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Improvement of Hydrocarbon Degrading Bacteria Isolated from Automobile Mechanic Workshop Contaminated Soil Using Rock Phosphate

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Abstract
Indiscriminate discharge of used petroleum products or spillage is a source of pollution to our environment hence, the need for a concerted effort in studying the possibility of using oil degrading bacteria for remediation. This study was aimed at investigating the effect of rock phosphate supplementation on bacteria degradation of crude oil contaminated medium. Bacteria species were isolated from contaminated soil in mechanic workshops and screened for their hydrocarbon degradation potentials using standard microbiological procedures. The screened isolates and their consortium were used for the degradation of crude oil using minimal salt medium supplemented with rock phosphate for 20 d. Parameters investigated were pH, fungal load, phosphate solubilization, available phosphorus and hydrocarbon content. The identified bacterial isolates were Pseudomonas fluorescens, Corynebacterium glutamicum, Micrococcus luteus, Escherichia coli and Bacillus subtilis. The screened isolates were Pseudomonas fluorescens and Bacillus subtilis. The degradation result showed that the highest bacterial load and available phosphorus content were from rock phosphate crude oil Mineral Salt Medium (RCMSM) and consortium with values 16.69±0.40 cfu/mL and 14.97± 0.03 mg/L respectively on day 16. The least values 6.00±0.19 cfu/mL and 0.71±0.06 mg/L were from crude oil Mineral Salt Medium (CMSM) P. fluorescens and CMSM consortium respectively. Statistically, data from RCMSM and consortium were significantly different from other media and inoculum used ($p< 0.05$). The results suggested that the consortium of the isolates could be used in remediating crude oil contaminated soils.

Keywords: Automobile mechanic workshops, bacterial isolates, crude oil, Rock Phosphate

Introduction
Pollution caused by petroleum and its derivatives is the most prevalent problem in the local environment [1]. The discharge of engine oil which is generated during oil-changing processes from vehicles or motorcycles is a major source of oil pollution in automobile mechanic workshops and its environs [2,3]. The presence of different substrates and metabolites in hydrocarbon contaminated soils has no doubt provided an environment for the growth development of quite a complicated microbial community [4].

Biodegradation of hydrocarbons by natural population of microorganisms is reported to be one of the primary mechanisms of eliminating petroleum pollution from the environment [5], which exploits the ability of various bacteria species to degrade hydrocarbons [6]. Bioremediation techniques have obvious advantages such as noninvasive, cost-effective, conserves soil texture and characteristics and environmentally friendly compared to physical and chemical methods of remediation [1]. Supplementation with selected nutrients (biostimulation) especially with growth limiting factors in the form of organic manure, nitrogen and rock phosphate has been documented to be successful and increase the rate of biodegradation of hydrocarbon pollutants. [7, 8].

Inorganic fertilizers have been applied as biostimulants, over the years, for enhanced bioremediation of hydrocarbon polluted sites. However, its excessive application has been implicated in negative consequences such as eutrophication and atmospheric pollution. Also, inorganic fertilizers are much expensive in developing countries due to their high demand in agriculture to improve crop yield [1]. These challenges and the quest for environmental sustainability have motivated researchers to search for an alternative substrates to replace inorganic fertilizers and to enhance bioremediation. Hydrocarbon degrading and Phosphate-solubilizing microorganisms with the addition of Rock phosphate are recognized as a promising alternative for bioremediation of polluted soil [3].

Phosphorus is an essential macronutrient needed by microorganisms for growth and metabolic activities [9]. The major source of phosphorus is rock phosphate (RP), a phosphate-bearing mineral which is a finite and non-renewable natural resource. Phosphate solubilizing bacteria (PSB) possess the capability to change the insoluble form of phosphorus into soluble one resulting in the availability of phosphorus for optimum growth and improvement of environments [10, 11]. This study was aimed at investigating the effect of rock phosphate supplementation on the degradation of contaminated crude oil medium by bacterial isolates.

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Materials and Methods

Characterization and Identification of Bacterial Isolates

Bacterial strains used in this study were isolated from automobile mechanic workshops soil, in Benin City, Edo State, using pour plate method inoculated on Nutrient agar amended with Nystatin to discourage fungi growth, The bacterial isolates were characterized and identified based on cultural and microscopy characterization using standard methods as described by Cheesbrough, [12]; Mills *et al*. [13] and Holt *et al*. [14].

Screening of Bacterial Isolates for Hydrocarbon Utilization

The identified bacterial isolates were screened for the ability to degrade crude oil using minimal salt medium (with 1 % crude oil as carbon source). Minimal salt medium was prepared according to the composition formulated by Mills *et al*. [13] as modified by Okpokwasili and Okorie [15]. The flasks were inoculated with bacterial isolates and incubated, under aerobic condition at 200 rev/min at temperature (28±2°C). The bacterial growth was monitored through measurement of optical density using spectrophotometer at 600 nm [16]. Two selected isolates with highest turbidity and their consortium were used for degradation of crude oil.

Biodegradation Potentials of Screened bacterial Isolates

The bacterial isolates which had highest optical density during the screening test were used for degradation of crude oil. The degradation of the crude oil was carried out in Mineral Salt Medium (MSM) according to the method of Mills *et al*. [13] as modified by Okpokwasili and Okorie [15]. The media used were designated as crude oil Mineral Salt Medium (CMSM) containing 1 % crude oil and MSM, the second rock phosphate crude oil Mineral Salt Medium (RCMSM) containing 2 g of rock phosphate, 1 % crude oil and MSM and the third control (uninoculated) containing 2 g of rock phosphate, 1 % crude oil and MSM. The first two media were inoculated with the respective screened bacterial isolates with 10^6 cfu/ml bacterial suspension. All the flasks were incubated at 28±2 ^oC in a Gyratory shaker at 150 rpm and analyzed at every 4 d interval for 20 d. Parameters analyzed at every 4 d interval were bacterial load, pH, phosphorus solubilization. available phosphorus content and hydrocarbon content.

Analysis of media

After each sampling time, the pH of samples was determined using a pH meter (Jenway 3051). The bacterial growth was monitored using pour plate technique on a nutrient agar plates.

Phosphorus solubilization was determined using aliquots of each sample were added separately to 0.12 ml of 0.05 M p-nitrophenyl phosphate (pNPP) solution, followed by 1 h incubation at 37°C. Control treatments containing only liquid medium were included in each experiment with pNPP added after incubation. The yellow colour as observed in the separate media was measured at 410 nm [17].

Available Phosphorus of the soil samples was determined using the method of Watanabe and Olsen, [18] with the aid of UV spectrophotometer at a wavelength of 712 nm.

After each incubation period, residual hydrocarbon contents of the soil samples were determined by toluene cold extraction method as described by Yeung *et al*. [19] and Adesodun and Mbagwu, [20].

Results

The optical density at 600 nm of the bacterial isolates during the screening for the degradation ability is shown in Table 1. There was increase in turbidity of the medium containing the isolates with the highest turbidity observed for *Pseudomonas fluorescens* (2.551) followed by *Bacillus licheniformis* (2.023) and the least *Corynebacterium glutamicum* (1.338).

Table 1: Ability of bacterial Isolates to degrade crude oil using spectrophotometer

Isolates	Optical density at 600 nm
Bacillus subtilis	2.023
Micrococcus varians	1.487
Corynebacterium glutamicum	1.338
Pseudomonas fluorescens	2.551
Escherichia coli	1.684

Changes in pH during the degradation of crude oil and rock phosphate amended samples inoculated with *P. florescens*, *B. licheniformis* and their Consortium is shown in Table 2. There was observable decline in the pH of all media. The highest pH value at day 20 was from the medium CNSM without inoculation (7.33±0.11) and the least CMSM C (5.01±0.06).

Table 2: Changes in pH of contaminated crude oil medium and Rock phosphate supplementation medium inoculated with *Pseudomonas fluorescens, Bacillus subtilis* and their consortium

Medium	**Degradation Period (d)**					
	0	4	8	12	16	20
CMSM BS	7.06±0.02	6.78±0.35	6.51±0.03	6.16±0.04	5.81±0.09	5.87±0.04
CMSM PF	7.11±0.08	6.97±0.13	6.62±0.10	5.99±0.16	5.73±0.05	5.72±0.05
CMSM C	7.07±0.09	6.65±0.23	6.38±0.09	5.55±0.08	5.27±0.11	5.01±0.06
RCMSM PF	7.31±0.07	6.02±0.21	5.74±0.22	5.15±0.11	5.07±0.42	5.02±0.09
RCMSM BS	7.28±0.12	6.43±0.31	5.48±0.17	5.26±0.51	5.15±0.16	5.05±0.11
RCMSM C	7.30±048	6.86±038	5.59±038	5.23±018	4.44±017	4.27±026
URCMSM	7.33±0.12	7.33±0.14	7.33±0.11	7.33±0.11	7.33±0.11	7.33±0.11

Key: CMSM BS: Crude oil and mineral salt medium plus *B. subtilis*
CMSM PF: Crude oil mineral salt medium plus *P. fluorescens*
CMSM C: Crude oil mineral salt medium plus consortium
RCMSM PF: Rock phosphate crude oil Mineral Salt Medium plus *P. fluorescens*
RCMSM BS: Rock phosphate crude oil mineral salt medium plus *B. subtilis*
RCMSM C: Rock phosphate crude oil mineral salt medium plus consortium
URCMSM: Uninoculated rock phosphate crude oil mineral salt medium

There was observable increase in the bacterial load for all the inoculated media with the highest increase observed on day 16 followed by a decrease on day 20 (Figure 1). The highest bacterial load observed on day 16 was from the medium RCMSM C while the least was from CMSM PF with values 16.69±0.40 and 7.34±0.07 cfu/mL respectively.

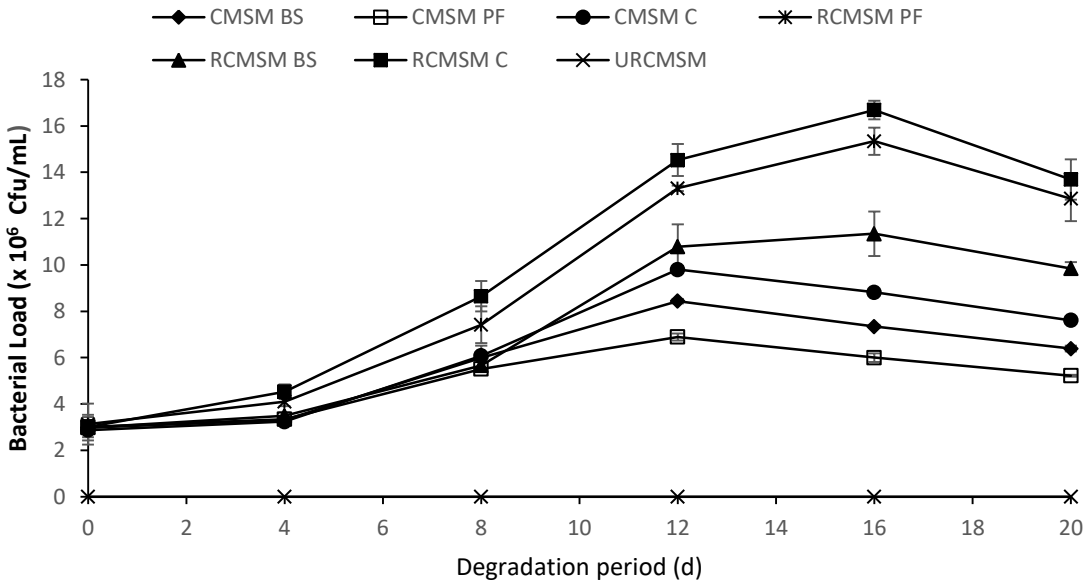

Figure 1: Bacterial load of contaminated crude oil medium and Rock phosphate supplementation medium inoculated with *Pseudomonas fluorescens, Bacillus subtilis* and their consortium
Key: CMSM BS: Crude oil mineral salt medium plus *B. subtilis*
CMSM PF: Crude oil mineral salt medium plus *P. fluorescens*
CMSM C: Crude oil mineral salt medium plus consortium
RCMSM PF: Rock phosphate crude oil mineral salt medium plus *P. fluorescens*
RCMSM BS: Rock phosphate crude oil mineral salt medium plus *B. subtilis*
RCMSM C: Rock phosphate crude oil mineral salt medium plus consortium
URCMSM: Uninoculated rock phosphate crude oil mineral salt medium

The ability of the bacterial isolates to solubilize phosphate is shown in Figure 2. There was increase in the phosphate solubilization of all inoculated Rock phosphate crude oil mineral salt media and the highest peak was at day 16. The highest phosphate solubilization was observed in the media containing the consortium (20.46±0.03 mg/L), *P. fluorescens* (16.59±0.83 mg/L) and *B. licheniformis* (14.81±0.38 mg/L). There was no observable increase in the phosphate solubilization for the Uninoculated rock phosphate crude oil mineral salt medium which remained unchanged from day 8 to 20.

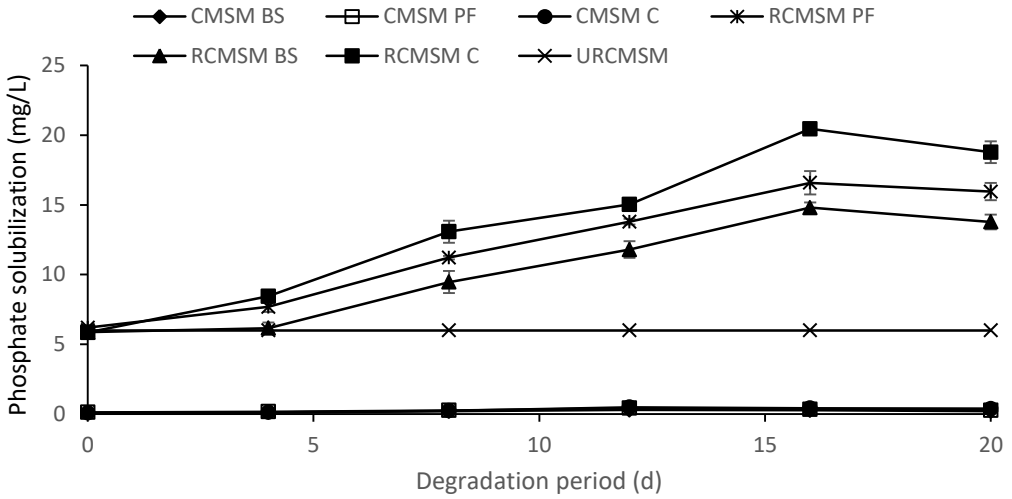

Figure 2: Phosphate solubilization of contaminated crude oil and Rock phosphate supplementation medium inoculated with *Pseudomonas fluorescens, Bacillus subtilis* and their consortium

Key: CMSM BS: Crude oil mineral salt medium plus *B. subtilis*
CMSM PF: Crude oil mineral salt medium plus *P. fluorescens*
CMSM C: Crude oil mineral salt medium plus consortium
RCMSM PF: Rock phosphate crude oil mineral salt medium plus *P. fluorescens*
RCMSM BS: Rock phosphate crude oil mineral salt medium plus *B. subtilis*
RCMSM C: Rock phosphate crude oil mineral salt medium plus consortium
URCMSM: Uninoculated rock phosphate crude oil mineral salt medium

Figure 3 shows the available phosphorus content of crude oil contaminated rock phosphate medium inoculated with *P. fluorescens*, *B. licheniformis* and their consortium. There was observable increase in the available phosphorus content of all inoculated media and peaked at day 16. The highest available phosphorus content was observed in the Rock phosphate crude oil mineral salt medium inoculated with consortium (14.97±0.03 mg/L), *P. fluorescens* (13.94±0.53 mg/L) and *B. licheniformis* (11.34±0.38 mg/L) while the Uninoculated rock phosphate crude oil mineral salt medium remained unchanged

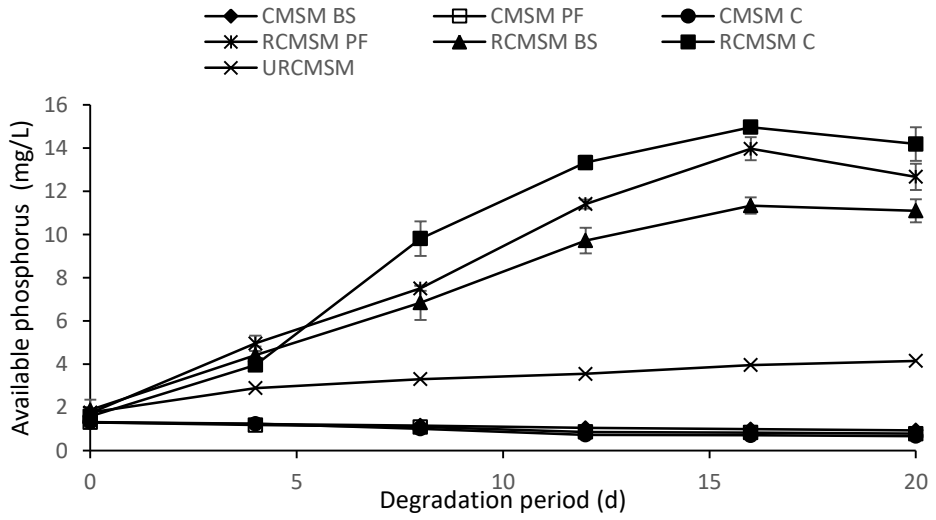

Figure 3: Available phosphorus content of contaminated crude oil and Rock phosphate supplementation medium inoculated with *Pseudomonas fluorescens, Bacillus subtilis* and their consortium

Key: CMSM BS: Crude oil mineral salt medium plus *B. subtilis*
CMSM PF: Crude oil mineral salt medium plus *P. fluorescens*
CMSM C: Crude oil mineral salt medium plus consortium
RCMSM PF: Rock phosphate crude oil mineral salt medium plus *P. fluorescens*
RCMSM BS: Rock phosphate crude oil mineral salt medium plus *B. subtilis*
RCMSM C: Rock phosphate crude oil mineral salt medium plus consortium
URCMSM: Uninoculated rock phosphate crude oil mineral salt medium

Hydrocarbon content of crude oil contaminated rock phosphate medium inoculated with *P. fluorescens*, *B. licheniformis* and their consortium is shown in Figure 4. There was observable decrease in the hydrocarbon content in all the inoculated media. The lowest hydrocarbon content was from the Rock phosphate crude oil mineral salt medium inoculated with consortium (706.31 ± 19.60 mL/L) and the highest was from Uninoculated rock phosphate crude oil mineral salt medium (2003.91 ±0 mL/L) on day 20.

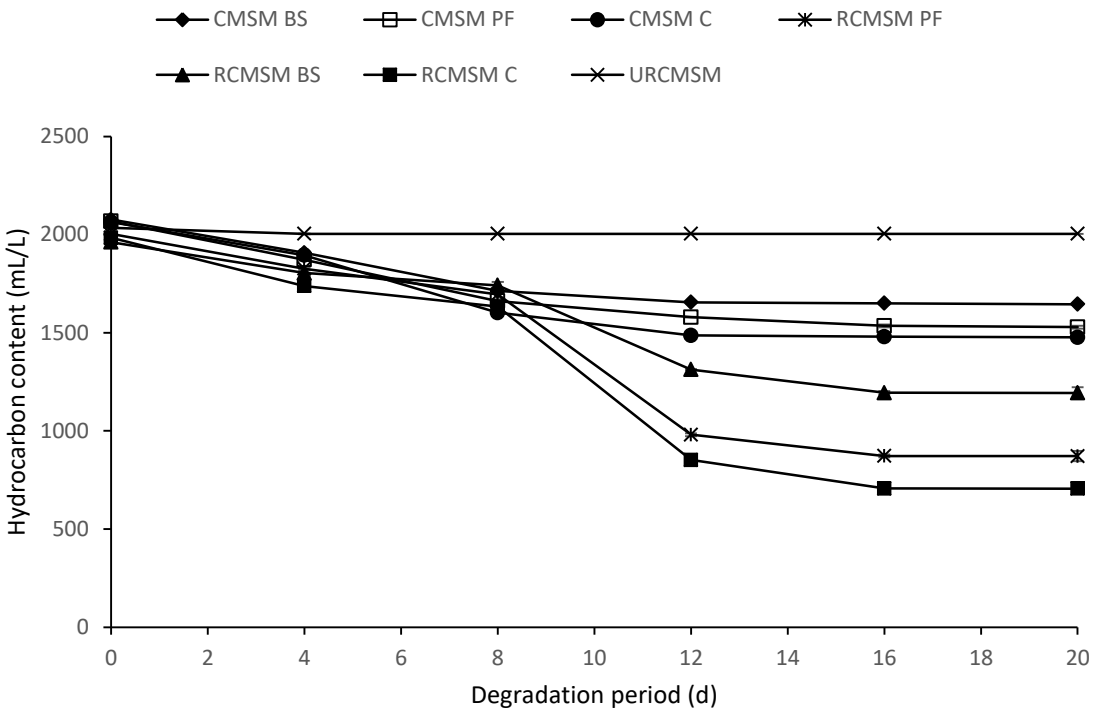

Figure 4: Hydrocarbon content of contaminated crude oil and Rock phosphate supplementation medium inoculated with *Pseudomonas fluorescens, Bacillus subtilis* and their consortium

Key: CMSM BS: Crude oil mineral salt medium plus *B. subtilis*
CMSM PF: Crude oil mineral salt medium plus *P. fluorescens*
CMSM C: Crude oil mineral salt medium plus consortium
RCMSM PF: Rock phosphate crude oil mineral salt medium plus *P. fluorescens*
RCMSM BS: Rock phosphate crude oil mineral salt medium plus *B. subtilis*
RCMSM C: Rock phosphate crude oil mineral salt medium plus consortium
URCMSM: Uninoculated rock phosphate crude oil mineral salt medium

Discussion

This study showed a general decline in pH of all crude oil contaminated media throughout the period of study with the media containing the consortium and rock phosphate amended medium showing more decline. The decline in pH denotes organic acid formation by phosphate solubilizing bacteria. This observation was reported by Khan *et al*. [21] to be the effect of the production of acidic intermediates which functioned to lower the pH of the media. The organic acids produced by phosphorus solubilizing bacteria chelate mineral ions and decrease the pH to bring phosphorus into solution [22].

In this study, bacteria load in the rock phosphate amended and unamended media increased in population with time. Nevertheless, the counts were higher in amended media compared to unamended media at respective intervals. An increase in the population of bacteria was observed during the study period regardless of the slight proliferation stall observed between day 8 and day 16. A similar study attributed the proliferation to additional bacteria populations, which utilized products from hydrocarbon breakdown [1]. The bacterial load of hydrocarbon contaminated media revealed that the addition of phosphorus source elevated the bacterial load as well as growth rate of all media. It was also evident that the medium containing the consortium had more bacterial load than that containing the pure isolates. Nakasaki *et al.* [23]; Adesodun and Mbagwu [20] indicated that phosphorus is a necessary nutrient for biodegradation of polluted environments which is required as phospholipids in synthesizing components of nucleic acids, cell membranes incorporation and for energy generation [24]. The decline in proliferation of bacteria observed at day 20 was due to depletion in nutrient,

which in turn reduced microbial activities (growth) in the amended media [1]. In another study, [25] reported that bacteria population tended to be highest during the mid-period and decreased gradually towards the end of study. Nevertheless, the observed decline in bacterial count was attributed to decreasing bioavailability of hydrocarbons to the microflora. Some of the bacterial genera, *Bacillus* and *Pseudomonas* investigated in this study have been reported by other researchers as hydrocarbon utilizing bacteria isolated during bioremediation of crude oil contaminated soils [8, 25, 26]. Alori *et al*. [9] also reported that *Bacillus* and *Pseudomonas* are among group of bacteria known to solubilize rock phosphate. The consortium of these bacteria sp had been shown to increase the availability of phosphorus in an environment (soil) thus improving food production through enhancing agricultural yield [27].

The rate of biodegradation of crude oil contaminated soil could be rapid although lack of growth rate limiting nutrient may decrease the reclamation process of the contaminated environment [6]. Biodegradation potential of microbiota of contaminated environments could be effectively enhanced by supplementation with essential nutrients such as nitrate, phosphate and sulphate sources [28]. The addition of growth limiting nutrients such as phosphorus has enhanced the rate of bioremediation and biodegradation of wastes and contaminated sites [29].

There was observable increase in phosphate solubilization and available phosphorus with rock phosphate amended medium but in contrast with unamended medium where there was decline in available phosphorus with slight increase in phosphate solubilization. This contrast was attributed to the fact that in unamended medium, there was no much residual phosphorus in the medium for the bacterial cultures to utilize, resulting in a decline. This is in agreement with the study of Khan *et al*. [21] who reported that during mineralization of rock phosphate, living organisms have the ability to transform the inorganic forms of phosphorus to organic phosphate which are then incorporated into their living cells. Phosphate solubilization rate is greatly influence by the ability of microorganisms to produce organic acids from the carbon source substrate [30], such as acetic, lactic and citric acid thus dropping the pH of the medium. These organic acids solubilize rock phosphate using acidification, chelation and exchange reactions [11]. Supplementation with rock phosphate is thus suggested to have provided additional source of phosphorus and with solubilization and utilization, net available phosphorus was higher when compared to the unamended medium [10, 21, 31, 32].

The result of hydrocarbon content during remediation period in this study revealed that amended medium with rock phosphate improved and increased the degradation of crude oil when compared to the unamended media. The medium inoculated with bacteria consortium showed considerable decrease in the hydrocarbon content when compared to the pure isolates. This implies that rock phosphate amended hydrocarbon polluted medium supported bacterial growth. This further mineralized and solubilized rock phosphate resulted in increased utilization of the residual carbon in the medium as sole carbon source for growth and energy generation. Similar result of increased biodegradation of petroleum hydrocarbon with the addition of phosphorus as a necessary nutrient [33, 34]. These characteristic reductions in hydrocarbon content were attributed to its utilization as sole carbon source by the bacterial isolates for growth and energy production which inevitability leads to the reclamation of the contaminated soil [35].

Conflict of interest

The authors declare no conflict of interest in this work

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