *Fundamentals of Analytical Chemistry*, 8th Edn. Brooks/Cole, U.K., pp: 337-349.
- Stotzky, C., 1965. Determination of Carbon Dioxide. In: *Methods of Soil Analysis Part 2; Chemical and Microbiological Properties*. Madison, C.A.B (Ed.). American Society of Agronomy Inc.
- Thijsee, G.J.E. and A.C. van der Linden, 1961. Iso-alkane oxidation by a *Pseudomonas*. *Antonie van leeuwenhoek*, 27: 171-179.
- Ward, D.M., R. M. Atlas, P.D. Boehm. and J.A. Calder, 1980. Biodegradation and Chemical evolution of oil from the Amoco Microbial Spill. *Ambio: A J. Human Environ. Res. Manag.*, 9: 277-283.
- Zajic, J.E. and B.Supplisson, 1972. Emulsification and degradation of Bunker C fuel oil by microorganisms. *Biotechnol. Bioengi.*, 14: 331-334.
- Zhang, S. and S.A. Crow Jr., 2001. Toxic effects of Ag (I) and Hg (II) on *Candida albicans* and *C. maltosa*: A flow cytometric evaluation. *Applied Environ. Microbiol.*, 67: 4030-4035.