ENHANCED DEGRADATION OF HYDROCARBONS IN SPENT ENGINE OIL CONTAMINATED SOIL BY *Pseudomonas aeruginosa* AND *Alcaligenes faecalis*

Supported by tetfund

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Received: January 29, 2020 **Accepted:** May 11, 2020

Abstract: This study was conducted to assess bacterial degradation of total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAHs) contents of spent engine oil (SEO) contaminated soil through the employment of *Pseudomonas aeruginosa* and *Alcaligenes faecalis* isolated from spent engine oil polluted soil from Dutse mechanic village, Nigeria. About 1.5 kg of autoclaved soil was deliberately contaminated with SEO at three levels. The soil was then supplemented with processed compost, powdered cocoa pod husk (CPH) and powdered cow dung (CD). Successively, the soil was bio-augmented with bacterial co-culture (150 ml). TPH and PAHs were estimated at the commencement, 5th and 10th week of the study. Factorial experiment laid out in a completely randomized design (CRD) was adopted. Results indicate that all the biostimulants employed did have significant effects on bacterial degradation of TPH and PAHs ($P<0.05$). Compost facilitated the most TPH reductions (756 and 250 mg\kg) on 5 and 10% SEO contaminated levels at 5th and 10th week, respectively ($P<0.05$). Powdered CPH only recorded the most significant PAHs reductions (37.7 mg\kg) on 15% SEO contamination level while compost recorded the most significant PAHs reduction (29.7 mg\kg) on 10% SEO contamination level compared with other biostimulants employed at the 5th week and 10th week, respectively ($P<0.05$). The significant feat that *Pseudomonas aeruginosa* and *Alcaligenes faecalis* co-culture recorded in this study has indicated its potential for utilization in achieving effective remediation of hydrocarbon related pollution.

Keywords: Biodegradation, PAHs, spent engine oil, microbes, pollution

Introduction

The technological advancements currently seen in the world over pertaining to the employment of products that are of hydrocarbon origin have progressively caused all forms of environmental pollutions related to hydrocarbons (Adeleye *et al.*, 2018). The public attention that polycyclic aromatic hydrocarbons (PAHs) which are synonymous with spent engine oil have generated over the years has been credited to the level of toxicity, mutagenicity, teratogenicity and carcinogenicity it has when found in air, soil, water and freshwater pollution (Bumpus, 1989; Clemente *et al*., 2001; Cerniglia and Sutherland, 2001; Ravindra *et al.*, 2008; Yerima *et al*., 2013; Umana *et al*., 2017). Numerous hydrocarbonoclastic microorganisms capable of degrading petroleum components have been isolated and reported in the literature. However, few of these hydrocarbon utilizers appear to be important for petroleum and its PAHs component degradation in the natural environments (Harayama *et al.*, 1999).

Hydrocarbon components most especially PAHs are mostly released into the environment through human activities such as combustion of fossil fuels, biomass (Omar *et al.*, 2002), and indiscriminate disposal of spent engine oil. Subsequently, through different pathways including percolation into the soil, atmospheric fallout, urban runoff and municipal or industrial effluents, it may find its way into surface waters (Zhu *et al.*, 2004). PAHs have been implicated to be extant natural constituents in fossil fuels and can be formed during the incomplete combustion of organic materials, and are consequently present in comparatively high concentrations in fossil fuel refining products (Bos *et al*., 1984).

It has been established by Atlas and Bartha (1983), that microbial biodegradation of hydrocarbon contents in the environment can be very slow for a number of factors; population of the hydrocarbon degraders, temperature and nutrient availability are not provided to the optimal and desirable level. Owing to the documented deleterious health possibilities that PAHs can exert on humans and the environment, its removal from environmental media requires sustainable and cheap approach. It is against these backdrops that this study was conducted to assess the removal of hydrocarbon contents by organic amendment enhanced *Pseudomonas aeruginosa* and *Alcaligenes faecalis* co-culture isolated from SEO polluted soil.

Materials and Methods

Description of the study area

This study was staged at the back of the Department of Soil Science, Faculty of Agriculture in Federal University Dutse campus (Lat $11^0$46'39''N and Long 9^0 20'3''E) Jigawa state, Nigeria. Britannica (2019) described the undulating relief of the study area as being covered by Sudan savanna agro-ecological zone.

Collection of fresh cocoa pod husks and cow dung

About 30 kg of fresh cocoa pod husks (CPH) was collected from a heap of CPH. About 30 kg Cow Dung (CD) was obtained from the liarage of the Abbatoir in Dutse, Nigeria.

Preparation and processing of powdered cocoa pod husk

About 15 kg CPH was air dried for thirty (30) days and crumpled into powder as described by Agbor *et al.* (2015). The powdered CPH was sieved into fine form and subsequently autoclaved at 121^0C for 15 min with a view to expunging influence of undesirable microbial lifeas done by Ezekoye *et al.* (2017).

Preparation and processing of powdered cow dung

Out of the collected CD, about 10 kg was sun-dried and rumpled into powder with the aid of pestle and mortar. It was correspondingly sieved into fine form, autoclaved at 121^0C for 15 minutes to remove unnecessary effect of unwanted microbial lifeas done by Ezekoye *et al.* (2017). It was

thereafter stored in a container and labeled as powdered CD only.

Generation and processing of compost

The compost employed in this study was generated from the procedure outlined by Adeleye *et al*. (2019). The compost was successively autoclaved at 121^0C for 15 min to remove peripheral impact of undesirable microbial life as done by Ezekoye *et al.* (2017). Subsequently, it was kept in a container and labelled appropriately.

Collection and processing of soil

As outlined by Agbor *et al*. (2015), about 250 kg top soil (0-25 cm depth) that having no history of pollution was collected from four different points close to the Department of Soil Science, Federal University Dutse main campus. The soil was subsequently air-dried and bulked to generate composite sample.

Collection of spent engine oil

About eight (8) liters of spent engine oil was obtained from one of the service pits in Mechanic Village Dutse, Jigawa State.

Preparation and contamination of soil

In reference to Soretire *et al.* (2017), 2 mm mesh size was employed to sieve the bulked soil. The soil was subsequently autoclaved at 121^0C for 15 min so as to sterilize undesirable microbial life. About 1.5 kg of the sterilized soil was placed in 36 polyethylene bags and 5, 10 and 15% SEO levels were added distinctly. The soil and the varying SEO contamination levels were carefully mixed, and allowed to stand intact for fourteen (14) daysto ensure the process of volatilization of the toxic components reported by Abioye *et al.* (2012); Agbor *et al.* (2015).

About ten (10) grams of spent engine oil (SEO) polluted soil at the depth of five (5) centimeters was collected from the mechanic village, Dutse. The ambient temperature recorded with the aid of a thermometer during the sampling of the spent engine oil polluted soil was 37.3^0C. Spent engine oil dumping at the site where the SEO was sampled has been going on for four (4) years (Personal communication with Head Mechanic at the workshop).

Isolation and identification procedures for spent engine oil degrading bacteria (SEODB)

SEO degrading bacteria; *Pseudomonas aeruginosa* and *Alcaligenes faecalis* were isolated according to the selective enrichment procedure described by Adeleye and Yerima (2019) while its identification was done by adopting the procedures outlined by Barrow and Feltham (1993). Biochemical tests conducted for the identification of the bacterial isolates were done following the procedures outlined by Olutiola *et al.* (2000); Choopun *et al.* (2002); Cheesebrough (2006); Ochei and Kolhatkar (2008); Wilson (2012); Hemraj *et al.* (2013); Himedia (2015); Pokhrel (2015); Microbeonline (2019).

Biodegradation assay

Thirty six (36) polyethylene bags were set up for biodegradation incubation assay. According to Nkereuwem *et al*. (2010); Ezekoye *et al. (*2017), except the twelve (12) experimental bags adopted as control, 150 g of each sterilized biostimulant; compost, powdered CPH only and powdered CD only, was added and comprehensively mixed with the soil contaminated with 5, 10 and 15% SEO levels. Except the nine (9) experimental bags adopted as control, all the other twenty (27) remaining experimental bags were augmented and thoroughly mixed with one hundred and fifty (150) mL *Pseudomonas aeruginosa* and *Alcaligenes faecalis* co-culture. Three (3) replicates were kept for each SEO contamination level. All the experimental bags were later incubated at room temperature for seventy (70) days as established by Chorom *et al.* (2010)**.**

As outlined by Ayotamuno *et al*. (2006): Chorom *et al.* (2010)**,** the contents of each experimental bag were turned over twice a week for effective aeration. The moisture content of the experimental bags was equally maintained twice a week through the addition of six (6) mL sterile distilled water as reported by Abioye *et al.* (2012). Periodic sampling for the estimations of TPH and PAHs was done at the commencement, fifth week and tenth week of the experiment.

Determination of total petroleum hydrocarbon and polycyclic aromatic hydrocarbons

Total Petroleum Hydrocarbon (TPH) and Polycyclic aromatic hydrocarbons (PAHs) were determined using gas chromatograph flame ionization detector (GC-FID) system adopting the procedures described by USEPA (2003).

Determination of physicochemical parameters of soils and biostimulants

Unpolluted soil, SEO polluted soil and biostimulants' samples were analyzed for pH and electrical conductivity (EC) in deionized water (1:2.5 w/v for soil and 1:5 w/v for biostimulants). Organic carbon was estimated by the adjusted Walkley-Black procedure described by Nelson and Sommers (1986). Total Nitrogen and Phosphorous were determined by employing Kjeldhal and Bray-1 method described by Reeuwijk (1993); Bremmer, (1996) respectively. Cation exchange capacity (CEC) was determined using the summation method described by Chapman (1965). The soil mechanical analysis was similarly determined using the hydrometer method outlined by Bouyoucos, (1962).

Statistical analysis

All data collected were subjected to Proc. GLM of GenStat version 17 and significant means were separated using Duncan Multiple Range Test (DMRT).

Results and Discussion

Physicochemical properties of soils and biostimulants

The results of the physicochemical properties of unpolluted soil, SEO contaminated soil, compost, powdered CPH and powdered CD indicate CEC values of 3.51, 1.05, 221.7, 166.15 and 82.1 cmol\kg, respectively (Table 1).

Table 1: Physicochemical properties of unpolluted soil, SEO polluted soil and biostimulants

Parameters	Unpolluted soil	SEO polluted soil	Compost	Powdered Cow Dung	Powdered CPH
Moisture content (%)	2.04	0.8	2.0	7.3	11.11
Ash content (%)	-	-	65	68.8	23
$pH_{(water)}$	6.5	6.8	9.45	8.15	7.6
Organic Carbon (%)	0.49	0.52	48.25	41.55	33.40
Total Nitrogen (%)	0.06	0.08	5.85	2.85	2.65
Available Phosphorous (mg\kg)	11.02	9.40	1.48	1.2	0.08
EC (ds\cm)	0.92	1.20	8.86	8.10	6.42
Exchangeable Bases (cmol\kg)					
Potassium	0.19	0.07	213.16	80	162
Calcium	1.82	0.63	4.8	0.2	1.6
Magnesium	0.92	0.18	3.24	1.5	2.45
Sodium	0.58	0.17	0.5	0.4	0.1
CEC	3.51	1.05	221.7	82.1	166.15
Particle Size (g\kg)					
Clay + Silt	420	200	--	-	-
Clay	100	120	-	-	-
Silt	320	80	-	-	-
Sand	580	800	-	-	-
Textural class	Sandy Loam	Loamy Sand	-	-	-

Table 2: Identification of the bacterial inoculants according to the colony and biochemical characteristics

Parameters	Status		Status
Colonial characteristics	Creamy white colonies		Creamy colonies with scanty growth
Gram staining	Gram Negative Rods		Gram Negative Rods in Chains
Biochemical Tests			
Motility	+	Oxidase	+
Growth on MacConkey agar	+	Nitrate	-
Oxidase	+	Simmon's Citrate	+
Nitrate	+	Christensen's Citrate	+
Simmon's Citrate	+	Urease	-
Christensen's Citrate	+	Glucose	-
Urease	-	Fructose	-
Gluconate	+	Maltose	-
Glucose	+	Mannitol	-
Fructose	+	Sucrose	-
Lactose	-	Xylose	-
Maltose	-	Casein hydrolysis	-
Sucrose	-	Tyrosine hydrolysis	+
Xylose	+	Growth on Centrimide agar	+
10% Glucose	+		
10% Lactose	-		
Identity of bacteria	*Pseudomonas aeruginosa*		*Alcaligenes faecalis*

Legend: + = Positive; – = Negative

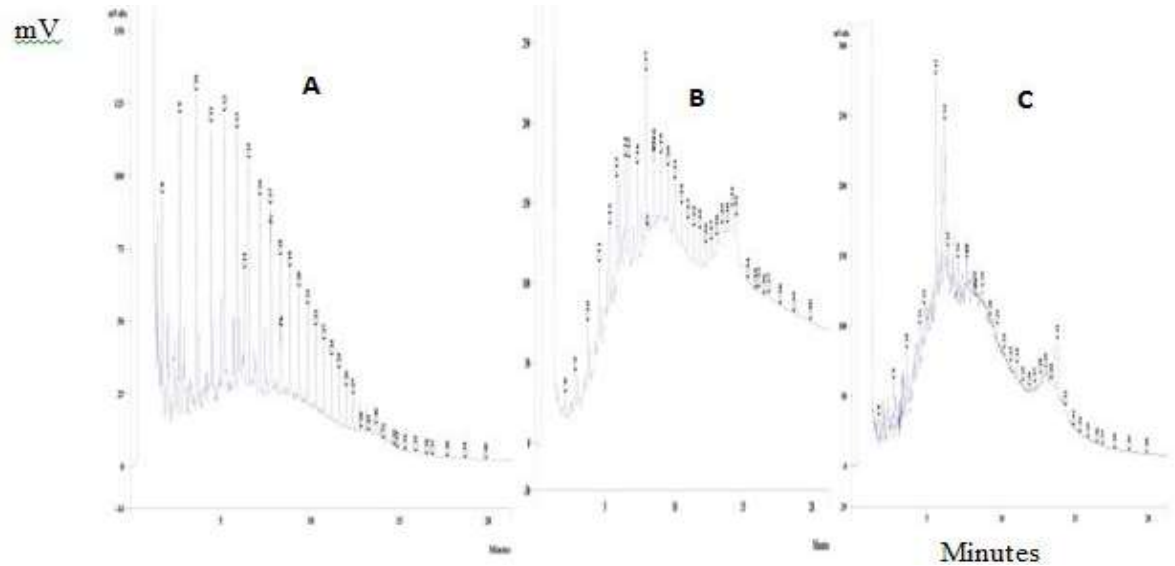

Fig. 1: Chromatograms showing (A) TPH fractions of 5% SEO (B) TPH fractions of 10% SEO and (C) TPH fractions of 15% SEO contamination levels before bacterial degradation

Isolation and identification of the innoculant bacteria

The results of selective enrichment medium adopted for the isolation of the bacterial inoculants from SEO contaminated soil on Trypticase soy agar led to the recovery of two principal bacterial isolates with colony characteristics presented and the results of the biochemical identification of the isolates are presented in Table 2.

Baseline concentrations of TPH and PAHs in varying SEO contaminated soil

Results generated on the TPH concentrations present in 5% (9934 mg\kg), 10% (10016 mg\kg) and 15% (10379 mg\kg) SEO contamination levels before bacterial degradation are depicted in Fig. 1. The results generated on the PAHs components of 5% (1065.9733 mg\kg), 10% (1726.5850 mg\kg) and 15% (1865.6340 mg\kg) SEO contaminated levels before bacterial degradation are presented in Tables 3. The results generated from the estimation of TPH and PAHs in the SEO contaminated soil studied have clearly demonstrated the sensitivity of GC-FID in estimating succinct concentrations of petroleum hydrocarbons in environmental samples.

Table 3: Polycyclic aromatic hydrocarbon contents in SEO contaminated soil before degradation

Hydrocarbon contents	5% (mg\kg)	10% (mg\kg)	15% (mg\kg)
Naphthalene	139.0226	0.9027	212.3526
Acenaphthylene	165.6564	146.4636	286.7352
Acenaphthene	119.8004	159.9880	306.9489
Fluorene	104.3942	192.6765	160.7632
Phenathrene	91.2870	210.1942	146.0150
Anthracene	140.0905	334.1136	212.1509
Fluoranthene	39.1378	154.8751	27.3495
Pyrene	33.3092	118.7443	11.9124
Benzo[a]anthracene	28.2368	150.1325	67.4873
Chrysene	14.035	38.5171	41.3368
Benzo [b] fluoranthene	41.8028	119.3219	103.1410
Benzo[k]fluoranthene	71.6423	14.2141	174.1245
Benzo [a] pyrene	40.9865	55.4440	91.4574
Indeno [1, 2, 3,c. d] pyrene	18.7666	12.1187	10.8305
Dibenzo [a, h] anthracene	17.8087	18.6788	12.9690
Totals	**1065.9733**	**1726.5850**	**1865.6340**

These results are in agreement with the report of Wenning and Martello (2014) on the acclaimed sensitivity and precision of the instrument. The detection of PAHs components by the GC-FID employed in this study is in line with the submissions of Irwin *et al.* (1997); Yerima *et al.* (2012) regarding their presence in petroleum hydrocarbons.

TPH biodegradation potential of Pseudomonas aeruginosa and Alcaligenes faecalis co-culture

Results generated regarding the influence of biostimulants on TPH degradation potential of *Pseudomonas aeruginosa* and *Alcaligenes faecalis*co-culture at the 5th week indicate that all the biostimulants significantly enhanced bacterial degradation of TPH ($P<0.05$).Compost had the most effects (756 mg\kg and 1577 mg\kg) compared with other biostimulants on 5 and 10% SEO contamination levels, respectively while powdered CPH only enhanced the most reduction (1181 mg\kg) attained by the biostimulants on 15% SEO contamination level (Fig. 2). However, at the 10th week when the experiment was terminated, compost significantly enhanced further reductions (305, 250 and 516 mg kg^{-1}) compared with other biostimulants on 5, 10 and 15% SEO contamination levels respectively (Fig. 3).

The TPH reduction achieved by powdered CPH only in this study is in line with the submissions of Agbor *et al.* (2012); Agbor *et al.* (2015), regarding its ability to attain such feat. Again, the significant reductions of TPH occasioned by the co-culture of *Pseudomonas aeruginosa* and *Alcaligenes faecalis* in this study can be attributed to their being enhanced optimally by the nutrients obtainable in the compost. This result is in agreement with the reports of several authors (Adedokun and Ataga, 2007; Agbor *et al.*, 2012; Agyarko and Asiedu, 2012; Offor and Iyagba, 2013; Otaraku and Anozie, 2013; Ezeaku and Egbemba, 2014; Hichman *et al.*, 2014; Ahamefule and Fawole, 2015; Romanus *et al.*, 2015) on the ability of organic amendments to release significant nutrients needed for optimum microbial metabolism into the soil when applied thereby bringing about significant TPH degradation.

As reported by Bharali*et al.* (2001): Zhang *et al.* (2005); Igwo-Ezikpe *et al.* (2009), the ability of *Pseudomonas aeruginosa* and *Alcaligenes faecalis*to produce biosurfactants when enhanced optimally with suitable nutrients might have led to the significant reductions of TPH achieved in this study.Diaz (2008) did report that enhancement of biodegradation process gives rise to the production of biosurfactant which might have ultimately helped the TPH degradation rate recorded in this study. Many authors (Vahaboglon *et al.*, 1996; Jorgensen *et al.*, 2000; Akpe *et al.*, 2013) have linked the ability of gram negative bacteria to utilize hydrocarbons with the presence of porins on their cell wall and availability of plasmid-borne or chromosomal gene meant for degradation of compounds.

In the past, several authors (Atlas, 1981; Bossert and Bartha, 1984; Sarkhoh *et al.*, 1990; Atlas and Bartha, 1992; Balba *et al.*, 1998) did categorize the duo of *Pseudomonas* and *Alcaligenes* amongst thecommonest genera of bacteria that are prolific hydrocarbon degraders. The SEO degradative prowess demonstrated by *Pseudomonas aeruginosa*and *Alcaligenes faecalis* co-culturein this study has further substantiated the submissions of these authors.

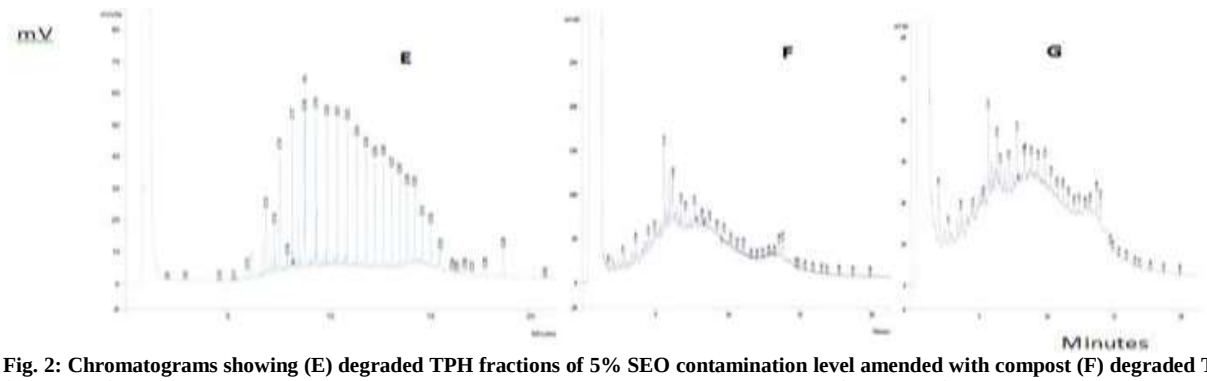

Fig. 2: Chromatograms showing (E) degraded TPH fractions of 5% SEO contamination level amended with compost (F) degraded TPH fractions of 10% SEO contamination level amended with compost (G) degraded TPH fractions of 15% SEO contamination level amended with powdered CPH only at the 5th week using *Pseudomonas aeruginosa* and *Alcaligenes faecalis* co-culture

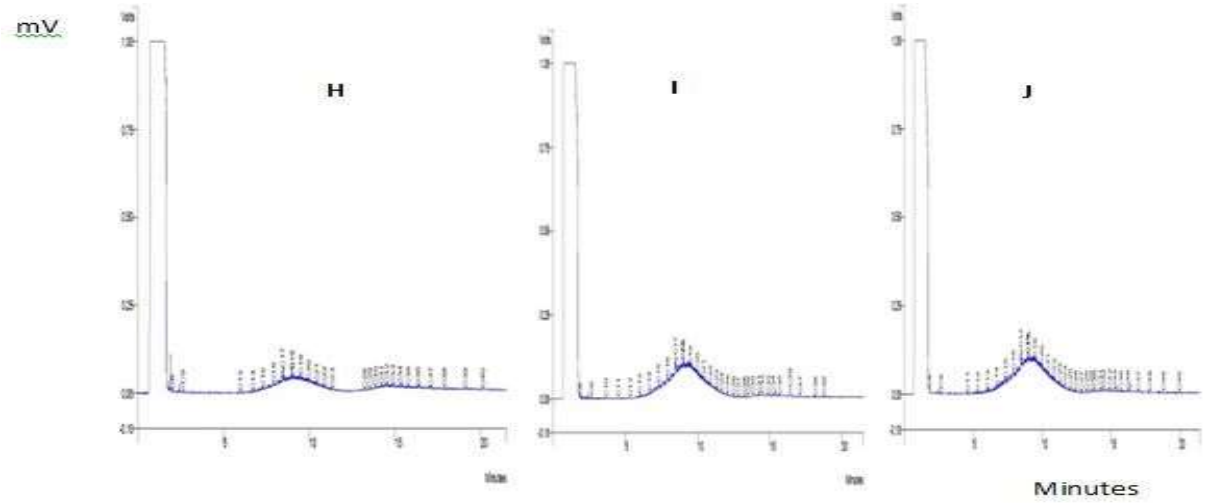

Fig. 3: Chromatogram showing (H) degraded TPH fractions of 5% SEO contamination level amended with compost (I) degraded TPH fractions of 10% SEO contamination level amended with compost (J) degraded TPH fractions of 15% SEO contamination level amended with compost at the 10th week using *Pseudomonas aeruginosa* and *Alcaligenes faecalis* co-culture

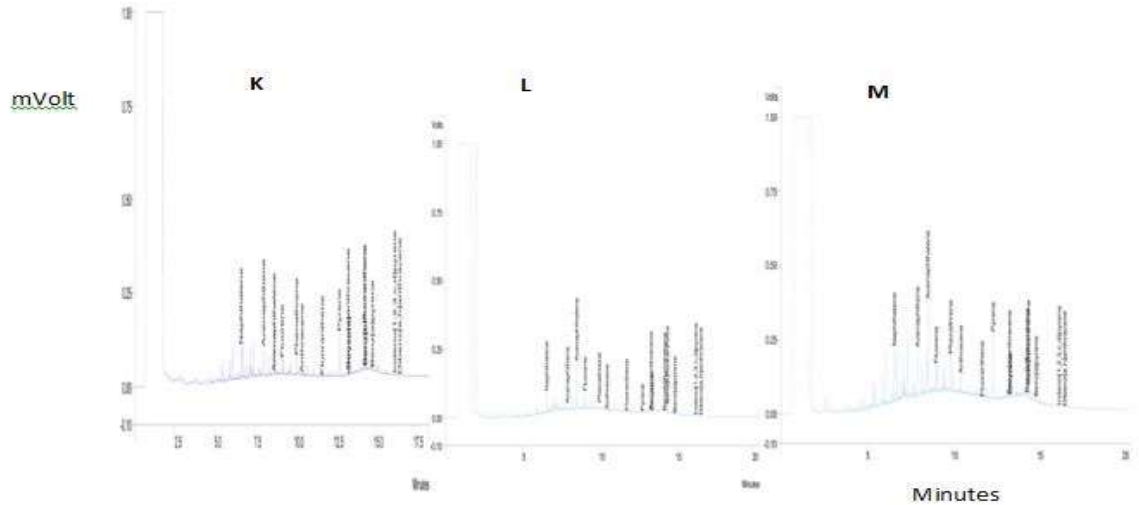

Fig. 4: Chromatograms showing (K) degraded PAHs fractions of 5% SEO contamination level amended with compost (L) degraded PAHs fractions of 10% SEO contamination level amended with powdered CPH only (M) degraded PAHs fractions of 15% SEO contamination level amended with powdered CPH only at the 5th week using *Pseudomonas aeruginosa* and *Alcaligenes faecalis* co-culture

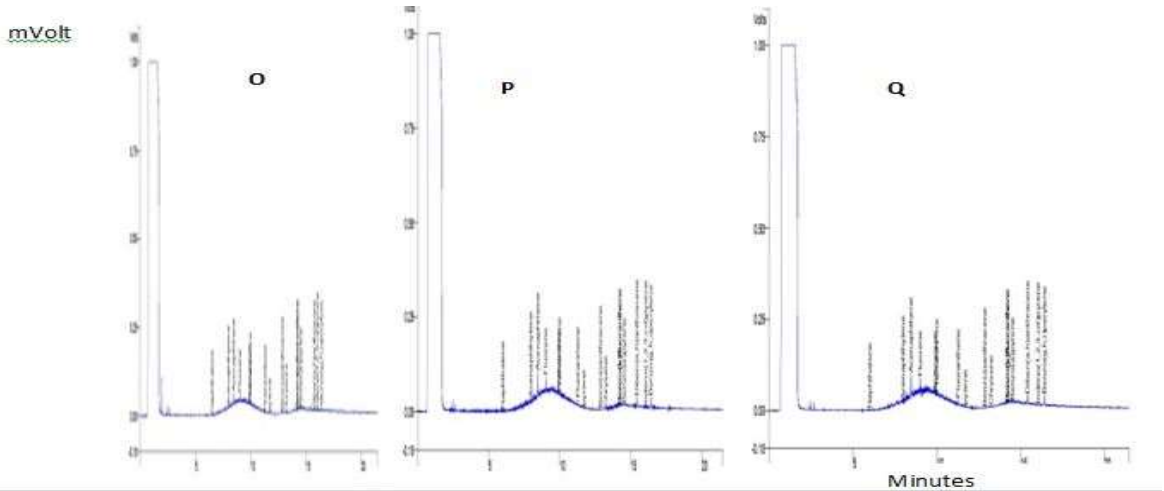

Fig. 5: Chromatograms showing (O) degraded PAHs fractions of 5% SEO contamination level amended with compost (P) degraded PAHs fractions of 10% SEO contamination level amended with compost (Q) degraded PAHs fractions of 15% SEO contamination level amended with powdered CPH at the 10th week using *Pseudomonas aeruginosa* and *Alcaligenes faecalis* co-culture

PAHs biodegradation potential of Pseudomonas aeruginosa and Alcaligenes faecalis co-culture

In terms of possible degradation of PAHs, at the 5th week, all the biostimulants significantly influenced bacterial degradation of the PAHs constituents of SEO contamination soil across SEO contamination levels ($P<0.05$). Compost enhanced the most degradation (170.0 mg kg^{-1}) on 5% SEO contaminated soil level compared with other biostimulants (Fig. 4). However, powdered CPH only enhanced the most reductions (385.4 and 371.7 mg\kg) in PAHs compared with other biostimulants on 10 and 15% SEO contamination levels respectively (Fig 4). At the 10th week, compost further influenced the most (64.9 and 29.7 mg\kg) reductions of PAHs compared with other biostimulants on 5 and 10% SEO contamination levels respectively while powdered CPH only recorded the most reduction (103.5 mg\kg) compared with other biostimulants on 15% SEO contamination level (Fig. 5).

The utilization and subsequent reduction of PAHs by the co-culture of *Pseudomonas aeruginosa* and *Alcaligenes faecalis* in this study is in agreement with the submission of Ogunbayo *et al.* (2012) that these constituents of petroleum hydrocarbons are known as substrates that support the growth of hydrocarbonoclastic microorganisms. The results obtained in this study are in line with the submissions of Kiyohara *et al.* (1982); Weissenfels *et al.* (1990); Lal and Khanna (1996); Viñas *et al.* (2005); Lakshmi and Velan (2011); Singha*et al.* (2017), on the capability of *Alcaligenes faecalis* to actively mineralize the PAHs contents of petroleum hydrocarbons leading to its degradation.Again, the ability of *Pseudomonas aeruginosa* and *Alcaligenes faecalis* to produce biosurfactant has been reported by Wong *et al.* (2004); Wyrwas *et al.* (2011) to enhance effective bioavailability of PAHs which might have led to the significant reductions attained in this study. The PAHs degradation capability exhibited by this bacterial co-culture is in support of the report of Ekpo and Udofia (2008) that implicated *P. aeruginosa* as possessing a more competent and active hydrocarbon degrading enzyme than many hydrocarbonoclastic bacteria.

Conclusion and Recommendation

In this study, bacterial degradation of TPH and PAHs was significantly enhanced by the addition of biostimulants in all the SEO contaminated soils at varying contamination levels studied. Owing to the significant reductions of the constituents that confer toxicity, mutagenicity and carcinogenicity on SEO when detected in soil, water and freshwater pollution, the combination of bioaugmentation and biostimulation is recommended as an efficient biotechnology tool to clean up hydrocarbon polluted environments. However, further research focusing on the molecular-based characterization of inherent hydrocarbon-degrading enzymes in the genome of the bacteria isolated and employed in this study for possible genotyping and DNA mapping is recommended.

Conflict of Interest

The authors declare that they have got no financial or non-financial conflict of interests regarding this manuscript.

Acknowledgement

The first author thanks the management of the Federal University Dutse, Nigeria for offering him a full tuition fee waiver towards running his Ph.D. programme out of which this research was conducted.

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