Biodegradation of Gamalin-20 by *Micrococcus* sp (Strain 189) in the Coastal Soils of Southeastern Nigeria

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Abstract: A soil microorganism capable of utilizing the widely used broad spectrum recalcitrant organo-chlorine insecticide, Gamalin-20, as primary carbon and energy source, was isolated from coastal soils of Akwete, Southern Nigeria. The procedure for isolation and screening of microbial strain involved microbial strain involved serial dilutions of soil samples and plating on Gamalin-20 Minimal Medium Agar (GMMA)**.** Isolates obtained were sub-cultured on Isolates were sub cultured on Nutrient Agar, Blood Agar, MacConkey Agar. Soil samples were analyzed for physico-chemical characteristics. Growth determination of microbial isolates from the primary isolation in three batches of the soil samples (1, 8 and 9) were obtained based on their cultural characteristics and physiological properties. The organism was characterized as *Micrococcus* sp strain 189. The implication of this finding for the mangement of petroleum-associated pollution is discussed.
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1. Introduction

The discovery and use of petroleum-derived synthetic organic chemicals as broad-spectrum pesticides have brought considerably relief to man from the scourge of insect-borne diseases such as malaria, and crop pests world wide (Barkley *et al*., 1989). These benefits derived from the use of hydrocarbons in the past two decades have been associated with negative impacts due to the fact that hydrocarbons including petroleum based herbicides, and pesticides, are not easily bio-degradable. The accumulation of the pollutants at certain levels may inhibit some biotic and microbial communities that are essential in some biogeochemical cycles which affects the productivity of the polluted ecosystems (Ekundayo and Obire, 1988; Rhodes and Hendricks, 1990; Iyaniwura, 1991).

The contamination of human habitats constitutes public health and socio economic hazards (Kobayashi and Rittman, 1982; Smith and Dragun, 1984). Organochlorine based pesticides such as dichlorodiphenyltrichane (DDT), chlordane, heptachlor, propachlor, Lindane (Gamalin-20), dieldrin, among others are highly problematic and their continuous use can lead to the emergence of tolerant strains of microbial organisms. Microbial communities exposed to hydrocarbons are known adapt, exhibiting selective enrichment and genetic changes resulting in increased proportions of hydrocarbon degrading bacteria and bacterial encoding hydrocarbon catabolic genes (Leathy and Colwell, 1990). Okerentugba and Ezeronye (2003), pointed out that adapted microbial communities have higher proportions of hydrocarbon degraders that can respond to the presence of hydrocarbon pollutants.

In Nigeria, one of the widely used pesticides in controlling agricultural pests especially in rubber tree *Hevea brasiliensis* Muell. Arg.) plantations is Gamalin-20. Unfortunately the use of this chemical has been grossly abused for fishing, or added to baites for catching wild animals. Unlike DDT, which has been shown to be degraded by a few microorganisms, little is known about microbial degradation of gamalin-20 in tropical coastal soils.

Organochlorines pesticides such as dichlorodiphenyltrichane (DDT), chlordane, heptachlor, propachlor, Lindane (Gamalin-20), dieldrin, etc., are highly problematic and their continuous use normally lead to the emergence of tolerant strains of microbial organisms. Microbial communities exposed to hydrocarbons are known to be adapted, exhibiting selective enrichment and genetic changes resulting in increased proportions of hydrocarbon degrading bacteria and bacterial encoding hydrocarbon catabolic genes (Leathy and Colwell, 1990). Okerentugba and Ezeronye (2003), pointed out that adapted microbial communities have higher proportions of hydrocarbon degraders that can respond to the presence of hydrocarbon pollutants.

The presence of certain microbial isolates can be of ecological significance as they can be multiplied and used in bio-remediation. In this regards

microorganisms may assume an enhanced role in the biodegradation of hydrocarbons (Okerentugba and Ezeronye, 2003). In particular, *Micrococcus* species ccurs in a wide range of environments, including human skin, water, dust, and soil. *Micrococcus* species like many other representatives of the Actinobacteria, can be catabolically versatile, with ability to utilize a wide range of unusual substrates, such as pyridine, herbicides, chlorinated biphenyls, and oil (Sims and O'ioughlin, 1992; Doddamani and Ninnekar, 2001).

As part of the strategies for managing the coastal soils of Nigeria, this study aimed to isolate *Micrococcus* species from soils at the Rubber Research Institute of Nigeria, Akwete substation, Nigeria, and assess their potential in biodegradation of Gamalin-20.

2. Materials and Methods

2.1 Study site:

Akwete is characterized by hot humid climate with a dominant rainy season and short dry season. Rainfall is fairly distributed with about 85 – 95% rainfall from March to October. Higher peak of the rainy period is in July with lesser peak in September. Temperature is usually high throughout the year with mean annual temperature of about 20ºC.

Akwete is part of the coastal plain sands of the Niger Delta Basin with an extensive red earths as loose ill-sorted sands underlying the recent deposits of the Niger delta. The soil type is predominantly loam, sandy clay loam, and sandy loam at 15-30 cm, 30-60 cm, and 60-90 cm, respectively.

Akwete RRIN substation was established by the former Eastern Nigeria regional government in 1960 as a demonstration station centre for the propagation and distribution of cassava, oil palm, coconut, as arm of Research and Training division of the Ministry of Agriculture, Umudike. Rubber was added in 1962. They were planted out in the field in 1962 and budded in 1964. Clones planted out were TJIR, PB 86, PR 107, GT 1, AVROS 1581, RRIC 45, PB/51, IAN 2880, HAR 1, RRIM series seedlings, and NIG 800.

2.2 Insecticide

Gamalin 20 was used in the early years of the station establishment but its use was discontinued after the ban of gamalin from circulation. The main pests prevalent in the study area are termites, bark feeding caterpillars, scale insects, spider mites, mealy bugs, weevils, stem boring beetles, thrips, among others and were controlled mainly with the use of gamalin 20. Other termicides such as methyl parathion, chloropyriphos, malathion, were rarely used aside gamalin 20 to controlled pest incidence.

Gamalin-20 was used for the study, in view of the fact that it was most commonly used to control pests. It was supplied by Tema chemicals, Ghana. Gamalin-20 is the trademark of the Imperial Chemicals Incorporated (ICI), England for the organochlorine insecticide Hexachlorocyclohexane (HCH) also called Lindane and sold as a 200 g/liter solution.

2.3 Isolation and identification of Gamalin-20 biodegrading microorganisms

Soil samples for the study were Study area designated locations 1, 8, and 9. The soil samples were collected from a depth of 0 – 5 cm and stored in a refrigerator at 4^0C prior to dilution procedure. 10 grammes each of freshly collected 10 soil samples were pulverized with mortar and pestle. Thereafter, 10-fold serial dilutions (10^{-1} to 10^{-10}) of the soil samples were made from a stock solution with 1g of soil sample dispensed in 20 ml of sterile distilled water, and after vigorous shaking, 0.1 ml of each dilution was plated onto Gamalin-20 Minimal Medium Agar (GMMA) (own formulation). The GMMA (in which Gamalin-20 was the sole carbon and energy source) had the following composition (w/v): Gamalin-20 (2.0%), $(NH4)_2$ $S0_4$ (1.0%) K_2HPO_4 (0.5%), KH_2 PO_4 (0.5%), NaCl (0.1%,), Agar (2.2 g), distilled water (100 ml), pH (7.0).

The inoculated medium was aerobically incubated at 27^0/C for 96 h. At the end of this period, the plates were examined for colony growth. Colonies that grew on the plates were then purified by streaking onto GMM agar and slants of the pure cultures prepared and stored at 4°C.

2.4 Subculture of isolates on different media

Isolates were sub cultured on Nutrient Agar, Blood Agar, MacConkey Agar alone, as well as on plates of these media supplemented with 0.5% Gamalin-20, to determine a medium for optima maintenance of the isolates. Identification of isolates was based on colony characteristics, Gram staining motility tests performed using the hanging drop method-Collin and Lyne (1978). The biochemical assay tests included methyl red, coagulase and catalase tests and utilization of mannitol and lactose.

2.5 Soil analysis

Soil samples were obtained from a depth of 0-5 cm depth from the immature rubber plantation of Akwete sub-station of RRIN. The soil samples were bulked and air dried on a laboratory bench at ambient temperature. The dried soil samples were then passed through a 2 mm mesh sieve (Endecott, England Ltd) and were analysed using standard methods for routine analysis (IITA 1979).

3. Results

3.1 Identification of Gammalin-20 biodegrading microbial isolate

Growth determination of microbial isolates from the primary isolation in three batches of the soil

samples (1, 8 and 9) were obtained based on their cultural characteristics and physiological properties (Table 1). All the three isolates were identified as *Micrococcus* sp strain 189. The growth of the organism was scanty in normal enrichment Blood agar, and Mac Conkey agar. However, it failed to grow on Nutrient agar. The organism grew profusely in all the media supplemented with Gamalin-20.

The characteristics of gamalin degrading microorganisms revealed colony morphology, microscopic appearances, biochemical reactions, sugar permanentation, and identification (Table 1). These characteristics ranged from colony morphology of convex elevation,with smooth and glisternin surface, and golden yellow pigmentation. The microscopic appearances showed discrete structure and difficult emulsificability while shape was circular and edge entire. For the biochemical reaction test, gram positive cocci micrococcus bacteria in chains and motile were obtained. The sugar permanentation test showed a variation in coagulase, methyl red, catalase, glucose, lactose and mannitol with micrococcus species identified as the prevalent gamalin 20 degrading organisms.

Soil analysis

Field observations revealed dark grayish brown, loamy sandy top
soils which were very weak granular to structure with abundant roots. The soils were drained coastal plain sands. Table 2 shows the physical and chemical characteristics of the soil samples analyzed. The clay content was 2.2%, and the soil 2.8%, while the sand content was 95%. The pH (H_20) of the soil was acidic (4.6), organic matter (2.85), available phosphorus (13.4 mg/kg), exchangeable cations were potassium (0.23), sodium (0.07), calcium (0.96), magnesium (0.32), cmol/kg. Exchangeable acidity was 1.68 while ECEC was moderate 3.26 cmol/kg, and high base saturation (48.31%) was obtained.

Weather records of Akwete sub-station

The monthly mean of climatic data recorded for Akwete sub-station, indicated that the highest rain fall occurred from the months of May to October and highest in July with 350.32 mm (Table 3). Records of maximum and minimum temperatures showed that temperatures declined generally from January to December and were only highest in February (34.96 ^{0} C max. and 17.72 ^{0} C min.).

DISCUSSION

A bacterial strain *Micrococcus* sp 189 was the only gamalin-20 degrading organism isolated from all three-soil samples in which growth was recorded from the ten soil samples tested. The study also revealed a striking observation of the organism almost total dependence on the utilization of Gamalin-20 for its growth and survival. This is indicated by the organism's inability to grow successfully in the enriched media such as Blood agar, and Mac Conkey agar, and its failure to grow on Nutrient agar, unless these media were amended with Gamalin-20. This implies therefore that Gamalin-20 served as most suitable substrate for the obligate growth of *Micrococcus* sp as its source for sole carbon.stein *et al*., 1991). In comparison the compound-DDT whose use has been greatly curtailed since 1975 and even banned in the US and other countries, however, is degraded by a few microbial genera (Fujimura *et al*., 1994; Fought *et al.*, 1996). This is contrary to Gamalin-20 (HCH), and Pentachloronitrobenzene (PCNB) whose chemical structures are highly resistant to biodegradation due to the presence of more halogen moieties (Barkay *et al*., 1989). The isolation of *Micrococcus* sp that has the capacity to degrade HCH is quite an interesting finding for such pesticide that was widely used in Nigeria for malaria (disease), and agricultural (insect) pest control.

The ability to isolate *Micrococcus* sp strain 189 from the designated soil environment amended with Gamalin-20, is evidence that *Micrococcus* organisms in such environment are active degraders. As a result of exposure, spontaneous mutation of existing *Micrococcus* organisms in the soil could have produced this strain with the relevant plasmid-mediated enzymes to biodegrade the Gamalin-20. Bacteria, generally, have evolved regulatory systems that ensure the synthesis of enzymes so that the initial attack on compounds is induced only when required (Okerentugba and Ezeronye, 2003).

In addition, some microorganisms have evolved highly effective systems for responding to a variety of potential growth substrate, and this corroborated by the obligate dependence of *Micrococcus* sp on the utilization of Gamalin-20. Okerentugba and Ezeronye (2003) pointed out that essential genes of bacteria are carried on a single chromosome but genes specifying enzymes required for the catabolism of some of the unusual substrates may be carried on the plasmids. Plasmids have been implicated in the catabolism of octane (Chakrabarty *et al*., 1973), naphthalene (Dunn and Gunsalus, 1973), camphor (Rheinwald *et al.*, 1973), and toluene (Williams and Murray, 1974), as well as a number of other compounds (Chakrabarty, 1976). Several mechanisms explained by Barkay *et al.* (1989), and Fought *et al*. (1996) give insight to the process of biodegradation.

The present study highlights the potential roles of bioremediation in the management of the coastal soils and waters in the Niger Delta that are persistently contaminated by hydrocarbons of

petroleum origin. The development of this technology could be one of the ways of managing the risk of pesticide accumulation. The goals of hydrocarbon pollution-free coastal environment as envisaged by Tzesos and Wang (1991) and Iyaniwura (1991) can be realized by this new bioremediation technology.

Table1. Characteristics of Gamalin 20 Degrading Organisms

Colony Morphology **Microscopic Appearances** **Biochemical Reactions**
Sugar Permanentation **Identification**

No	Size No	Elevation	Surface	pigment	Structure	Emulsifiability	Shape	Edge	Gram Rtn	Moltility	Coagulase	Methyle Red	Catalase	Glucose	Lactose	Mannitol
1	08	Convex	Smooth & Glisterning	Golden Yellow	Discrete	Difficult	Circular	Entire	+ cocci in Pairs	+	-	-	+	+	-	Micrococcus sp
2	08	Convex	Smooth & Glisterning	Golden Yellow	Discrete	Difficult	Circular	Entire	+ cocci in Pairs	+	-	-	+	+	-	+Micrococcus sp
3	08	Convex	Smo oth & Glisterning	Golden Yellow	Discrete	Difficult	Circular	Entire	+ cocci in Pairs	+	-	-	+	+	-	+Micrococcus sp

Table 2. SOIL ANALYSIS OF THE TOP SOIL AT AKWETE RRIN SUBSTATION

Mechanical analysis			
Sand (%)	Silt (%)	Clay (%)	Textural Class
95	2.8	2.2	sand

Chemical Composition										
pH	OM	Avail. P	Exchan. Cations (cmol/kg)				Exchang. Acidity		ECEC	B.S
(H_2O) %	mg/kg	k	Na		Ca		Mg		cmol/kg	(%)
4.6	2.85	13.4	0.23	0.07	0.96	0.32	1.68	3.26		48.31

Table 3. Monthly mean of Climatic Data at Akwete RRIN Substation

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Rainfall (mm)	27.54	19.23	123.03	157.06	271.75	249.52	350.32	318.99	321.02	215.73	92.41	18.73
Temperature(^{0}C)												
Maximum	33.45	34.96	33.75	33.30	32.77	31.03	29.29	29.87	30.20	30.17	31.86	31.67
Minimum	15.90	17.72	17.66	17.54	16.93	16.25	16.93	16.93	16.63	16.90	16.33	14.67

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