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COMPATIBILITY AND FORMULATION OF DIESEL DEGRADING CONSORTIA USING BACTERIA ISOLATED FROM CONTAMINATED SOIL

Umar Zubairu Darma*, Aisha Zango Mansir and Yunusa Yahaya Riko
Department of Microbiology, Umaru Musa Yar'adua University, Katsina
*Corresponding Author: zubairu.umar@umyu.edu.ng0806 595 6543

ABSTRACT
Soil contamination with diesel spillage is an increasing environmental challenge that damages living ecosystems. Efficiency of single bacterium in degrading diesel oil pollutants is faced with slow performance limitation. Therefore, the use of consortia is shown to be better, due to synergism, multi-enzymatic activity and potential for diversified catabolic functionalities. This study is aimed at formulating effective bacterial consortia that can degrade diesel in polluted environments. Four diesel degrading bacteria as Bacillus subtilis, Staphylococcus aureus, Micrococcus roseus and Rhodococcus specie were isolated and used for consortia formulation. Purity testing was performed on the isolates prior to consortia formulation, before their compatibility was tested by cross-spreading them on nutrient agar. Consortia formulation was made using Bacteria resting cells in Phosphate Buffer Saline based on compatibility testing and mathematical permutations. For on their ability to survive diesel on Bushnell-Haas Agar (BHA), consortia 2, 9 and 11 showed the best results among which consortium 11 was chosen as the best, considering growths on the medium within a 72 hrs period. The growth of the organisms before consortia formulation and after was also evaluated, which suggest that the consortium perform better than individual strains. Analysis of Variance showed significant statistical differences ($p<0.05$) between constituents of consortia, and diesel degradation on 2% (v/v) BHA. The degradation performances of the various consortia on BHA were furthermore separated by Duncan's Multiple Range Test. The colony counts obtained indicate that degradation was performed better by the consortia than individual strains. The findings of the study contribute towards illuminating inter-microbial relationships and microbial ecology especially within groups of diesel degrading bacteria. Further studies are imperative, to maximally harness the potentials of these bacteria for applications in large scale diesel biodegradation.
Key Words: Consortia, Diesel, Bacterial Biodegradation, Haemocytometry.

INTRODUCTION

Under the orchestration of techno-industrial breakthroughs, globalisation and population explosion, energy consumption is ever on the increase, universally, necessitating further exploration and utilisation of fossil fuels, such as diesel (Musa, 2019; Obi *et al*., 2016). As a case study, diesel fuel is consumed by stationary engines and in boilers, reciprocating engines, some generators, gas turbines, pipeline pumps, gas compressors, tractors and heavy duty vehicles, steam processing units in electric power plants and water heating facilities, locomotives, some aircraft, military vehicles, construction equipment among others (DSS, 2019). To put this in perspective, projecting from diesel consumption data provided by NBS (2019) for the first quarter of 2019, Katsina State alone will consume more than 34 million litres of diesel in 2019, up from the roughly 13 million consumed in 2016.

The cosmopolitan nature and ubiquitous presence of pernicious, spilled petrochemicals - including diesel - in the environment had been exhaustively reported in literature (Dixit *et al*., 2018). Diesel fuel is majorly composed of aliphatic hydrocarbons, with trace amount of aromatics (<10%) (Chaudary *et al*., 2019), however, individual chemicals in these groups present in diesel are greatly diverse (Ciric *et al*., 2010). Chromatographically, diesel is composed of Linear Saturated Hydrocarbons (n-alkanes or n-paraffins); Isoalkanes/isoparaffins (branched chain saturated hydrocarbons); Cycloalkanes/naphthalenes (saturated cyclic alkanes); and Aromatics, such as the PAHs (Naila, 2015; Yuniati, 2017).

These pollutants themselves, or their chemical derivatives, covertly or overtly find their way onto the environment during the prospection, exploration, refining, transportation, dispensing, utilisation and storage of diesel (You *et al*., 2018). Environmental proximity and presence of these xenobiotics is associated with attendant carcinogenicity, mutagenicity and consistent bioaccumulation across the food chain (Mahmoud and Bagy, 2018). Elaborately, the transfer of toxic, complex aliphatics, aromatics, nitrogen, sulphur and metals that originate from diesel spills into the food chain is advocated as the raison d'être (Yetti *et al.,* 2016) for xenobiotics' bioaccumulation in spillage-affected areas.

Generally, bye-products of petrochemical origin are regarded as priority pollutants as was vividly manifested in their declaration by the United States' Environmental Protection Agency (USEPA) as priority pollutants (Yuniati, 2017). The extent of damage elicited by these pollutants can be lethal/sub-lethal, acute or chronic depending on the severity, dosage, time/duration of exposure and the particular organism affected (Patowary *et al.,* 2016).

Remediating spoilage-affected environment is challenging, furthermore the recalcitrant and persistent nature of the pollutants aggravates the problem; besides, timeframe of their stay in the deteriorated environments is elongated and aries with the identity of the particular chemical constituent (Al-Hawash *et al*., 2018). As such, bioremediation, and specifically biodegradation, is advocated as the best methods of environmental reclamation of contaminated sites (Atlas and Bertha, 2018) due to its many advantages over conventional physicochemical methods of removal, such as excavation, dispersion, sorption, volatisation, and abiotic transformation, which, nevertheless, have drawbacks of being expensive, destroy soil structure, and produce toxic chemical by-products. The advantages include effectiveness, efficaciousness, efficiency, applicability, inexpensiveness, environmental-friendliness *inter alia* (Wang *et al*., 2019).

A consortium refers to an agglomeration of two or more diverse microbial species living together (Nawong *et al*., 2018), with consequent benefits of vigor, efficaciousness and modularity. Consortia can also be found living together as polymicrobial entities in microbial biofilms (dos Santos *et al*., 2018)

Since pollutants rarely exist singularly in contaminated environment, effective biodegradation requires the employment of a consortium (aggregate/group) of microorganisms, each capable of degrading one or more of the constituents of the contaminants or the use of a microorganism capable of degrading all the pollutants, with the latter being rare to find and the former desirable in biodegradation scenarios (Lee *et al*., 2019).

The ideal consortium shall consist of variegated microbes capable of interacting seamlessly and having diverse properties and numerous applications (Bradacova *et al*., 2019). Hypothetically, interactions between microorganisms in any consortium can be of two types: antagonistic (which are iniquitous to at least one member of the consortium) or synergistic (which are benevolent and complementary) (Kumar and Jagadeesh, 2016).

In diesel polluted environments, which are often characterized by the existence of miniaturized microenvironments, with different physicochemical conditions, survival and degradation activity by a single strain is virtually impossible, however, by using a microbial consortium harboring different microbes, biodegradation can be carried out efficaciously and easily (Lee *et al*., 2018).

The advantages of a microbial consortium over single strains in biodegradation experiments also include structural resilience, resistance and functional resilience (Sydow *et al*., 2016) and these are indispensable in temporal shifts in environmental conditions during diesel biodegradation. The multiplicity of catabolic genes responsible for biodegradation in diverse consortia is also advocated as a merit of consortia use in biodegradation (Xu *et al*., 2018) Furthermore, as opined by Garrido-Sanz *et al.* (2019), consortia have the advantages of overlapping substrate requirements and these can function extensively in the cascade of diesel biodegradation. Moreover, as espoused by Sarkar *et al*. (2013), the mechanism of synergistic approach of consortia to biodegradation depends on the growth factors of the organisms and co-metabolism function. According to Bento *et al*. (2005), the enhanced activity of consortia in diesel degradation scenarios is enhanced by the possession of multiple "metabolic mechanisms, surface active agents and emulsifiers". Finally, the isolation of high numbers of microorganisms from an environment is commonly taken as an index that those organisms degrade the particular contaminant of the environment from which they were isolated (Ajao *et al*., 2014).

These factors underline the rationale behind conducting this study, which aims to formulate, test the compatibility of and evaluate the diesel degradation performance of diesel degrading bacteria consortia isolated from Katsina metropolis using Bushnell-Haas Agar supplemented with 2% diesel as a sole carbon source.

MATERIALS AND METHODS

Materials

Diesel used in Bushnell-Haas Agar medium as a carbon source was purchased from Nigeria National Petroleum Cooperation (NNPC) Mega Station, Katsina. All chemicals used were of analytical or general purpose laboratory reagent grade and bacteria were isolated from the diesel contaminated soil within Katsina state.

Diesel Degrading Bacterial Isolates and Purity Testing

The isolates used in consortia formulation were previously putatively identified, as guided by the Cowan and Steell's Manual (Barrow and Feltham, 2004), via colonial morphology, cellular morphology (by Gram staining) and biochemical characterization. Purity testing was carried out as modified from Wang *et al*. (2011). Cultures of previously identified diesel degrading bacteria isolated from Katsina Metropolis were sub-cultured onto fresh nutrient agar (BioLab, Budapest Hungary) plates (prepared using the commercially prepared powdered medium according to manufacturer's instructions and then autoclaved at 121^0C for 15 minutes before being used to ensure pure cultures of individual strains are being used (Ukaegbu-Obi *et al*., 2017).

Preparation of Phosphate Buffer Saline

Phosphate Buffer Saline (PBS) was prepared by dissolving NaCl (8g), KCl (0.2g) (Loba Chemie, Mumbai), Na_2HPO_4 (1.44g) and KH_2PO_4 (BDH, UK) 0.24 g, in 1 litre of distilled water and autoclaved at 121^0C for 15 minutes (Umar *et al*., 2017).

Compatibility Testing

The four isolates used in the study, i.e. *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus roseus*, and *Rhodococcus* species were plated at one position on a Nutrient Agar plate, and incubated at 37^0C for 24 hours. Appearance of mixed bacteria growth designates that the isolates are compatible with one another.

Bacteria Inoculums Preparation

Bacterial inoculums were prepared using the four selected diesel degrading isolates (*Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus roseus* and *Rhodococcus* species) which served as the inoculums during the diesel biodegradation experiments (Ghazali *et al.,* 2004). Pure colonies from each bacterial culture plate were suspended in the sterile PBS for cells quantification using haemocytometer.

Quantification of Live Cells via Haemocytometry

The quantification of bacterium cells per milliliter was performed by further diluting the PBS suspended cells with 0.4% trypan blue (NIEHS, 2019). The cells were subsequently counted microscopically using Neubauer British Standard Haemocytometer (Marienfield, Germany) until 0.4×10^5 cells per ml was obtained (Chen *et al.,* 2011). Based on observation of cells using x40 and x100 objective lenses of a compound binocular microscope, living cells were calculated using the following formula:

Total viable cells (cells/ml) = $X \times Y \times Z \times 10^4$

Where:

X is the volume of the suspended bacterium cells used for microscopy (0.1 ml);

Y is the dilution factor of cells in trypan blue;

Z is the average number of living cells counted per square and

10^4 is the conversion factor of 0.1 mm^3 (area of square) to ml.

Formulation of Diesel degrading Consortia

Formulation of diesel degrading consortia was carried out in accordance with the protocols of Sarkar *et al*. (2011). Mathematical permutation approach was used to determine number of replications for each composition in a particular consortium (Chen *et al.,* 2011; Rosen, 2007). Experimental repetitions were determined by the following permutation formula adopted from Laisin *et al*. (2012):

$$E = \frac{n!}{(n-r)!r!}$$

Where:

E is the total number of experimental repetitions,

n is the total number of bacteria involved (4),

r is the number of bacteria per consortium (2, or 3, or 4) while,

! is the factorial sign.

The number of replications, r, was successively computed for the following scenarios:

Consortium Testing and Evaluation of Diesel Degradation Using Bushnell-Haas Agar

Eleven different consortia combinations involving the isolates designated A (*Bacillus subtilis*), B (*Staphylococcus aureus*), C (*Micrococcus roseus*) and D (*Rhodococcus* sp.), viz. (A and B; A and C; A and D; B and C; B and D; C and D); (A, B, and C; A, B and D; A, C, and D; and B, C and D); and (A, B, C and D), were made by combining equal proportions (1ml) of the individual bacteria resting cells.

Bushnell Haas Agar (BHA) was prepared using KH_2PO_4 (1g); K_2HPO_4 (1g); NH_4NO_3 (1g); $MgSO_4$ (0.2g); $FeCl_3$ (0.05g); $CaCl_2$ (0.02g); Agar-Agar (15g) dissolved in one liter of distilled water and sterilized at 121^0C for 15 minutes (Siddique and Adam, 2002). Fresh diesel (2 litres) which was incorporated into the medium as a carbon source (Yetti *et al.*, 2016; Atlas, 2005), at a concentration of 2% v/v was purchased from NNPC Mega Station, Katsina, and filter sterilized before use. One hundred microliter of each of the formulated consortia were then separately inoculated on BHA medium containing 2% v/v diesel concentration in duplicates (Mwamura, 2017). These consortia were designated 1-11, respectively. Incubation was done at 37^0C for 72 hours. Colony forming units were recorded from all the treatments on 24-hours basis and used to assess the effectiveness of the combined strains, as modified from Karthika *et al.* (2014). This criterion was used in selecting a final consortium that comprised the entire four bacterial strains in a single experiment.

Data Analysis

Microsoft Excel (2007) Data Analysis ToolPak was used to calculate the one-way Analysis of Variance (ANOVA) to determine sufficient variations between consortia constituents (Berk and Carey, 2010). Subsequently, the individual means were separated using Duncan's Multiple Range Test (DMRT).

RESULTS

Compatibility Testing

Luxuriant and robust growth of all the four species of bacteria involved in the compatibility testing was observed which confirmed the compatibility nature of all the isolates (Figure 1).

Diesel Degradation Response of Inocula from the Formulated Consortia

The results of the diesel degradation performance of the various consortia evaluated in terms of growth in CFU/ml obtained on BHA supplemented with 2% v/v diesel showed that the growth is dependent on the time of incubation. Results after 48 hours of incubation (Table 1) were higher than those obtained after 48 hours of incubation (Table 2), but the variation is not statistically significant ($p<0.05$), with a p-value of 0.96156. There exist significant differences between the various consortia constituents as evaluated by one-way analysis of variance ($p<0.05$), with a p-value of <0.05. The results were further anlaysed using a post-hoc test (Duncan's Multiple Range Test), which segregated the consortia into clusters of similarly performing strains.

It is evident from the tables that consortium 11, consortium 9 and consortium 2 are the best performing in terms of CFU/ml obtained from the BHA cultures after 72 hours, with yields of 1.7×10^4, 3.3×10^3 and 3.0×10^3 respectively.

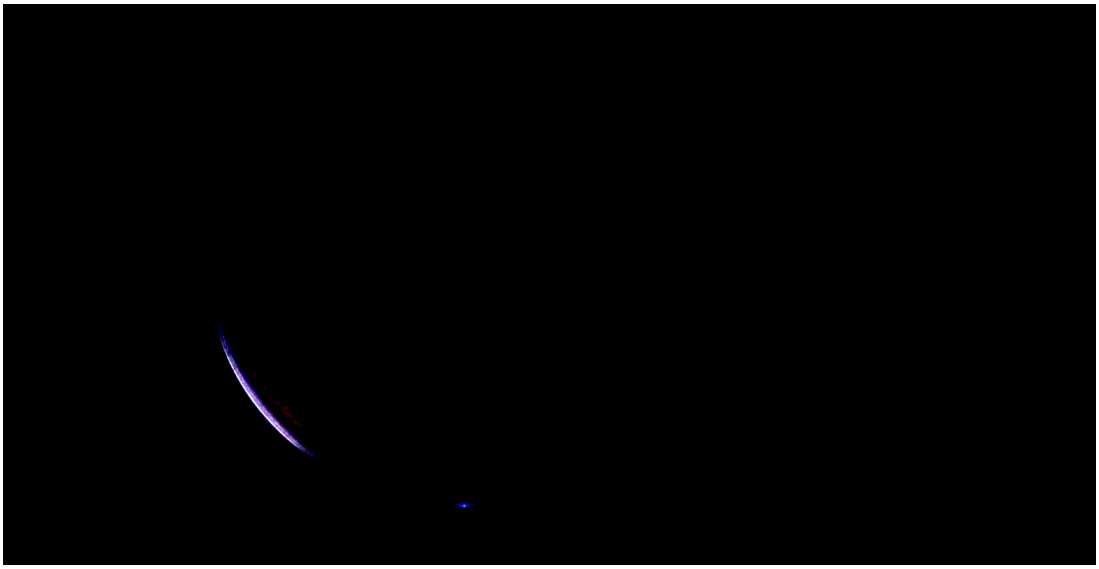

Figure 1: Plate showing robust mixed culture growth of the four isolates indicating their compatibility with one another.

Key: 1= *Bacillus subtilis* strain which appeared large, rough, mucoid and white, 2= *Micrococcus roseus* strain which appeared pinkish/reddish, circular, raised and small, 3= *Rhodococcus* species having large, flat, irregular and red colonies; and 4= *Staphylococcus aureus* which appeared small, yellowish, raised and circular.

The results of the performance of single strains of consortia compared with the chosen consortium (CST 11) indicate the improvement obtained in terms of degradation performance between the single strains and the consortium 11 containing four organisms (Fig 2.0).

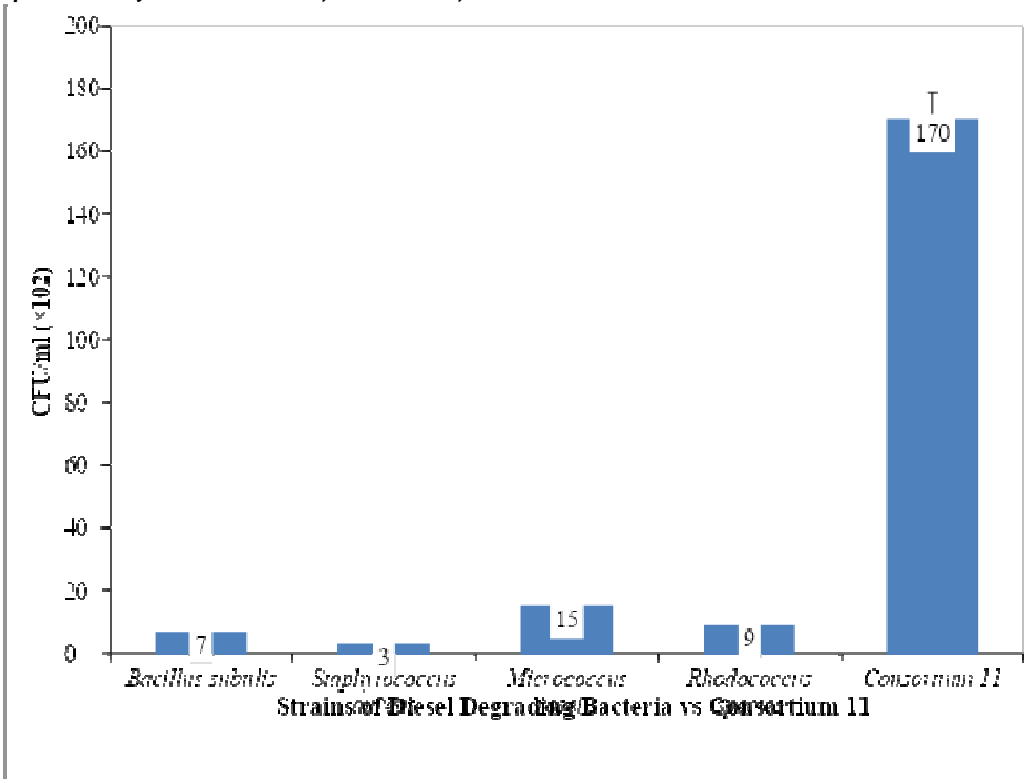

Figure 2: Comparison of diesel degradation response of individual strains and a consortium harboring all the strains measured as CFU/ml growth on BHA after 72 hours of incubation.
NB: Consortium 11 = *B. subtilis, S. aureus, M. roseus* and *Rhodococcus* sp. Error bars depict percentage errors (5%).

Table 1: Diesel degradation response in CFU/ml obtained from different consortium compositions made of *Bacillus subtilis, Staphylococcus aureus, Micrococcus roseus* and *Rhodococcus* species after 48 hours of incubation on 2% (v/v) Bushnell-Haas Agar.

Combination	Consortium	CFU/ml ± S.D.
1	*B. subtilis* and *S. aureus*	$2.0 \times 10^2 \pm 7.07^e$
2	*B. subtilis* and *M. roseus*	$3.0 \times 10^3 \pm 2.83^b$
3	*B. subtilis* and *Rhodococcus* sp.	$4.4 \times 10^2 \pm 14.14^e$
4	*S. aureus* and *M. roseus*	$1.6 \times 10^3 \pm 22.63^c$
5	*S. aureus* and *Rhodococcus* sp.	$3.0 \times 10^2 \pm 33.94^e$
6	*M. roseus* and *Rhodococcus* sp.	$4.8 \times 10^2 \pm 13.94^e$
7	*B. subtilis, S. aureus* and *M. roseus*	$1.6 \times 10^3 \pm 11.31^c$
8	*B. subtilis, S. aureus* and *Rhodococcus* sp.	$1.1 \times 10^3 \pm 22.63^c$
9	*B. subtilis M. roseus* and *Rhodococcus* sp.	$6.0 \times 10^1 \pm 227.69^f$
10	*S. aureus, M. roseus* and *Rhodococcus* sp.	$8.0 \times 10^2 \pm 22.63^d$
11	*B. subtilis, S. aureus, M. roseus and Rhodococcus* sp.	$1.6 \times 10^4 \pm 90.51^a$

NB:- Similar superscripts in the same column denote no significant differences ($p<0.05$) between mean diesel degradation performances between consortia, different superscripts denote significant differences ($p \leq 0.05$) between the means. S.D. = Standard deviation.

Table 2: Diesel degradation response in CFU/ml obtained from different consortium compositions made of *Bacillus subtilis, Staphylococcus aureus, Micrococcus roseus* and *Rhodococcus* species after 72 hours of incubation on 2% (v/v) Bushnell-Haas Agar.

Combination	Consortium	CFU/ml ± S.D.
1	*B. subtilis* and *S. aureus*	$3.0 \times 10^2 \pm 7.07^f$
2	*B. subtilis* and *M. roseus*	$3.0 \times 10^3 \pm 2.83^b$
3	*B. subtilis* and *Rhodococcus* sp.	$6.4 \times 10^2 \pm 14.14^d$
4	*S. aureus* and *M. roseus*	$1.9 \times 10^3 \pm 22.63^c$
5	*S. aureus* and *Rhodococcus* sp.	$7.8 \times 10^2 \pm 33.94^d$
6	*M. roseus* and *Rhodococcus* sp.	$6.7 \times 10^2 \pm 13.94^d$
7	*B. subtilis, S. aureus* and *M. roseus*	$1.7 \times 10^3 \pm 11.31^c$
8	*B. subtilis, S. aureus* and *Rhodococcus* sp.	$1.4 \times 10^3 \pm 22.63^c$
9	*B. subtilis M. roseus* and *Rhodococcus* sp.	$3.3 \times 10^3 \pm 227.69^b$
10	*S. aureus, M. roseus* and *Rhodococcus* sp.	$4.8 \times 10^2 \pm 22.63^e$
11	*B. subtilis, S. aureus, M. roseus and Rhodococcus* sp.	$1.7 \times 10^4 \pm 90.51^a$

Key: S.D. = Standard deviation. Consortium in bold is the chosen consortium.

NB:- Similar superscripts in the same column denote no significant differences ($p<0.05$) between mean diesel degradation performances between consortia, different superscripts denote significant differences ($p\leq0.05$) between the means.

DISCUSSION

The importance of compatibility testing is advocated by Kumar and Jagadeesh (2016), who stated that a major limitation of consortia is failed performance on the field, due to incompatible nature of the individual strains of microorganisms present in the consortia. However this study involved the consortium compatibility testing which proved that the organisms can grow together synergistically when cultured on nutrient agar, and therefore, the combined isolates are suitable for formulation of bacteria consortia accordingly, thus elimination that possible drawback. The compatibility of the strains may also be aided by the fact they are all Gram positive, since antagonism (via bacteriocin production, for example) is more likely to occur amongst significantly different microbial groups.

The status of some the bacteria used in formulating the diesel degrading consortia as diesel degraders had already been confirmed in previous literature: *Bacillus subtilis* in Rehman *et al.* (2015), from contaminated soils of Mansehra, Pakistan; *Staphylococcus aureus* by Karthika *et al.* (2014), *Micrococcus* species by Nikhil *et al.* (2013), who, in addition, isolated *Pseudomonas* sp. from garage soil, in India. The potential penchant of *Rhodococcus* sp. for degrading alkanes had already been stated, in the same vein, Lee *et al*. (2006) had already reported a novel species of *Rhodococcus* capable of such degradation.

The advantages of utilizing all four microbes in a single consortium are readily discernable. *Micrococcus* species had already been proved to be a fast growing microorganism capable of degrading many organic and other compounds, (Nikhil *et al*., 2013) and this research confirmed that the isolated *Micrococcus roseus* grows profusely and in a fast manner. *Staphylococcus aureus* is capable of growing in diversified environments, likewise *Bacillus subtilis*. Ijah and Antai (2003) had already earmarked *Bacillus* species as the most prominent bacterial species isolated from diesel contaminated soils, and the most effective and efficient.

The contributions of *Rhodococcus* sp. cannot be overemphasized, since it expresses the best ability for possessing and utilisng genes for diesel degradation, including alkB gene, among the four chosen strains.

All the strains alone showed relatively lower performance (10^1 to 10^3 CFU/ml) compared to the consortium harbouring all the strains (10^4 CFU/ml). This can be attributed to the limitations in terms of metabolic capability and absence of multiplicity of enzyme systems in single strains of microorganisms compared to groups/aggregates.

It had been suggested that residual oils have been seen to supplant the growth of 2nd and 3rd strains of microflora subsequent to degradation of the original oil by a single strain (Mnif *et al*., 2015), and this may be responsible for increases in CFU/ml values over time in the formulated consortia.

It is obvious that the result of CFU/ml obtained from the haemocytometry (4×10^4) is lower than that obtained after growing the organisms in diesel for 48 hrs. This showed that the organisms require time to adapt to the environment change and secrete enzymes and carryout catabolism on the diesel substrate for energy generation. However, it is obvious that increase in time is associated with increases in CFU/ml.

Consortium 9 showed significant ability, since it generated 3.3×10^1 CFU/ml within 72 hours. However, it was noticed that only 6.0×10^1 CFU/ml was observed after 48 hours. This can possibly be because some of the fast growing bacterium/bacteria in the consortium were unable to degrade certain constituents of the diesel straightaway, and can only do so after a lag of 48 hours, during which the other slow growing bacterium/bacteria had sufficiently broken down, degraded or metabolized the diesel constituents to the extent that the fast growing member(s) can act on it, or act on the product of the metabolism of the first strain. Consortium 2 also showed potential. It generated a yield of 3×10^3 CFU/ml within 48 hours. It is worth pointing out that the consortia can expedite degradation of diesel within 48-72 hours. However, the best performing consortium was consortium 11 habouring all the strains, which generated 1.7×10^4 CFU/ml after 72 hours. The least growth was obtained in consortium 9, which had been explained previously, and consortium 1, consisting of *Bacills subtilis* and *Stapylococcus aureus* which yielded only 3.0×10^2 CFU/ml after 72 hours.

The findings of this study that the consortia performed better than single strains are in agreement with many previous literatures. Nikhil *et al.* (2013) had shown that diesel degradation by *Pseudomonas* and *Micrococcus* species is significantly greater than using either strain singly. Sarkar *et al*. (2013) also reported higher rates of BTEX (benzene, toluene, ethyl benzene and xylene) degradation using a microbial consortium than using a single strain.

The reasons for these observations are obvious. Greene *et al.* (2002) reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons, such as crude oil or diesel fuel. Such mixed cultures display metabolic versatility and superiority to single cultures (Hamme *et al.,* 2000). Consequently, a microbial conglomerate containing various microbes that manufacture the degradative enzymes for diverse ingredients of the decomposition pathway is considered to be well suited to the degradation of aromatic hydrocarbons. During biodegradation, microbes may not be directly implicated in the degradation channel, but, for the degradation of aromatic hydrocarbons, they may produce some micronutrients or surface-active agents which may aid such a process (Barathi and Vasudevan, 2001; Ozaki *et al.,* 2007).

It is frequently reported that a single isolate has a high biodegradation rate for only a specific fraction of hydrocarbons (Chen *et al*., 2011). However, biodegradation of a hydrocarbon contamination by a consortium of microorganisms is advantageous because a variety of hydrocarbons would be affected simultaneously increasing the removal efficiency in a timely manner. Moreover, the co-metabolism of the diesel components in a consortium is another possibility which can enhance the process. Ghazali *et al*. (2004) had previously formulated consortia using *Bacillus* and *Pseudomonas*.

CONCLUSION

This research had demonstrated the compatibility of *Bacillus subtilis, Staphylococcus aureus, Micrococcus roseus* and *Rhodococcus.* Diesel degradation performance measured using the index of growth in CFU/ml in 2% (v/v) BHA of the consortium harbouring all four strains was better than the single strains and all other formulated consortia. The findings of the study contribute towards illuminating inter-microbial relationships and microbial ecology especially within groups of diesel degrading bacteria. Further studies, including optimization of culture and physicochemical conditions for the chosen consortia are imperative, towards maximally harnessing the potentials of these bacteria for applications in large scale diesel biodegradation in spillage affected environments.

Conflict of Interest

We declare none.

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REFERENCES

Ajao, A.T., Yakubu S.E., Umoh V.J. and Ameh J.B. (2014). Enzymatic Studies and Mineralization Potential of *Burkholderia cepacia* and *Corynebacterium kutscheri* Isolated from Refinery Sludge. *Journal of Microbiology Research*, *4*: 29-42.

Al-Hawash, A.B., Zhang, X. and Ma, F. (2018). Removal and Biodegradation of Different Petroleum Hydrocarbons Using the Filamentous Fungus *Aspergillus* sp. RFC-1. *MicrobiologyOpen, 8*:e619: 1-14.

Atlas, R. (2005). *Handbook of Media for Environmental Microbiology*, 2nd Edition, London, UK: Taylor & Francis Group. Pp: 42, 76.

Atlas, R. and Bartha, R. (2018). Biodegradation of Petroleum in Soil Environment at Low Temperatures. *Journal of Microbiology*, *17*: 1652-1657.

Barathi, S. and Vasudevan, N. (2001). Utilization of Petroleum Hydrocarbons by *Pseudomonas fluorescens* Isolated from Petroleum Contaminated Soil. *Environment International 26*: 413-416.

Barrow, G.I. and Feltham, R.K.A. (2004). *Cowan and Steel's Manual for the Identification of Medical Bacteria*. Third Edition. Cambridge, UK: Cambridge University Press.

Bento, F.M., Camargo, F.A.O., Okeke, B.C. and Frankenberger Jr., W.T. (2005). Diversity of Biosurfactant Producing Microorganisms Isolated from Soils Contaminated with Diesel Oil. *Microbiological Research*, *160*: 249-255.

Berk, K.N. and Carey, P. (2010). *Data Analysis with Microsoft Excel Updated for Excel 2007*. Boston, Massachusetts: Brooks/Cole, Cengage Learning.

Bradacova, K., Floria, A.S., Bar-Tal, A., Minz, D., Yerminyahu, U., Shawahna, R., Kraut-Cohen, J., Zolti, A., Erel, R., Dietel, K., Weinmann, M., Zinnerman, B., Berger, N., Ludewig, U., Neumann, G. and Poster, G. (2019). Microbial Consortia versus Single-Strain Inoculants: An Advantage in PGPM-Assisted Tomato Production? *Agronomy, 9*(105): 1-25.

Chaudary, D.K., Bajagain, R., Jeong, S-W. and Kim, J. (2019). Biodegradation of Diesel Oil and *n*-alkanes (C18, C20, and C22) by a Novel Strain *Acinetobacter* sp. K-6 in Unsaturated Soil. *Environmental Engineering Research, 2020*: 1-27. Available online via: https://doi.org/10.4491/eer.2019.119, accessed 12.07.2019.

Chen, S.C., Frett, E., Marx, J., Bosnakovski, D., Reed, X., Kyba, M. and Kennedy, B.K. (2011). Decreased Proliferation Kinetics of Mouse Myoblasts Overexpressing FRG1. *PLoS ONE*, *6*(5):e19780.

Ciric, L., Philip, J.C. and Whiteley, A.S. (2010). Hydrocarbon Utilization Within a Diesel-Degrading Bacterial Consortium. *FEMS Microbiology Letters, 303*: 116-122.

Diesel Service and Supply, DSS (2019). *Why Use Diesel*? Available Online via: https://www.dieselserviceandsupply.com/why_use_diesel.aspx, accessed 13.07.2019.

Dixit, H., Lowry, M., Mohsin, U., Moond, M., Kumar, S., Chokriwal, A. and Gupta, S. (2018). Screening and Identification of Diesel Oil Degrading Bacterial Isolates from Petroleum Contaminated soil of Barmer, *Journal of Pharmaceutical, Chemical and Biological Sciences,* 6:34-40.

dos Santos, A.L.S., Galdino, A.C.M., de Mello, T.P., Ramos, L.S., Branquinha, M.H., Bolognese, A.M., Neto, J.C. and Roudbary, M. What are the Advantages of Living in a Community? A Microbial Biofilm Perspective. *Memorias do Instituto Oswaldo Cruz, 113*(9): e180212: 1-7.

Garrido-Sanz, D., Redondo-Nieto, M., Guirado, M., Jiminez, O.P., Millan, R., Martin, M. and Rivilla, R. (2019). Metagenomic Insights into the Bacterial Functions of a Diesel-Degrading Consortium for the Rhizoremediation of Diesel-Polluted Soil. *Genes*, *10*(456): 1-19.

Ghazali, F.M., Rahman, R.N.Z.A., Salleh, A.B. and Basri, M. (2004). Biodegradation of Hydrocarbons in Soil by Microbial Consortium. *International Biodeterioration and Biodegradation, 54*: 61-67.

Greene, A.E., Kay, J.G., Stehmeier, L.G. and Voordouw, G. (2002). Microbial Community Composition at an Ethane Pyrolysis Plant Site at Different Hydrocarbon Inputs. *FEMS Microbiology Ecology*, *40*: 233-241.

Hamme, J.D., Odumeru, J.A. and Ward, O.P. (2000). Community Dynamics of a Mixed-Bacterial Culture Growing on Petroleum Hydrocarbons in Batch Culture. *Canadian Journal of Microbiology, 46*: 441-450.

Ijah, U.J.J. and Antai, S.P. (2003). Removal of Nigerian Light Crude Oil in Soil Over a 12-month Period. *International Biodeterioration and Biodegradation, 51*: 93-99.

Karthika, R., Gopinath, L.R., Archaya, S. and Bhuvaneswan, R. (2014). Isolation of Diesel Degrading Bacteria, Identification of Catechol Gene and its Biogas Production. *IOSR Journal of Environmental Science, Toxicology and Food Technology*, *8*: 76-82.

Kumar, K.H. and Jagadeesh, K.S. (2016). Microbial Consortia-Mediated Plant \Defense Against Phytopathogens and Growth Benefits. *South Indian Journal Of Biological Sciences, 2*(4): 395-403.

Laisin, M., Okoli, O.C., Ujumadu, R. and Nsiegbe, N. (2012). r-Permutation and r-Combination Techniques for k-Inclusion

Condition. *Journal of Physical Sciences and Innovation*, *4*: 5-11.

Lee, Y., Jeong, S.E., Hur, M., Ko, S. and Jeon, C.O. (2018). Construction and Evaluation of a Korean Native Microbial Consortium for the Bioremediation of Diesel Fuel-Contaminated Soil in Korea. *Frontiers in Microbiology*, *9*, 2594: 1-11.

Lee, Y., Lee, Y. and Jeon, C.O. (2019). Biodegradation of naphthalene, BTEX, and aliphatic hydrocarbons by *Paraburkholderia aromaticivorans* BN5 isolated from petroleum-contaminated soil. *Nature Scientific Reports*, *9*(860): 1-12.

Mahmoud, G.A-E. and Bagy, M.M.K. (2018). Chapter 12 - Microbial Degradation of Petroleum Hydrocarbons. In, V. Kumar *et al*. (eds.) *Microbial Action on Hydrocarbons*. Singapore: Springer Nature.

Mnif, I., Mnif, S., Sahnoun, R., Maktouf, S., Ayedi, S. and Ellouze-Chaabouni, S. (2015). Biodegradation of Diesel Oil by a Novel Microbial Consortium: Comparison Between Co-inoculation with Biosurfactant-producing Strain and Exogenously Added Biosurfactants. *Environmental Science and Pollution Research, 22*: 14852–14861.

Musa, S.I. (2019). Isolation and Identification of Diesel Oil-degrading Bacteria in Used Engine Oil Contaminated Soil. *Journal of Applied Science and Environmental Management*, *23*: 431-435.

Mwamura, A. (2017). *Screening, Isolation and Characterization of Hydrocarbonoclastic Bacteria from Oil Contaminated Soils*. Unpublished MSc. (Biochemistry) Thesis submitted to the University of Nairobi, Kenya.

Naila, T. (2015). *Isolation and Study of Optimum Growth Condition of Diesel Degrading Bacteria from Soil*. Unpublished Bsc. Dissertation Submitted to BRAC University, Dhaka, Bangladesh.

Nawong, C., Umsakul, K. and Sermwittayawong, N. (2018). Rubber Gloves Biodegradation by Consortium, Mixed Culture and Pure Culture Isolated from Soil Samples. *Brazilian Journal of Microbiology*, *49*: 481-488.

NBS - National Bureau of Statistics, Nigeria (2019). *Petroleum Products Importation and Consumption (Truck Out) – Q1 2019*. Available online via: https://nigerianstat.gov.ng/download/945, accessed 12.07.2019.

NIEHS – National Institutes of Environmental Health Sciences, USA (2019). *Cell Viability Testing with Trypan Blue Exclusion Method*. Available Online via: https://brd.nci.nih.gov/brd/sop/download-pdf/941, accessed 11.07.2019.

Nikhil, T., Deepa, V., Rohan, G. and Satish, B. (2013). Isolation, Characterisation and Identification of Bacteria from Garage Soil and Comparison of Their Bioremediation Potential. *International Research Journal of Environment Sciences, 2*: 48-50.

Obi, L.U., Atagana, H.I. and Adeleke, R.A. (2016). Isolation and Characterisation of C rude Oil Sludge Degrading Bacteria. *SpringerPlus, 5*(1946): 1-13.

Ozaki, S., Kishimoto, N. and Fujita, T. (2007) Change in the Predominant Bacteria in a Microbial Consortium Cultured on Media Containing Aromatic and Saturated Hydrocarbons as the Sole Carbon Source. *Microbes and Environment*, *22*: 128-135.

Patowary, K., Patowary, R., Kalita, M.C. and Deka, S. (2016). Development of an Efficient Bacterial Consortium for the Potential Remediation of Hydrocarbons From Contaminated Sites. *Frontiers in Microbiology*, *7*: 1-14.

Rehman, Z.U., Khan, K., Faisal, S., Kamal, R., Ahmad, S., Irfan, M.K.H., Safa, I.U. and Kumar, T. (2015). Isolation and Identification of Diesel Degrading Bacteria from Oil Contaminated Soil in Mansehra, Pakistan. *International Journal of Scientific and Engineering Research, 6*: 1438-1661.

Rosen, K.H. (2007). *Combinatorics of Permutations*. 1st Edition, Boca Raton, Florida: Chapman and Hall/CRC.

Sarkar P., Rai A.R. and Ghosh, S. (2013) Degradation of Aromatic Hydrocarbon (BTEX) by a Solvent Tolerant Bacterial Consortium. *Journal Urban Environmental Engineering*, *7*: 274-279.

Sarkar, P., Meghvanshi, M. and Singh, R. (2011). Microbial Consortium. A New Approach in Effective Degradation of Organic Kitchen Wastes. *International Journal of Environmental Science and Development, 2*(3): 170-174.

Siddiqui, S. and Adams W.A (2002). The Fate of Diesel Hydrocarbons and their Effect on the Germination of Perennial Ryegrass. *Environmental Toxicology*. *17*: 49-62.

Sydow, M., Owsianiak, M., Szczepaniak, Z., Framski, G., Smets, B.F., Ławniczak, Ł., Lisiecki, P., Szulc, A., Cyplik, P. and

Chrzanowski, Ł. (2016). Evaluating Robustness of a Diesel-Degrading Bacterial Consortium Isolated from Contaminated Soil. *New Biotechnology, 33*(6): 852-859.

Umar, Z.D., Aziz, N.A.A., Zulkifli, S.Z., Muskhazli, M. (2017). Rapid biodegradation of polycyclic aromatic hydrocarbons using effective *Cronobacter sakazakii* MM045 (KT933253). *MethodsX*, *4*, 104-117.

Ukaegbu-Obi, K.M., Iweha, O.N. and Okonkwo, E.C. (2017). Bacterial Community Dynamics of Diesel-Polluted Soil and their Bioremediation Potential. *FUTO Journal Series, 3*: 161-165.

Wang, Y., Wang, Q. and Liu, L. (2019). Crude Oil Degrading Fingerprint and the Overexpression of Oxidase and Invasive Genes for n-hexadecane and Crude Oil Degradation in the *Acinetobacter pittii* H9-3 Strain. *International Journal of Environmental Research and Public Health, 16*(188): 1-14.

Wang, Z., Liu, X., Chen, L., Hu, X. and Liu, F. (2011). Degradation of Diesel with Microorganisms in Rhizosphere of *Carex phacota Spr*. *Procedia Environmental Sciences*, *8*: 61–67.

Xu, X., Liu, W., Tian, S., Wang, W., Qi, Q., Jian, P., Gao, X., Li, F., Li, H. and Yu, H. (2018). Petroleum Hydrocarbon-Degrading Bacteria for the Remediation of Oil Pollution Under Aerobic Conditions: A Perspective Analysis. *Frontiers in Microbiology*, *9*, 2885: 1-11.

You, Z., Zhou, H., Zhang, S., Kim, H., Chiang, P.C.M Yun, W., Zhang, L. and He, M. (2018). Comparison of Petroleum Hydrocarbons Degradation by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. *Applied Sciences, 8*: 1-19.

Yetti, E., Wijaya, H., Thontowi, A. and Yopi, A. (2016). Isolation of Oil Degrading Bacteria from the Terrestrial sites of Minas, Riau and the Prescreening of the Isolates on the Simple Polycyclic Aromatic Hydrocarbon. *Proceedings of the 6th International Symposium for Sustainable Humanosphere*: 237-243.

Yuniati, M.D. (2017). Bioremediation of Petroleum Contaminated Soil: A Review. *IOP Conf. Series: Earth and Environmental Science*, *118*: 012063.