Ecofriendly and Cost Effective Carbon Dot and Microbial Culture in Remediation of Petroleum Hydrocarbon Spill on Soil

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Abstract: Bioremediation technology plays a crucial role in ensuring ecological security when addressing petroleum hydrocarbon contamination in the environment. Its advantages include cost-effectiveness, high efficiency, minimal environmental impact, and the absence of secondary pollutants. Recently, carbon dots (CDs) have gained attention due to their exceptional properties, including quantum size, strong light absorption, tunable luminescence, high quantum yield, biocompatibility, low toxicity, and environmental friendliness. This study aims to evaluate the effectiveness of carbon dots in combination with microbial culture for the remediation of hydrocarbon-contaminated soil. Soil analysis revealed that the contaminated sample was acidic (pH 5.75±1), whereas the control sample had an alkaline pH of 7.84±1. Carbon dots synthesized using biomass was characterized via Transmission Electron Microscopy and Energy Dispersive X-ray, showing a particle size of 10 nm and elemental composition. The synthesized CDs exhibited photoluminescence under ultraviolet light. A bacterial consortium consisting of *Bacillus thuringiensis*, *Bacillus velezensis*, and *Stenotrophomonas* species was used to stimulate biodegradation.Gas Chromatography-Flame Ionization Detector (GC-FID) analysis over 86 days (sampled every four weeks) revealed a significant reduction in Total Petroleum Hydrocarbon (TPH). Carbon dots (CD) alone had an insignificant TPH value of (1.37±2), while microbial culture (MC) had (13.90±7).The study confirms that combining carbon dots with microbial culture significantly enhances hydrocarbon degradation in contaminated soil.

Key word: Biostimulation, Carbon dot, Transmission Electron Microscope, Energy dispersive X-R**ay**

INTRODUCTION

The top soils are the most multifaceted and varied ecosystem in the world. In addition to providing humanity with 98.8% of its food, soils supply a broad range of other services, from carbon storage and greenhouse gas guideline, to flood alleviation and providing support for our expansive cities (Khalid-Sayed, *et al*., 2021).Soil contamination is becoming more and more serious, mainly due to the reduction of soil area and pollution by chemical compounds such as pesticides, crude oil, heavy metals, persistent organic matter, and acidic substances (Dora, 2019)

The most extensive pollutants found in the environment are sourced from crude oil and pesticides, during processing, transportation and storage; they release these products to the environment (toxic chemicals). They are harmful and don't break down easily, so they can persist. (Glaser, *et al*., 2001; Habib, *et al*., 2017).

The methods of remediating polluted soil are mainly divided into physical, chemical, biological, and plant methods. Physical remediation technologies mainly include soil leakage, thermal desorption, steam extraction, and off-site landfill (Wang *et al*., 2010). Cleaning up polluted soil can involve the use of chemicals to make the pollutants less harmful. But the procedure is expensive and can lead to secondary pollutants in the environment (not ecofriendly). Scientists have tried using different chemicals, like stabilizing agents, to clean up pollution (Okerentugba 2016). The end result is not completely palatable.

However Bioremediation technologies are expanding in terms of applicability, compared to other remediation methods, due to their high efficiency rate, low cost, and non-toxic end products. However, microbial capacity and action, on soil amendments and oxygen conditions, are the key factors in bioremediation. Petroleum hydrocarbon polluted soil typically has a small quantity of

microbes, low porosity, and few nutrients, limiting the efficiency of microorganisms in actual application (Jin *et al*., 2021; Jones *et al*., 2021; Khalid-Sayed et al., 2021). Since their initial discovery in 2004, carbon dots (CDs) have attracted a lot of attention (Sun *et al*., 2021). This is a result of their exceptional benefits, which include superb fluorescence, biocompatibility, environmental friendliness, a lack of photo - bleaching, and ease of production. By possibly addressing both soil organic matter Green House Gases (GHG) emissions and food insecurity simultaneously, particularly in moist soils, carbon dot is being addressed. Tropical climates make it challenging to practice sustainable agriculture since some soils quickly degrade their organic matter, leaving them with few stabilizing minerals. However, the application of additive is only effective due to the short post-amendment time (Glaser *et al*., 2001). One study generated a single carbon dot and found that its application led to higher crop yields for four years after amendment. This suggests that the carbon dot may be a more effective and long-lasting soil conditioner due to its stable environment (Kuzyakov *et al*., 2014). The top-down and bottom-up approaches have been used to synthesize nanoparticles. Physical approaches with a top-down approach include attrition ball milling, physical vapour deposition (PVD), lithography, and pyrolysis (thermal evaporation). The bottom-up approach uses both chemical and biological methods; the former include sol-gel, chemical vapour deposition (CVD), co precipitation, micro emulsions, hydrothermal, sonochemical, and microwave processes, while the latter make use of lower-celled organisms (algae, bacteria, fungi, yeast, and actinomycetes), plant extracts, enzymes, and agricultural wastes (Ledwani and Sangwai, 2020).

It is highly recommended to use phytonano technology, also known as biosynthesis of nano materials from plant materials, to produce eco-friendly, straightforward, and affordable nano materials that are scalable, biocompatible, and simple to synthesize using water as the universal solvent. These nano materials are superior, consistent, and feature smaller, longer-lasting particles (Ijaz *et al*., 2020).

The bulk density, particle size distribution, porosity, structure, and texture of soil are all improved by carbon dot (Ding 2016; Xu *et al*., 2012]. A rise in soil carbon, pH, and CEC are only a few of the chemical aspects of soil that are affected (Laghari *et al*., 2016). The large surface area and porous nature of carbon dots help to account for the higher retention of nutrients and water (Atkinson *et al*., 2010; Xu *et al*., 2012). The application of carbon dot can improve the regulation of inorganic nitrogen and lessen ammonia evaporation (McHenry, 2011). Carbon dot has generated a lot of attention recently for use in a variety of environmental applications, including the elimination of pollutants, carbon sequestration, and soil improvement. Due to its many unique characteristics, carbon dot is a dependable, economical, and environmentally friendly chemical for the removal of a variety of impurities. Given that the green revolution has significantly reduced the ongoing organic and heavy metal contaminations in the food chain and the surrounding environment, fast-growing industries and increasing variety of agrochemical-based crop manufacturing practices have become increasingly important (WHO, 2017). As a result, there is grave public concern for the preservation of the environment and individual health. Among other conservative techniques, chemical precipitation, ion exchange, adsorption (using activated carbon), and membrane separation technologies are frequently used to remove importunate contaminants from aqueous and gaseous phases. These techniques are expensive and frequently produce a sizable amount of chemical waste that has no useful purpose. Unstable organic compounds (VOCs), aromatic dyes, agrochemicals, antibiotics, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and other organic contaminants can all be removed

using carbon dot, a low-cost carbonaceous material (Beesley *et al*., 2011; Xu *et al*., 2012; Zheng *et al.,* 2018) and a series of inorganic contaminants from aqueous, sgaseous, and/or solid phases (e.g., heavy metals, ammonia, nitrate, phosphate, sulfide, etc.) (Ahmad *et al*., 2014; Sun *et al*., 2021). The term ”Carbon dot” refers to a derivative of thermochemical exchange, which includes heating, gasification, torrefaction, and hydrothermal carbonization of carbonaceous biomass, including agricultural residues, algal biomass, forest residues, manures, activated sludge, energy crops, digestate, etc. at high temperatures (300-900℃) and under oxygen-limiting conditions.

These carbon molecules act independently to improve soil quality by increasing soil carbon sequestration and reducing emissions of Green House Gases (GHGs) (Zhang *et al*., 2018). This aim of this work is to ascertain the efficacy of Carbon Dot (CDs) and microbial culture in remediation of petroleum hydrocarbon spill in an oil-contaminated site of an abandoned artisanal refinery in Obi-Ayagha of Ughelli South, Delta State, Nigeria.

MATERIALS AND METHODS

Site Description and Sample Study Area: The project is being conducted at the Hydrocarbon Pollution Research/Training facility at the Integrated Institute of Environment and Development (IIED), located in Obi-Ayagha, Ughelli South, and Delta State. Using a soil auger, 50gram of soil sample from the surface strata ($0 - 15m$) were taken from locations where artisanal refinery activities had historically contaminated soil with crude oil (polluted soil sample). It was gathered during the dry season, which is the first quarter of the year. The wetlands in the Delta, which total 70,000 km^2 (27,000 sqm) and cover 20,000 km^2 are divided into four natural zones. These wetlands were predominantly generated by silt deposition. Mangrove swamp forests, freshwater swamps, lowland rain-forests, and coastal barrier islands are examples of such ecosystems. The Niger Delta typically experiences equatorial weather on its southern shore and sub-equatorial weather in the north. The monthly mean temperature ranges between 25℃ and 29℃, while the annual precipitation totals fall between 2000mm and 4000mm, with relative humidity exceeding 70%.The Niger Delta is cantered on environmental contamination since it has impacted rural economic operations. The Biomass (Coconut husk) was sourced locally in Obogu Village located in Udu Local Government Area of Delta State. Isolates were sourced from the impacted soil taken from the abandoned artisanal refinery site located in Obi-Ayagha, Ughelli south, in Delta State.

Total Petroleum Hydrocarbon (TPH) Analysis: Total Petroleum Hydrocarbon (TPH) analysis was conducted using a Gas Chromatograph-Flame Ionization Detector (GC-FID). The analysis took place at Thermo Steel Laboratory in Warri, Delta State, Nigeria. Ten grams (10g) of soil samples (both impacted and control) were transferred into an extraction bottle. The sample was spiked with 0.1ml of squalene (internal standard).It was dried with anhydrous sodium sulphate. For the Extraction, the dried sample was extracted using a 3:1 mixture of n-hexane and dichloromethane. The extraction process was facilitated using a sonicator for the purification and analysis, the extract was cleaned using a silica gel column.1.0µl of the purified extract was injected into a calibrated Gas Chromatograph (GC) equipped with a capillary column. Data processing was done using DATA APEX CLARITY software. The quantification of Total Petroleum Hydrocarbon (TPH) was performed using GC-FID (Agilent 7890).

Characterization and Synthesis of Carbon Dot: Hydrothermal Synthesis Method: The hydrothermal method was adopted with modifications from existing synthesis methods Boadu *et al*., 2021; Tang *et al*., 2012; Wang *et al*., 2010; Xu, *et al.,* 2014)

About 50g of coconut shaft powder (biomass) was weighed and mixed with

1000ml of distilled water. The homogenate was boiled for 2 hours at 100°C. Fluorescence Observation: After filtration, the filtrate was exposed to an ultraviolet (UV) lamp, producing green fluorescence. For the drying and Carbon Dot Collection the filtrate was transferred to a conical flask and sealed with foil paper to make it airtight. The residue was poured into Petri dishes and dried in an oven at 105°C. The carbon dots were deposited on the Petri dish surface, scraped off, and stored in sample bottles for further analysis.

Transmission Electron Microscope (TEM) Techniques: Staining and Dehydration: Double staining was performed before dehydration and sectioning. Heavy metals such as uranium, lead, or tungsten were used to enhance contrast between different structures in the specimen. Discoloration before hydration was conducted at the block stage. After sectioning, specimens were briefly exposed to an aqueous solution for staining.

Microbiological Analysis: Total Microbial Population Estimation: One gram (1g) of each soil sample was suspended in 9 ml of sterile distilled water under aseptic conditions. The dilution (0.3 ml) was plated on suitable media: Plate Count Agar (PCA) for heterotrophic bacteria (THBC). Mineral Salt Agar (MSA) for hydrocarbon-utilizing bacteria (HUB). The number of individual colonies was recorded as colony-forming units (CFU/g), (Ataikiru, et al., 2017; Bushnell, and Haas, 1941; Okerentugba, 2016).

Screening and Identification of Bacterial Isolates: Turbidometric Method: Measures turbidity (cloudiness) of bacterial cultures to quantify bacterial growth. The amount of scattered light is directly proportional to the number of bacterial cells in the culture. Bacteria isolates were suspended in nutrient broth. Initial turbidity was measured using a turbidimeter. The culture was incubated at 37°C for 24 hours. Final turbidity measurement was taken, and growth was determined by subtracting the initial reading from the final reading.

Spectrophotometric Approach: Used to quantify bacterial growth and estimate hydrocarbon degradation.Absorbance was measured at 600 nm using a spectrophotometer following the method of (Habib *et al.*, 2017; Okorhi-Damisa and Tudararo-Aherobo, 2023).

Identification of Isolates (Culture dependent and Non-culture dependent method): Genomic DNA extraction, sequencing, and bioinformatics were carried out at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Identification was done by extracting the geneomic DNA from the samples using the ZymoBIOMICS DNA Microprep Kit (Zymo Research, Catalogue No. D4031).The isolates identified and used all had high degrading capabilities, hence we have *Lysinibacillus fusiforms*, *Bacillus velezensis* and *Bacillus thurigiensis* (Tudararo-Aherobo and Okorhi-Damisa, 2023). Consortia of the organisms used were scaled up using the nutrient broth. They were identified based on their macroscopic, microscopic and biochemical characteristics using both the culture dependent and culture independent method (BAM, 1998).

RESULTS AND DISCUSSION

The pH of the impacted soil was 5.75±1 (acidic), while the pH of the unaffected soil was 7.84±1 (alkaline) (Table 2). Phyto-nanotechnology was used to synthesize carbon dots (CD).This method reduces toxic chemical usage and produces eco-friendly nanomaterials. Carbon dots contain potassium, magnesium, calcium, copper, zinc, and iron, enhancing their potential agricultural benefits (Ijaz, 2020; Panwar *et al.*, 2012). Significant levels of potassium and minor concentrations of magnesium, calcium, copper, zinc, and iron were found in carbon dot (CD), which may boost the fertilizer's value. With few adjustments, the hydrothermal process was used to process the biomass (coconut shaft (Saeed and Kamel 2021; Okieimen *et al*.,2020; Tan *et al.*, 2017).

In Figure 2, EDX findings revealed 69.10% for C, 7.20% for O, 2.22% for Na, 3.20 for Fe, 0.23 for Ca, and 0.33 for Si. This simply demonstrated that the plant extract did more than just act as a bioreductant; it also stabilized and capped the nanoparticle, which may be attributed to the biomolecules of the plant extract. Soil carbon provides nutrients through mineralization, helps to aggregate soil particles (structure), and provides resilience to physical degradation. It also increases microbial activity, water storage, and availability to plants, and protects the soil from erosion. Figure 3 depicts the Cocci-shaped, separated, and spherically packed carbon dot from transmission electron microscopy (TEM). Along with validating growth direction and crystal quality in agreement with the results of (Aljaafari *et al.*, 2020), the image also showed cocci appearance of the carbon dot. Figure 7 illustrates how the uncovered eye of the UV-visible spectroscopy depicts the biomass synthesis, demonstrating its colour change from brown to greenish. With an absorption peak at 342 nm, UV-visible spectroscopy was used to confirm the CD production. Table 3 Culture independent (molecular) and dependent method were adopted in the isolation and identification of the bacteria, hence. *Bacillus thuringiensis*, *Bacillus velezensis*, and *Lysinibacillus fusiformis* were isolated and identified. Using the nutrient broth, consortia of the identified organisms were scaled up. They were identified based on macroscopic, microscopic, and biochemical traits (BAM, 1998), hydrocarbon utilizing bacteria has been shown to have an antagonistic effect on the harmful effects of crude oil. These bacteria have been screened and used as environmental remediation agents, which help to speeds up the removal of these contaminants from the environment, by altering their exterior mechanism and secreting bioemulsifiers to improve their contact to target the hydrocarbon substrates, these bacteria increase the holding capacity of cells according to (Kaczorek *et al*., 2012; Krasowska and Sigler, 2014). Natural attenuation, biostimulation, and bioaugmentation methods were applied. Various setups were observed over 0–84 days. The setup was created in three equal portion, and it was observed in the laboratory at room temperature (25-27℃). During this period some parameters were monitored, analysed and result realized using the Gas chromatography flame induction detector (GC-FID) revealed that the control (Natural attenuation) were in the range of 1071.62±0.54 and 84.27±0.01, for the 1.0% whereas the 10% recorded 12504.50±0.71 and 200.08±0.16 within the period of exposure, drastic reduction of TPH was observed within the early and the final stage of the laboratory experiment. For the biostimulation set up, Composite microbial culture (CMC) with three concentrations, A were within the range of 948.46±0.07 and 29.7±0.01, while B was within 110.07±0.10 and 21.95±0.08, C was within 99.99±0.01 and 13.88±0.04, the reduction of the TPH was so drastic that it was almost within detectable limit especially with concentration A as this is a true fact that microbes has catalytic effect on hydrocarbons. For Carbon dot, concentration A were within 1026.52±0.01 and53.88±0.00, while for the B they are 455.71±0.15 and 11.54±0.01, for C 182.38±.011 and 1.37±0.01. For the biostimulation and bioaugumentation, concentration A were within the range of 1006.76±0.06 and 45.10±0.01, for B it recorded 553.67±0.22 and 15.63±0.04, for C it was 214.77±0.15 and 4.13±0.01 respectively, Crude oil are complex mixtures of aliphatic and aromatic compounds that require diverse bacterial genus with dissimilar catabolic genes to completely mineralize them. This is due to the fact that various bacterial species have various catalytic enzymes, which causes a wide range in their contributions to fossil fuel-contaminated locations (Chaerun, *et al*., 2004; Varjani and Gnansounou, 2017).

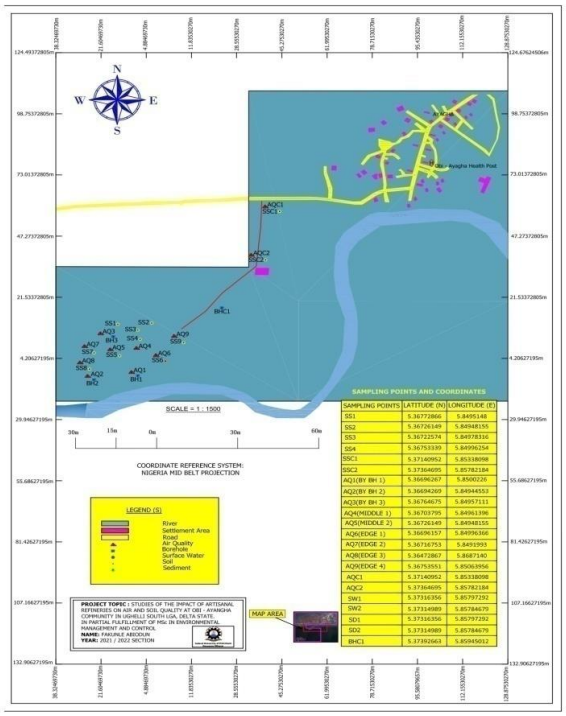

Figure 1: GPS Map of the Study Area (Obi-Ayagha Community, Delta State, Nigeria)

Table 1: Coordinates of the sampling sites within the sampled locations

Sample Collection Site	Type of Sample	Latitude	Longitude	Temperature/Soil texture
Obi-Ayagha abandoned artisanal refinery Site	Hydrocarbon Contaminated Soil	5.3674330	5.8499400	Loamy/clay 28.30 −29.00
Obogu village	Coconut husk	5.456756.5	5.634906	Loamy soil 28.1^0C-29^0C
FUPRE campus Garden	Natural pristine Soil	5.570334.5	5.840970	Loamy/clay 28.30 −29.00

Table 2: Soil pH

Soil type	pH
Impacted soil: 5.75 ± 1	acidic
Unaffected soil: 7.84 ± 1	alkaline

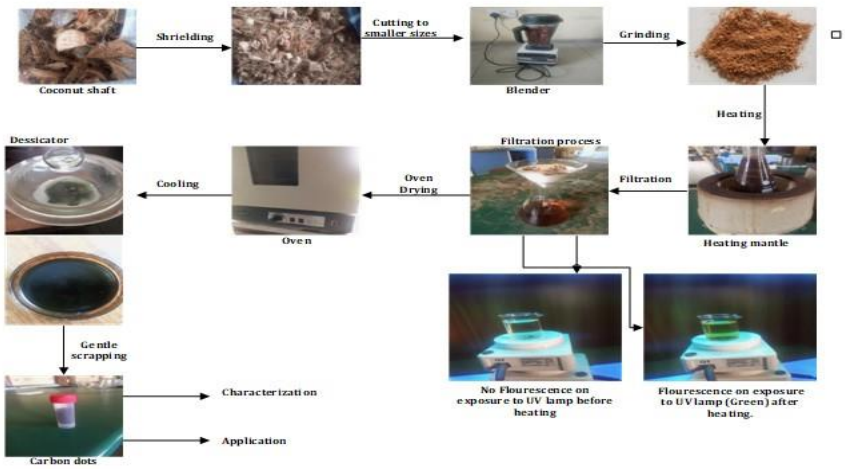

Plate 1: Biosynthesis of Carbon Dots

Table 3: Characterization of the Biomass

Carbon Particles (Coconut husk)	Characterization Techniques	
	Transmission Electron Microscope (TEM) Revealed the particle size (10)	
Carbon dot (C-dot)	Characterization Techniques	Results
	UV-Vis spectroscopy	Green fluorescence of carbon dot under ultraviolet light
Carbon dot (C-dot)	EDX (Energy dispersive X-Ray)	Displayed the various elements present in the carbon dot
	Transmission Electron Microscope (TEM)	showed separated and spherical packed nanoparticles in cocci-shaped form

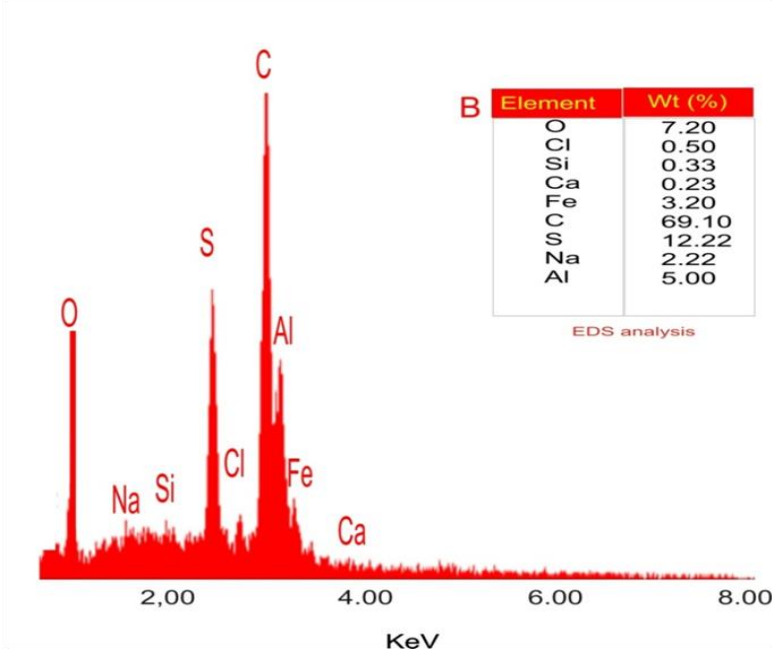

Figure 2: Energy dispersive X-Ray (EDX) analysis obtained from the Biomass

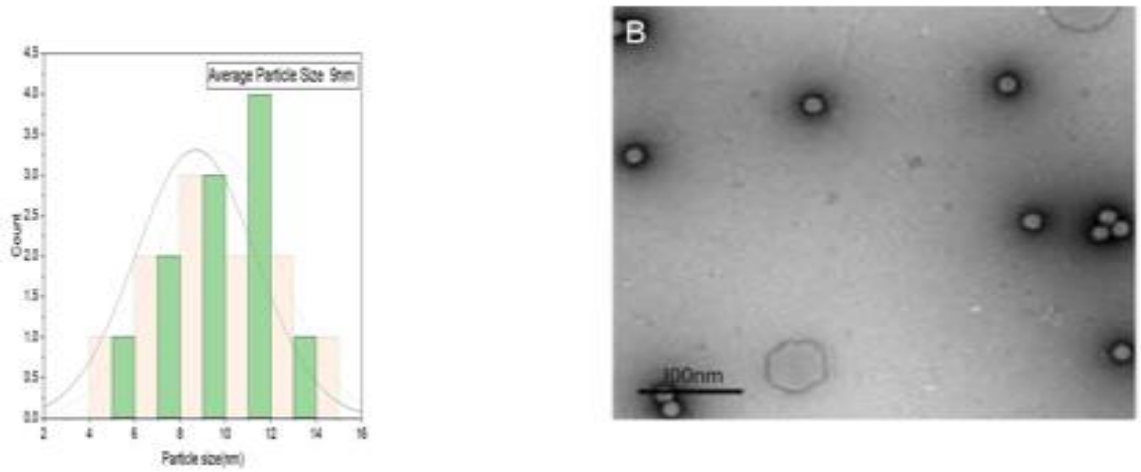

Figure 3: TEM micrograph of carbon dot from Coconut shaft

Table 4: Identity of the Bacterial Isolates

SAMPLE IDENTITY	PERCENTAGE	GenBank ACCESSION NUMBER
Bacillus thuringiensis	97.18%	CP044978.1
Lysinibacillus fusiformis	94.84%	FJ418643.1
Bacillus velezensis	84.83%	CP045835.1

Table 5: Experimental Design

	Setup		
Natural attenuation	100	10	1.0
	100	190	199.9
Total	200	200	200
Natural Attenuation (Field) Sample			
Biostimulation (CD) Nutrient	12	1.2	0.12
Impacted soil	8	0.8	0.08
Pristine soil	180	198	199.8
Total	200	200	200

Key: 10% = 100g/kg = 100,000.00g; 1% = 10g/kg = 10,000.00g

Table 6: Natural attenuation (Natural and Contaminated soil) 1000g (100,000mg/l and 10,000mg/l)

Exposure Period (Days)	A (1%)	B (10%)
0	1071.62±0.54	125.04.50±0.71
28	423.76±0.03	756.39±0.19
56	119.79±0.26	267.41±0.24
84	84.27±0.01	200.08±0.16

Table 7: Biostimulation (Composite Microbial Culture) 1000g

Exposure Period (Days)	A	B	C
0	948.46±0.07	110.07±0.10	99.99±0.01
28	600.13±0.91	84.90±0.01	60.25±0.01
56	54.73±0.01	47.76±0.01	25.55±0.01
84	29.7±0.01	21.95±0.08	13.88±0.04

Key: A=60;40:900=100,000mg/kg; B=6:4:990=10,000mg/kg; C=0.6;0.4:999=1000mg/kg

Table 8: Biostimulation (Carbon DOT) 1000g

Exposure Period (Days)	A	B	C
0	1026.52±0.01	455.71±0.15	182.38±0.011
28	859.97±0.04	98.45±0.07	55.81±0.01
56	182.38±0.11	32.80±0.01	16.50±0.71
84	53.88±0.00	11.54±0.01	1.37±0.01

Key: A=60;40:900=100,000mg/kg; B=6:4:990=10,000mg/kg; C=0.6;0.4:999=1000mg/kg

Table 9: Biostimulation and Bioaugumentation 1000g

Exposure Period (Days)	A	B	C
0	1006.76±0.06	553.67±0.22	214.77±0.15
28	951.52±0.03	254.80±0.04	90.77±0.01
56	162.30±0.71	70.52±0.08	45.05±0.04
84	45.10±0.01	15.63±0.04	4.13±0.01

Key: A=50;30:920=100,000mg/kg; B=8:2:990=10,000mg/kg; C=0.8;0.2:999=1000mg/kg

CONCLUSION

Bioremediation studies have showed that the organic amendment in combination with the Microbial culture is an excellent procedure for cleaning up an environment soiled with Hydrocarbons, Carbon dots have been applied in biomedical, environmental treatment, and in other vital areas, however with the rapid development in the field of environmental treatment and protection, the

recently updated CDs synthesis and applications are extremely effective. This practice is safe, eco-friendly and cost effective, and the fact has also been established that areas dented by oil spill could be returned to its usual state within a short period. Carbon dot and microorganisms (HUB) plays a major role in Bioremediation, their nutrient requirement (Carbon, nitrogen, phosphorus) and environmental conditions (oxygen or other electron acceptor, temperature, pH etc) should be determined. Soil metagenomics and molecular characterization of bacterial isolates from the soil has revealed the composition of the soil microbial communities and compare our remarks with the soil wellbeing.

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