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Screening for Crude Oil Degrading Bacteria in Liquid Organic Waste (Effluent Samples)

Azuka Ramanus Akpe*, Afe Omolola Ekundayo and Frederick Ikechukwu Esumeh
Department of Microbiology, Ambrose Alli University, P. M. B. 14 Ekpoma, Edo State, Nigeria

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Abstract. The screening for crude oil degrading bacteria in some liquid organic wastes (cassava mill effluents, rubber effluents and oil palm mill effluents) was carried out. Hydrocarbon utilising bacteria were isolated on mineral salt agar using vapour phase technique. The samples yielded 20 bacterial isolates from 13 different genera. Cassava mill effluent and rubber effluent had the highest number (7), while oil palm effluent had the least number (6) of bacterial isolates. The isolates that had the highest occurrence (occurring in all samples) were *Pseudomonas aeruginosa* and *Escherichia coli.* Of these 13 genera 9 were gram negative, while only 4 were gram positive. The total heterotrophic bacterial (THB) count and total hydrocarbon utilisers (THU) from all the effluent samples ranged from 3.0×10^4 to 6.0×10^7 cfu/mL and 2.3×10^2 to 4.2×10^3cfu/mL, respectively. The counts of hydrocarbon utilisers were obviously lower than the heterotrophic counts, although the differences in counts were found to be statistically non-significant ($P > 0.05$). Rubber effluents and oil palm mill effluents had the highest number of hydrocarbon utilisers with three isolates each. The active hydrocarbon utilisers encountered in this study included *Serratia marscescens, Bacillus cereus, P. aeruginosa, Enterobacter aerogenes* and *Bacillus subtilis.* Presence of nutrients and crude oil degrading bacteria in these effluents suggests that these effluents can be used to enhance bioremediation through their use as biostimulation and bioaugmentation agents.

Keywords: hydrocarbon utilisers, bacterial isolates, effluents, crude oil, physicochemical properties

Introduction

The global increase in petroleum exploration, production and usage has resulted to increased discharge of products and operational materials into the environment (Mandri and Lin, 2007). Environmental (air, soil and fresh water) pollution by petroleum and petrochemical products has attracted much attention in recent decades. This is because most of these products especially, the polycyclic aromatic hydrocarbons (PAHs) are toxic, mutagenic and carcinogenic (Clemente *et al.,* 2001).

Prolonged exposure to high concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Mishra *et al.,* 2001). In addition, PAHs have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the environment. Crude oil pollution of oil and surface water has been prevalent in Nigeria, and other oil producing countries since the commencement of soil exploration and development of petroleum industry (Okoh *et al.,* 2001; Song *et al.,* 1986). Bioremediation method is one of the most promising technologies, currently in use or under development. The microbial by-product of oil biodegradation becomes part of the natural food chain with much of the degraded hydrocarbon material further metabolised by marine organism or incorporated in soil humus with accumulation to toxic materials in the environment (Ijah and Antai, 2003).

Lack of essential nutrients such as nitrogen and phosphorus is one of the major factors affecting biodegradation of hydrocarbon by microorganism in soil and water environment. Therefore, the addition of organic or inorganic nitrogen-rich nutrients (biostimulation) is an effective approach to enhance bioremediation process (Walworth *et al.,* 2007). Positive effects of nitrogen amendments on degradation have been widely demonstrated (Abioye *et al.,* 2009).

Some liquid organic wastes such as cassava mill effluents, oil palm mill effluents, and rubber effluents have diverse chemical composition and some of the constituents may be deleterious to microbial growth. Therefore, screening for the presence of crude oil degrading bacteria in these effluent samples was carried

*Author for correspondence; E-mail: lordromis@yahoo.co.uk

out as well as the physicochemical properties of the samples were observed. This will determine their possible use to enhance bioremediation.

Materials and Methods

Sources of samples, media used and sterilisation procedures. Total 10 samples, each of cassava mill effluents, rubber effluents and oil palm mill effluents were collected from various locations in Ekpoma, Edo State, Nigeria. The crude oil used was chevron escravos, crude oil obtained from chevron tank farm at escravos. The mineral salt medium was used as described by Mills *et al.* (1978) and modified by Okpokwasili and Amanchukwu (1988). Bacteriological agar (oxoid) was added to obtain a solid medium at a rate of 1.5%, when necessary. The general purpose media used included commercial preparations of oxoid nutrient agar, nutrient broth, MacConkey agar, peptone water, urease agar and citrate agar.

Media were sterilised by autoclaving at 121 °C for 15 min. Crude oil used for biodegradation studies was filter-sterilised using sterile 0.22 µm pore size membrane (Type: MILLEX-GS Millipore Corporation, Bedford, MA01730 Rev. 9/94 12172). This method was adopted since petroleum contains volatile components, which evaporate if sterilised by autoclaving. In addition, crude oil naturally contains hydrocarbon utilising microorganisms which if autoclaved, will be killed releasing their carbon nutrient into the medium. These organic compounds from the organisms might be preferred by the experimental microorganisms for growth and as a result, produce false positive result on the utilisation of petroleum hydrocarbon. Glass wares were sterilised at 160 °C for one hour using hot air oven.

Determination of total heterotrophic and total hydrocarbon utilising bacterial numbers and types. ***Bacterial enumeration.*** The total heterotrophic bacterial count in the samples were determined by making ten-fold serial dilution of the samples on normal saline (0.85% w/v, sterile NaCl). Then 1 mL of the appropriate dilution was pour plated in duplicates on the surface of the appropriate medium. The plates were then incubated for 24-48 h at a temperature of 37 °C, and the colonies were counted that were developed on the plates. Also, mineral salt agar medium of Mill *et al.* (1978) as modified by Okpokwasili and Amanchukwu (1988) was used for the enumeration of hydrocarbon utilising bacteria. Chevron escravos crude oil soaked in sterile 9 cm Whatman (No.1) filter paper and placed in dish cover served as carbon source. Thus the hydrocarbon was supplied to the inoculum by vapour-phase transfer. After incubation at room temperature for 1-5 days, colonies formed were count.

Bacterial characterisation and identification. The biochemical and phenotypic characteristics used to characterise and identify isolates included gram staining, colonial appearance, motility, urease, catalase, indole oxidase, citrate, methyl red, voges proskaeur and sugar fermentation. These tests were performed using the methods of Harley and Prescott (2002) and Gerhardt (1994) and identified based on observations of Holt *et al.* (1994), and Barrow and Feltham (1986).

Determination of physicochemical properties of samples. Methods for the determination of physicochemical properties of samples (cassava mill effluents, oil palm mill effluents and rubber effluents) were used as outlined by APHA (1985). The pH meter used was pocket-sized HANA $pHep^{+}$ HI 98108 with automatic temperature compensation. Conductivity values were determined using conductivity meter (Jenway 4010, UK) and temperatures were measured using standard mercury thermometer.

Total organic carbon was determined by dichromate wet oxidation method of Walkley and Black as modified by Dhyan *et al.* (1999). Nitrate content was determined using the macro Kjeldahl digestion method of Brady and Weil (1999) and available phosphorus was determined using the method reported by Olsen and Sommers (1982). Sulphate was determined using the turbidometric method, while oil and grease were determined by the partition gravimetric method.

Sodium and potassium were determined using flame photometric method, while calcium and magnesium were determined by using the method of Brady and Weil (1999). The metal contents were determined using an atomic absorption spectrophotometer (AAS) (Perkin Elmer AA Unit Model: 3100 Serial Number: 148157)

Results and Discussion

The bacterial isolates from various samples showed that 20 isolates from 13 different genera were obtained in this study (Table 1). Cassava mill effluents and rubber effluents had the highest and same number (7) of bacterial isolates, while oil palm mill effluents had the least number (6) of bacterial isolates. The isolates that had the highest occurrence (occurring in all samples)

Table 1. Bacterial isolates from effluent samples

Cassava mill effluent	Rubber effluent	Oil palm mill effluent
Klebsiella pneumoniae	*Pseudomonas aeruginosa*	*Serratia marscescens*
Lactobacillus spp.	*Streptococcus faecalis*	*Escherichia coli*
Pseudomonas aeruginosa	*Bacillus subtilis*	*Bacillus cereus*
Staphylococcus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*
Alcaligenes faecalis	*Achromobacter* spp.	*Staphylococcus aureus*
Escherichia coli	*Proteus mirabilis*	*Acinetobacter* spp.
Enterobacter aerogenes	*Staphylococcus saprophyticus*	

were *Pseudomonas aeruginosa* and *Escherichia coli.* The presence of these different genera from these samples aligns with the widely documented fact that bacteria are present in almost any ecological niche (Harley and Prescott, 2002). Of these 13 genera, 9 were gram negative, while only 4 were gram positive. The preponderance of gram negative bacteria in this study is similar to the earlier report of Foght and Westlake (1987) that both gram positive and gram negative bacteria are encountered in the degradation of contaminants with gram negative bacteria dominating. This finding also correlates the work of Esumeh *et al.* (2009) and Agbonlahor *et al.* (1993) that isolated only gram negative organisms suggesting that they are better degraders of crude oil compared with their gram positive counterparts.

One of the most predominant isolate in this study i.e., *Pseudomonas* spp., has been noted for its biochemical versatility with the ability to grow on diverse substrates and chemicals (Chikere and Chijioke-Osuji 2006; Devereux and Sizemore, 1982). Some of the isolates obtained in this present study were encountered by Ogbulie *et al.* (2010); Esumeh *et al.* (2009); Chikere and Chijioke-Osuji (2006) and Akpe (2003).

Table 2 shows a list of hydrocarbon utilising bacteria from various samples. Rubber effluents and oil palm mill effluents had the highest number of hydrocarbon utilisers with three isolates each. The active hydrocarbon utilisers encountered in this study includes *Serratia marscescens, Bacillus cereus, P. aeruginosa, Entero-bacter aerogenes* and *B. subtilis.* The hydrocarbon utilising genera encountered in this study have been reported earlier. *Pseudomonas* spp., are often isolated from hydrocarbon contaminated sites. They have broad activity for hydrocarbons and can degrade many alkanes, alicyclics and aromatics. Utilisation of hydrocarbon - based substrate has also been reported by species of *Staphylococcus, Aeromonas, Proteus, Corynebacterium, Streptococcus, Bacillus, Micrococcus,* and *Alcaligenes* (Iyagba *et al.,* 2008; Plohl *et al.,* 2002; Benka - Cooker and Olumagin, 1999; Hughes *et al.* 1984).

The total heterotrophic bacterial (THB) count and total hydrocarbon utilisers (THU) from all the effluent samples ranged from 3.0×10^4 to 6.0×10^7 cfu/mL and 2.3×10^2 to 4.2×10^3cfu/mL, respectively (Table 3). The counts of hydrocarbon utilisers were obviously lower than the heterotrophic counts, although the differences in counts were found to be statistically

Table 2. Hydrocarbon utilising bacteria from various samples using vapour phase technique

Samples	Hydrocarbon utiliser
Oil palm mill effluent	**S. marscescens, E. coli, *B. cereus, *P. aeruginosa*
Rubber effluent	**P. aeruginosa*, *S. faecalis, *B. subtilis*
Cassava mill effluent	*K. pneumoniae*, **E. aerogenes*

* = very active crude oil degraders with colonies appearing within 24 h of incubation in mineral salt medium containing crude oil.

Table 3. Mean total heterotrophic and hydrocarbon utilising bacterial counts

Samples	Average total heterotrophic bacterial count	Average total hydrocarbon utilisers' count
Oil palm mill effluent	$3.0 \times 10^4 \pm 0.09$ cfu/mL	$2.3 \times 10^2 \pm 0.04$ cfu/mL
Rubber effluent	$6.4 \times 10^5 \pm 0.085$ cfu/mL	$3.4 \times 10^2 \pm 0.025$ cfu/mL
Cassava mill effluent	$6.0 \times 10^7 \pm 0.035$ cfu/mL	$4.2 \times 10^3 \pm 0.055$ cfu/mL

$P > 0.05$.

non-significant (P>0.05). This result was found to be similar with reported by earlier Eziuzor and Okpokwasili (2009), and Okpokwasili and Oton (2006), and the lower number of hydrocarbon utilisers compared to the heterotrophic population, suggested that all organisms that can cause the decay of biological substrates could not degrade crude oil (Atlas and Bartha, 1993). Also, crude oil contains some fractions that may greatly affect the survival of other microorganisms because hydrocarbons are known to contain volatile toxic components, which can inhibit their growth (Obire, 1993). It is only crude oil degraders that can easily adapt to such changes. However, in a previous study of crude oil polluted and unpolluted soil samples by Chikere and Chijioke-Osiji (2006), it was found that hydrocarbon utiliser population were higher than the heterotrophs.

The physicochemical properties of the samples are shown in Table 4. It was observed that the pH of samples ranged from acidic range of 4.46 to near neutrality 5.29. The most acidic sample was cassava mill effluent (pH 4.46), while the least acidic was rubber effluent (pH 5.29). The high acidity of cassava mill effluent was connected with the fermentative activities of microorganisms on the sugars and starch present in the effluent. The conductivity values ranged from 0.04 ms/cm to 7.12 ms/cm with rubber effluent having the least and cassava mill effluent having the highest. The metal level was found to be very low even in some cases it was not detected. The metal contents of samples were below reported pollution levels (Nweke *et al.,* 2006; Aydinalp and Cresser, 2003; Chen *et al.,* 1999). Hence, samples were not considered metal-polluted.

Nitrate level was highest in cassava mill effluents (45 mg/mL) and lowest in rubber effluents (0.80 mg/mL). The potassium level ranged from 7.99 mg/mL in rubber effluent samples to 29.40 mg/mL in cassava mill effluents. Magnesium was highest in rubber effluent (9.15 mg/mL) and lowest in oil palm mill effluent (4.15 mg/mL). Among the effluent samples, oil palm mill effluents had the highest content of iron (28.70 mg/mL) followed by cassava mill

Table 4. Physicochemical properties of cassava, rubber and oil palm mill effluents

Parameters	Cassava mill effluent (mg/mL)	Rubber effluent (mg/mL)	Oil palm mill effluent (mg/mL)
pH	4.46	5.29	4.70
Conductivity ms/cm	7.12	0.04	1.55
TDS	462.80	28.40	405.50
Nitrate	45.00	0.80	NT
Total Nitrogen	NT	NT	76560.00
Nitrite	15.00	NT	NT
Sulphate	45.00	3.40	NT
Hardness	214.00	54.80	NT
Calcium	75.75	4.92	48.90
Magnesium	6.08	9.15	4.15
Potassium	29.40	7.99	25.20
Sodium	650.00	2.46	5.10
Chromium	0.01	NT	ND
Manganese	6.57	NT	2.20
Iron	13.21	0.80	28.70
Nickel	0.02	NT	ND
Zinc	0.53	1.50	0.30
Copper	2.77	NT	0.86
Lead	0.10	ND	ND
Cadmium	0.01	ND	ND
Alkalinity	NT	48.50	NT
Oil and Grease	NT	NT	3800.00
Phosphorus	NT	NT	162.00

ND = not detected; NT = not tested; TDS = total dissolved solids.

effluents (13.21 mg/mL), the least iron content was recorded in rubber effluent (0.80 mg/mL). The presence of nitrate, potassium and other inorganic salts and elements in the effluents explains, why bacteria could grow in them and suggests the possible role of these effluents in biostimulation and bioaugmentation of crude oil contaminated soil. Also the application of these effluents in this regard will help to solve waste disposal problems in the environment. Treatment of oil polluted soil is necessary to protect water supplies, human health and environmental quality (Chang *et al.,* 1996). Hence, the use of these effluents as amendments in crude oil polluted sites is recommended to facilitate bioremediation.

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