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Phenol and Heavy Metal Tolerance Among Petroleum Refinery Effluent Bacteria

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ABSTRACT:

Bacterial isolates from petroleum refinery effluent were evaluated for growth in increasing doses of phenol and heavy metal ions. All the test organisms were able to grow in mineral salt medium with phenol concentration of 15.0 mM (≈ 1412.0 mg/l) except *Pseudomonas* sp. RBD3. *Aeromonas* sp. RBD4, *Staphylococcus* sp. RBD5 and *Pseudomonas* sp. RBD10 showed the highest tolerance to 15.0 mM of phenol followed by *Corynebacterium* sp. RBD7 while Escherichia coli RBD2 and *Citrobacter* sp. RBD8 showed the least tolerance to 15.0 mM of phenol. The minimum inhibitory concentrations (MICs) ranged from 1.0 mM for mercury and 4.5 mM for chromium, nickel, lead and copper. The bacterial strains were most susceptible to mercury toxicity. Viable counts of the organism on mineral salt-phenol agar showed a typical growth pattern for inhibitory substrate. The threshold concentration is 0.5 mM for *Bacillus* sp. RBD1, *Escherichia coli* RBD2, *Bacillus* sp. RBD6, *Citrobacter* sp. RBD8, Streptococcus sp. RBD9, *Pseudomonas* sp. RBD11 and *Escherichia coli* RBD12 and 1.0 mM for *Pseudomonas* sp. RBD3, *Aeromonas* sp. RBD4, *Staphylococcus* sp. RBD5, *Corynebacterium* sp. RBD7 and *Corynebacterium* sp. RBD10. The results suggest that microorganisms isolated from petroleum refinery effluent are potentially useful for detoxification of phenol impacted systems in the presence of heavy metals.

Keywords:
Phenol, heavy metals, refinery effluent bacteria.

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INTRODUCTION

Petroleum refinery effluents are wastes liquids that resulted from the refining of crude oil in petroleum refinery. The effluents are composed of oil and grease along with many other toxic organic and inorganic compounds (Diya'uddeen *et al.,* 2011). Among the toxic components of these effluents are heavy metals. Heavy metals include cobalt, chromium, nickel, iron, manganese, zinc, etc. They usually form complexes with different non metal donor atoms which account for their participation in various microbial metabolisms in the environment (Kamnev, 2003). Some of these heavy metals such as cobalt, chromium, nickel, iron manganese, zinc, etc. are required in trace amount by microorganisms at low concentration as nutrients, since they provide vital co-factors for metalloproteins and enzymes and are known as essential metals while others such as cadmium, mercury, lead, etc have no physiological functions and are known as nonessential metals (Sevgi *et al.,* 2010). At high concentration both essential and nonessential heavy metals exert an inhibitory action on microorganisms by impairing the essential functional groups as well as modifying the active conformation of biological molecules. This results in reduction of microbial activity leading to increased lag phase as well as slow growth rate (Aleem *et al.,* 2003).

It is expected that petroleum refinery effluents will contain some of these metals in reasonable quantity as well as aromatic compounds such as phenols. Organic and inorganic mixed pollutants are known to be commonly present in industrial effluents and also other contaminated sites. In this case, apart from affecting the viability of the microbiota, the metal activity may have synergistic effect on biodegradation processes of the aromatic compounds. Thus studies related to the association of the bacterial tolerance properties to metals and degradation of phenolic compounds may be relevant to applications in bioremediation processes (Silva *et al.,* 2007).

Discharge of these metals into natural waters at increased concentration in refining operations can have severe toxicological effects on aquatic environment and humans. Heavy metals as well as phenol are known to be harmful pollutants emanating from industrial wastewaters that have negative effects on microorganisms. These metals are in the form of inorganic and metallo-organic compounds while phenol appears to be a soluble component of the industrial effluents (Nwanyanwu and Abu, 2010; Hernandez *et al.*, 1998). These environmental pollutants which are environmentally mobile tend to accumulate in organisms, and become persistent because of their chemical stability or poor biodegradability (Emoyan *et al.*, 2005). Contamination of wastewater with high concentration of heavy metals caused a significant decrease in the numbers of bacteria in biological system (Otokunefor and Obiukwu, 2005). It is obvious that heavy metals are one of the toxic contaminants in wastewaters and causes disorder in biological wastewater treatment (Sa'idi, 2010). Microorganisms being ubiquitous in nature have been reported to be found in inhospitable habitats such as petroleum refinery effluents, coke effluents, etc (El-Sayed *et al.*, 2003; Hidalgo *et al.*, 2002) as the effluents are characterized by the presence of phenols, metal derivatives, surface active substances and other chemicals (Suleimanov,1995). Bruins *et al.,* (2000) in their work reported that organisms in such inhospitable environment must have developed metal resistance systems in an attempt to protect sensitive cellular components. On the other hand, utilization of phenol and other pollutant is enhanced by adaptation and production of appropriate enzymes by organisms for the removal of the toxicants (Nwanyanwu *et al.,* 2012).

This study investigated the tolerance to heavy metals and phenol by bacterial population in petroleum refinery effluent.

MATERIALS AND METHODS

Sample collection

Petroleum oil refinery effluent was collected from Biological treatment plant unit (Rotary biodisk, RBD) of Port Harcourt oil refinery complex and transported to the laboratory for physicochemical analysis which includes pH, total dissolved solids, biological oxygen demand (BOD), chemical oxygen demand (COD), phosphate (PO_4), nitrate (NO_3), oil and grease, phenol, electrical conductivity and heavy metals content. The methods used for the analysis were as shown elsewhere (Nwanyanwu *et al.,* 2012).

Microbiological analysis

Microbiological counts were estimated by plating 0.1 ml of the 10^2 - 10^6 decimally diluted effluent samples in physiological saline on appropriate agar plates. Total heterotrophic bacterial count was done on nutrient agar plates while phenol-utilizing bacterial count was done on phenol-agar medium of Hill and Robinson (1975). The inoculated plates were incubated for 24 h at 30°C for the heterotrophic bacterial count and 72 h for phenol-utilizing bacteria count.

Isolation and identification of bacterial strains

The discrete bacterial colonies that developed on phenol-agar medium were purified, characterized biochemically and identified as described by Nwanyanwu *et al.,* (2012).

Preparation of inoculum

The organisms were grown in nutrient broth medium contained in Erlenmeyer flasks (100 ml) at 28±2°C for 48 h. Thereafter, the cells were harvested and washed in sterile deionized distilled water. The cell suspensions were standardized by adjusting the turbidity to an optical density of 0.1 at A_{540}.

Screening of isolates for phenol tolerance

Into 5.0 ml mineral salt broth medium contained in 15.0 ml screw capped glass culture tubes were added aliquots of phenol stock solution (200 mM). The tubes were sterilized by autoclaving at 121°C for 15min and allowed to cool at room temperature (28±2°C). Thereafter, 0.1 ml aliquot of cell suspensions were seeded into the tubes and incubated at 30°C for 96 h. The final concentrations of phenol in the tubes ranged from 0.1-100 mM. Controls included cells in mineral salt medium without phenol and mineral salt medium supplemented with phenol but without cells. Development of turbid culture depicted tolerance to phenol stress. Isolates that exhibited phenol tolerance from 5.0 mM and above were used for further phenol and heavy metal toxicity assay.

Table1: Physicochemical and microbiological analyses of biological treatment unit of petroleum refinery wastewater

Parameter/ unit	Value
pH	8.18
Elect. conduct (µs/cm)	485
Oil and grease (mg/l)	15.0
TDS (mg/l)	250
BOD (mg/l)	8.0
COD (mg/l)	76.0
Phenol (mg/l)	13.6
PO_4^{2-} (mg/l)	0.14
NO_3^- (mg/l)	1.20
Metal concentration	
Zn^{2+} (mg/l)	0.02
Cu^{2+} (mg/l)	<0.02
Cr^{2+} (mg/l)	0.05
Pb^{3+} (mg/l)	<0.01
Ni^{2+} (mg/l)	0.02
Cd^{2+} (mg/l)	<0.01
Microbial load	
THBC (CFU/ml)	2.52×10^8
TPUBC (CFU/ml)	1.14×10^8
% TPUBC (%)	45.24

THBC = Total Heterotrophic bacterial count;
TPUBC = Total phenol-utilizing bacterial count

Growth on phenol-mineral salt agar

The isolates were tested for their ability to grow on mineral salt agar medium (MSM) amended with increasing phenol concentrations. An aliquot (100 µl) of decimally diluted standardized inoculum of each isolate in physiological saline was spread plated onto surface of MSM plates with 2.0-20 mM of phenol concentrations. Control included cells in MSM plates without phenol. The culture was incubated at 30^{o}C for 72 h (Kahru *et al*, 2002). The number of the colony that developed was enumerated as colony forming unit per ml (CFU/ml).

Minimum inhibitory concentration (MIC) determination

Stock solutions of Cd, Zn, Hg, Cu, Pb, Ni, Co and Cr as salts of $CdCl_2$, $ZnSO_4$, $HgCl_2$, $CuSO_4$, $PbCl_2$, $Ni(NO_3)_2$, $CoCl_2.6H_2O$ and $K_2Cr_2O_7$ were prepared in deionized distilled water. All the chemicals used were analytical reagent grade.

The minimum inhibitory concentrations (MIC) of eight heavy metal ions at which no growth was observed were determined at pH 7.2 against each bacterial isolate using tube dilution method (Hassen *et al*., 1998) with little modifications. Graded concentrations of each heavy metal ranging from 0.05 mM to 10.0 mM were prepared in tryptone soy broth (TSB) contained in screw capped culture tubes. The supplemented TSB-heavy metal medium was sterilized by autoclaving at 121^{o}C for 15 min. On cooling to room temperature (28±2^{o}C), the tubes were seeded with 100 µl of the bacterial suspension and incubated at 30^{o}C for 72 h. Inoculated medium free of heavy metal ions and uninoculated medium with metal ions served as positive and negative controls respectively. The MIC of the metal to the test isolates is the lowest concentration that totally inhibited growth of the organisms.

RESULTS AND DISCUSSION

The physicochemical and microbiological properties of the petroleum refinery effluent are shown in Table 1. Phenol-utilizing bacteria represented 45.24% of the microbial load of biodisk effluent. The high population of phenol-utilizing bacteria obtained could be related to natural selection and adaptation to phenol at the unit. The concentration of heavy metals in the effluent present in the effluent may be as a result of physicochemical treatment (oxidation and reduction, chemical precipitation, etc) given to the raw wastewater before been channeled into the biological treatment unit.

The result of screen test for phenol tolerance is shown in Table 2. With the exception of *Pseudomonas* sp. RBD3 that tolerated phenol up to 10 mM, all the organisms are able to tolerate phenol stress up to 15.0 mM. The growth of the isolates in the medium with phenol concentrations above 10.0 mM may be attributed to previous exposure to phenolic raw wastewater influent into the biological treatment unit (RBD). This is in line with the report of Santos *et al*., (2001) in which they related the growth of *Trichosporom* sp. in phenolic amended medium of 10.0 mM concentration to previous phenolic wastewater shock load from stainless steel industry. Moreso, the tolerance of the organisms to high concentration of phenol (15.0 mM) may be the ease with which the isolates open the phenol ring for its subsequent uptake as carbon and energy source (Ajaz *et al.,* 2004). Gurujeyalakshmi and Oriel (1989) in their work have reported that *Bacillus stearothermophilus* strain BR219 could grow on phenol at levels up to 15 mM. In contrast, growth inhibition of *Bacillus*, *Pseudomonas* and *Citrobacter* species at phenol concentration above 1.0 mM has been reported by many authors (Obiukwu and Abu, 2011). Janke *et al.,* (1981) reported inhibition of phenol hydroxylase activity in *Pseudomonas* species at 0.25 mM phenol concentration. Yang and Humphrey (1975) found that the growth of *Pseudomonas putida* was strongly inhibited above phenol concentration of 0.5 mM. Buswell and Twomey (1975) reported that growth of

Table 2: Phenol tolerance of the test isolates in different concentrations of phenol

Bacteria	Growth in mineral salt broth with added phenol										
	Phenol concentration (mM)										
	0.1	0.2	0.5	1	2	5	10	15	20	50	100
Bacillus sp. RBD1	+	+	+	+	+	+	+	+	-	-	-
Escherichia coli RBD 2	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp. RBD 3	+	+	+	+	+	+	+	-	-	-	-
Aeromonas sp. RBD 4	+	+	+	+	+	+	+	+	-	-	-
Staphylococcus sp. RBD 5	+	+	+	+	+	+	+	+	-	-	-
Bacillus sp. RBD 6	+	+	+	+	+	+	+	+	-	-	-
Corynebacterium sp. RBD7	+	+	+	+	+	+	+	+	-	-	-
Citrobacter sp. RBD8	+	+	+	+	+	+	+	+	-	-	-
Streptococcus sp. RBD9	+	+	+	+	+	+	+	+	-	-	-
Pseudomonas sp. RBD10	+	+	+	+	+	+	+	+	-	-	-
Corynebacterium sp. RBD11	+	+	+	+	+	+	+	+	-	-	-
Escherichia coli RBD12	+	+	+	+	+	+	+	+	-	-	-

+ = growth ; - = no growth

Bacillus stearothemophilus was inhibited at phenol concentration above 5.0 mM.

The effect of increasing doses of phenol (0.05 - 15.0 mM) on the population of the test organisms are shown in Figure 1. Generally, the viable counts increased with the concentration of phenol until a certain concentration when the growth of the organisms was inhibited. The growth of the organisms on phenol followed a substrate inhibition pattern. Increasing phenol concentration resulted in decrease in microbial growth and eventually very minimal growth was detected at the highest phenol concentration (15.0 mM) in all the test organisms. The growth of *Bacillus* sp. RBD1, *Escherichia coli* RBD2, *Bacillus* sp. RBD6, *Citrobacter* sp. RBD8, *Streptococcus* sp. RBD9, *Pseudomonas* sp. RBD11 and *Escherichia coli* RBD12 with a total viable count of 7.1 x 10^6, 8.0 x 10^6, 7.2 x 10^6, 7.8 x10^6, 7.5 x 10^6, 8.8 x 10^6, 7.4 x 10^6 and 7.4 x 10^6 CFU/ml respectively were stimulated at phenol concentrations up to 0.5 mM (≈ 47.06 mg/l). Similarly, at phenol concentration up to 1.0 mM (≈ 94.11 mg/l), the growth of *Pseudomonas* sp. RBD3, *Aeromonas* sp. RBD4, *Staphylococcus* sp. RBD5, *Corynebacterium* sp. RBD7 and *Corynebacterium* sp. RBD10 with a total viable count of 8.4 x 10^6, 7.5 x 10^6, 7.2 x 10^6 and 8.4 x 10^6 CFU/ml respectively, were stimulated. Thereafter, the total viable counts progressively decreased as the phenol concentration increases. This growth pattern is typical of in an inhibitory substrate like phenol. The inhibition of bacterial growth by phenol is well-documented. However, some bacteria are more tolerant to phenol than others. For instance, the growth inhibition constant (K_i) for bacteria degrading phenol have been reported as 54.1mg/l (0.57 mM) (Monteiro *et al*., 2000), 129.79 mg/l (1.379 mM) (Kumar *et al.,* 2005), 2434.7 mg/l (25.87 mM) (Arutchelvan *et al*., 2006) and 7.818 mM (Wei *et al*., 2008). In this study, all the test organisms tolerated phenol up to 10.0 mM (≈ 941 mg/l) and with the exception of *Pseudomonas* sp. RBD 3, all the bacterial strains tolerated 15 mM (≈ 1412 mg/l). This is in line with the report of Worden *et al.,* (1991) that *Bacillus stearothermophilus* BR219

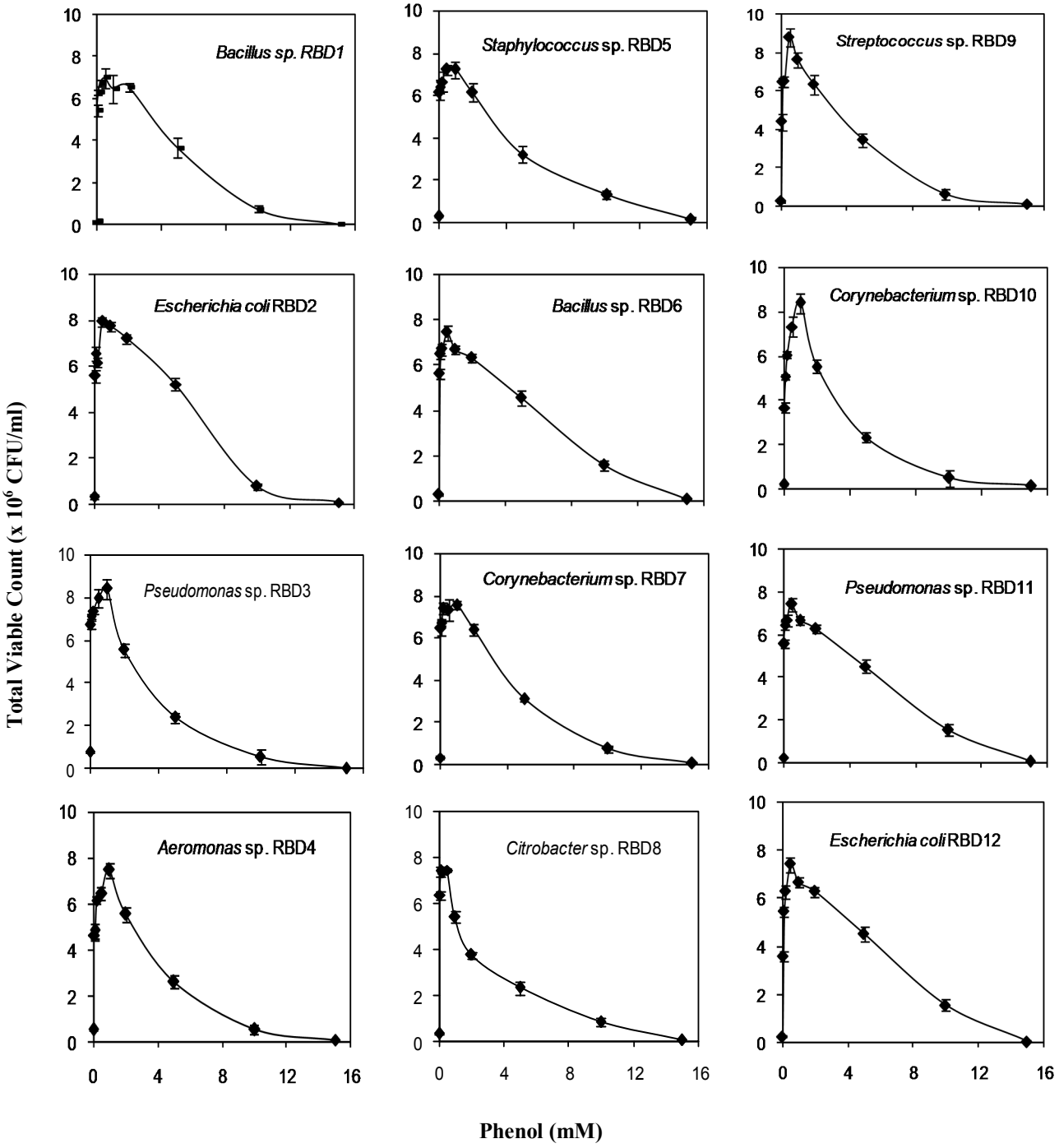

Figure 1: Growth of bacteria on mineral salt agar medium supplemented with increasing doses of phenol.

tolerated phenol concentration of 15.0 mM. Similarly, *Corynebacterium* species was reported to resist 15 mM phenol while *Staphylococcus, Corynebacterium, Bacillus* and *Proteus* were found to resist 10 mM of phenol (Ajaz *et al*., 2004). However, many authors have reported inhibition of microorganisms at such high phenol concentration (Hossein and Hill, 2006; Kotturi *et al*, 1991). Li and Humphrey (1989) as well as Gurujeyalakshmi and Oriel (1989) have reported microbial growth inhibition at relatively low concentrations of 2.0 mM and 0.25 mM respectively.

Table 3: Minimal inhibitory concentrations of heavy metals

Organism	MIC of metal (mM)							
	Cd	Zn	Hg	Cu	Pb	Ni	Co	Cr
Bacillus sp. RBD1	3.5	2.0	1.5	4.0	4.5	3.5	2.0	4.0
Escherichia coli RBD2	3.5	2.5	1.0	4.0	3.0	4.0	2.5	3.5
Pseudomonas sp. RBD3	4.0	3.0	1.5	4.5	3.0	4.5	3.0	4.5
Aeromonas sp. RBD4	3.5	3.0	1.0	3.0	4.0	4.0	2.0	4.0
Staphylococcus sp. RBD5	4.0	2.0	1.0	3.5	3.0	4.0	3.0	4.0
Bacillus sp. RBD6	3.0	2.5	1.5	3.5	4.0	4.0	3.0	4.0
Corynebacterium sp. RBD7	3.0	2.0	1.5	3.0	3.5	3.5	2.5	3.5
Citrobacter sp. RBD8	3.5	1.5	1.0	2.5	3.0	3.0	2.0	2.5
Streptococcus sp. RBD9	4.0	2.0	1.0	3.5	2.5	4.0	3.0	2.5
Pseudomonas sp. RBD10	3.5	3.0	1.5	4.5	4.0	4.5	3.5	4.0
Corynebacterium sp. RBD11	2.5	2.0	1.0	4.0	4.0	3.0	4.0	4.5
Escherichia coli RBD12	3.0	1.5	1.0	3.0	3.5	3.5	2.5	3.0

The tolerance levels of refinery wastewater phenol-utilizing bacteria to heavy metals expressed as minimal inhibitory concentrations (MIC) are shown in Table 3. The test isolates in this study showed similar trend of susceptibilities to heavy metal ions based on minimal inhibitory assay. The high MIC values obtained in the study may be as a result of long term exposure of the organisms to metal ions in the refinery effluent. Highest MIC values were exhibited in Chromium, Copper and Nickel while the least MIC was shown in mercury among the isolates with a maximum value of >3.0 mM and minimum value of <2.0 mM. *Pseudomonas* sp. RBD3 showed maximum MICs value range of 1.5 - 4.5 mM whilst *Escherichia coli* RBD12 showed minimum MICs value range of 1.0 - 3.5 mM in all the metals tested. The MICs are higher than that reported by El-Deeb **(**2009) for some phenol-degrading bacteria. However, the MIC values are similar to the values reported elsewhere (Nieto *et al*., 1989, Nweke *et al*., 2006a, Akinbowale *et al.,* 2007). The MIC of metal ranging from 0.5 - 2.5 mM, 1.25 - 2.5 mM, 5.0 - 12.0 mM, 1.0 - 1.25 mM, 0.25 - 1.0 mM and 1.25 - 5.0 mM against hydrocarbon-utilizing bacteria was reported for cadmium, chromium, lead, cobalt, mercury and copper respectively (Nweke *et al*., 2006a). These reported MICs in most cases corroborates the values observed in this study. The MIC in growth inhibition assay is analogous to the concentration of metal ion that exhibited 100 % inhibition in dehydrogenase activity assay. Thus, the MIC of zinc against river water planktonic bacteria have been reported as 1.558 ± 0.037 mM, 1.283 ± 0.068 mM, 2.469 ± 0.045 mM and 1.328 ± 0.094 mM for *Escherichia*, *Proteus*, *Micrococcus* and *Pseudomonas* species respectively (Nweke *et al.,* 2006b). Likewise, the concentration of zinc that gave 100% inhibition of dehydrogenase activity in sediment *Bacillus* and *Arthrobacter* species are 1.442 ± 0.062 mM and 1.199 ± 0.042 mM respectively (Nweke *et al*., 2007). Also, Hassen *et al.,* (1998) have reported MIC values of 0.1, 0.8, 1.5, 1.6 and 1.8 mM for Mercury, Cobalt, Zinc and Cadmium, Copper and Chromium respectively on *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Staphylococcus aureus*, *Streptococcus* sp. and *Bacillus thurieniensis*. Hassen *et al.,* (1998) in their work reported 3.0 mM chromium as the MIC for

Pseudomonas aeruginosa S8 and *Citrobacter freundii* S24. The variation in the tolerance of heavy metal could be attributed to the bacterial strain involved, assay technique or culture conditions. However, the study has proved that heavy metals such as mercury, zinc and lead do indeed have toxic effect on bacteria. Although it may vary from one species to another, there is no doubt that heavy metals do inhibit bacterial growth.

Metals as toxic contaminants of various environmental sites have been reported to have adversely affected potential biodegradation processes occurring in the environment (Said and Lewis, 1991). Amor *et al.,* (2001) reported that the level of metal inhibition of microbial growth depends on concentration as well as nature of the metal and the type of microbial species. Sandrin and Maier (2003) reported that metals such as copper, zinc, cadmium, chromium, nickel, mercury and lead are known to inhibit biodegradation of organic pollutants by microorganisms. Phenol biodegradation have also been reported to be inhibited by metals (Nakamura and Sawada, 2000; Alves de Lima *et al*., 2007, El-Deeb, 2009). Due to accumulative behaviour of heavy metals, the effluents from petroleum refinery industries could constitute enriched media to propagate and spread microbial populations which are resistant to metallic ions. Thus, microorganisms isolated from petroleum refinery effluent having combined abilities to grow in high concentration of phenol medium and resistance to metals is potentially useful for detoxification of phenolic wastewater co-contaminated with heavy metals.

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