Species of *Pseudomonas* and *Bacillus* Isolated from Refined Oil-contaminated Soil Showed the Potential to Efficiently Degrade Diesel

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INTRODUCTION

Large-scale environmental contamination is a direct result of the dramatic increase in both petroleum exploration and consumption [1]. Soil pollution reports frequently include diesel oil because of the complex hydrocarbon contaminant it is. Diesel oil is a mixture of alkanes and aromatic compounds [2]. Almost 1.7-8 million metric tons of diesel are reported to be released into the water and soil environment annually across the world [3]. The build-up of petroleum products in the environment poses serious risks to human health and the safety of biotic and abiotic components of the ecosystem [4]. Therefore, both vegetable oil and diesel fuel have been shown to have profound effects on plant roots, halting their development [5]. Unlike the mechanical method, which is very expensive and time-consuming, the best approach for reclamation of contaminated soil is bioremediation which uses microorganisms to degrade the toxic compounds in the environment [6,7]. Numerous isolates of bacteria and fungi have been reported to degrade crude oil from soil polluted with petroleum hydrocarbons and these include *P. aeruginosa*, *P. putida*, *Proteus sp.*, *Acinetobacter Iwofi*, *Alcaligenes eutrophus*, *Bacillus subtilis*, *Micrococcus luteus*, *Aspergillus spp.*, *Penicillus spp.*, *Rhizopus spp.* and *Candida spp.* [8]. Bioremediation is a low-cost method for removing petroleum hydrocarbons from the environment due to its widespread applicability and its ability to eradicate the contaminant entirely [9]. It is common practice to employ a variety of microbial communities, especially soil bacteria, to aid in the cleanup of a petroleum-contaminated area [10].

Degradation rates of hydrocarbons are highly sensitive to their physical form, chemical make-up and concentration [8]. Biodegradation of diesel has been shown to be sensitive to a variety of environmental conditions, including nutrients, pH, temperature and hydrocarbon concentration. Hydrocarbon concentration in diesel oil degradation has been overlooked

ABSTRACT

The high rise in the exploration and usage of petroleum products have led to widespread contamination in the environment. Hence, the continuous search for microorganisms with the potential to mineralize these pollutants is necessary. This study was conducted to isolate, identify and determine the diesel degradation potential of bacteria from oil-contaminated soil collected from three filling stations in Azare, Katagum LGA, Bauchi State, Nigeria. The diesel degrading bacteria were identified using standard protocols. The isolates were screened spectrophotometrically for their potential to utilize 1% diesel (v/v) as their sole carbon and energy source and the best candidate was used for determining the effect of diesel concentration on its biodegradation. The results revealed the presence of three bacterial species including *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Bacillus subtilis*. *P. Putida* showed the highest diesel degradation at 120 h with an absorbance of 2.27±0.03 at 600 nm followed by *P. aeruginosa* (2.23±0.03) and *Bacillus subtilis*. The best degradation was observed at 1% diesel concentration (v/v) followed by 2% and the least was recorded at 3% with the absorbance of 2.40±0.00, 2.27±0.03 and 2.20±0.00 respectively. The detection of these potential degraders is crucial in the light of the lingering search for efficient hydrocarbon degraders for efficient bioremediation since their degradative capability could be enhanced for deployment in the bioremediation of diesel-contaminated environments.

KEYWORDS

Diesel Degradation *Pseudomonas aeruginosa* *Pseudomonas putida* *Bacillus subtilis*
despite its importance. The purpose of this work was to conduct a preliminary screening of diesel degrading bacteria isolated from diesel contaminated soil under varying hydrocarbon concentrations.

MATERIALS AND METHODS

Collection of samples

Soil samples were collected from three (3) different filling stations including, Madobi, A.A Rano, and Katagum petroleum at Azare, Katagum L.G.A, Bauchi State. The samples were collected at each site by digging up the soil with a shovel for about 10cm and transferred directly into clean, sterile containers. Also, a contaminant-free soil sample was collected from the botanical garden of Bauchi State University, Gadau [10].

Isolation and identification of diesel degrading bacteria

We spread 100 µL of serially diluted diesel-contaminated soil samples on nutrient agar plates and incubated them at 37 °C for 24 h. Under the same conditions as described by Palanisamy et al. [11], we sub-cultured the colonies that had formed on the agar plates to obtain the pure colonies. Bacterial isolates were identified using standard biochemical protocols [12].

Screening of diesel degrading bacteria

The bacteria isolated from different diesel-contaminated soil were screened based on their ability to degrade diesel oil supplemented in the Mineral Salt medium (MSM). The MSM contained the following compositions: NH₄NO₃ – 3 g/L, K₂HPO₄.3H₂O - 0.5 g/L, KH₂PO₄ - 0.5 g/L, and trace amounts of CuSO₄.4H₂O - 0.002 g/L, MgSO₄.7H₂O - 0.008 g/L, FeSO₄.7H₂O - 0.002 g/L, and CaCl₂.2H₂O - 0.002 g/L. Briefly, as described by Palanisamy et al. [11], bacterial isolates were grown in Mineral Salt Medium with 1% diesel oil (v/v) as the only carbon source and incubated for 3 days at 37°C. Using a spectrophotometer, the optical density at 600 nm of the growing isolates was measured at set time intervals. The absorbance was measured and recorded after the spectrophotometer had been calibrated to zero with formulations identical to those used in the treatment (except for the addition of bacterial isolates).

Effect of diesel concentration on biodegradation

Substrate for a study on the impact of diesel concentration on the biodegradation process was MSM amended with 1%, 3%, and 5% concentrations of diesel oil. Bacterial strains were inoculated into the flasks, and the containers were then shaken in an incubator at 37 degrees Celsius (120 rpm). The both the tests and controls were both maintained in identical conditions. The impact of concentration on diesel biodegradation was investigated by collecting and analysing residual diesel oil samples at regular 6-hour to 120-hour intervals [11].

RESULTS

Bacterial isolates and their diesel degradation potential

In this study, three (3) bacterial species were identified which include Pseudomonas putida, Bacillus subtilis and P. aeruginosa. These bacteria were further used in the degradation of diesel which was assessed spectrophotometrically at the wavelength of 600nm. The degradation of 1% diesel (v/v) revealed that Pseudomonas putida had the highest degradation potential followed by Pseudomonas aeruginosa with the absorbance of 2.27±0.03 and 2.23±0.03 respectively while Bacillus subtilis had the lowest degradation potential of 2.00±0.00 after 120 h of incubation (Table 1).

Table 1. Degradation potential of diesel degrading bacterial isolates at 1%v/v diesel.

| Isolates    | Time (h) | 0 | 24 | 48 | 72 | 96 | 120 |
|-------------|----------|---|----|----|----|----|-----|
| Control     |          | 0.32±0.00 | 0.43±0.00 | 0.61±0.00 | 0.65±0.00 | 0.85±0.01 | 1.16±0.09 |
| P. putida   |          | 0.34±0.00* | 0.65±0.00* | 0.86±0.00* | 0.97±0.00* | 1.88±0.00* | 2.27±0.03* |
| B. subtilis |          | 0.34±0.00* | 0.64±0.00* | 0.75±0.00* | 0.95±0.00* | 1.75±0.00* | 2.06±0.00* |
| P. aeruginosa |          | 0.35±0.00* | 0.65±0.00* | 0.84±0.00* | 0.97±0.00* | 1.71±0.00* | 2.23±0.03* |

Note: Results are expressed as mean ± SEM. Results are statistically significant at p<0.05 as compared with the control group.

Effect of concentration on the biodegradation of diesel (1%, 2%, and 3%)

The best diesel degrading bacteria, Pseudomonas putida was used for this study. The highest degradation of diesel was observed at 1% concentration (2.40±0.00) after 120 h followed by 2% v/v (2.27±0.03). However, the least degradation was recorded at the concentration of 3% v/v with an absorbance of 2.20±0.00 (600 nm) optical density at 120 h (Table 2). There was a significant difference between the statistical values.

Table 2. Effect of concentration on the degradation of diesel by Pseudomonas putida.

| Diesel (%) | Time (h) | 0 | 24 | 48 | 72 | 96 | 120 |
|-----------|----------|---|----|----|----|----|-----|
| Control   |          | 0.45±0.01 | 0.63±0.01 | 0.66±0.00 | 0.83±0.00 | 0.95±0.03 | 1.35±0.00 |
| 1%        |          | 0.59±0.00* | 0.86±0.02* | 1.65±0.00* | 2.23±0.00* | 2.36±0.00* | 2.40±0.00* |
| 2%        |          | 0.55±0.01* | 0.78±0.00* | 1.54±0.00* | 2.00±0.00* | 2.13±0.03* | 2.27±0.03* |
| 3%        |          | 0.52±0.02* | 0.79±0.02* | 1.53±0.01* | 1.99±0.01* | 2.10±0.00* | 2.20±0.00* |

Note: Results are expressed as mean ± SEM. Results are statistically significant at p<0.05 as compared with the control group.

DISCUSSION

Three bacterial species capable of degrading 1% (v/v) diesel oil were isolated from different diesel-contaminated soil within Azare, Katagum LGA Bauchi State, Nigeria. These isolated bacteria included P. putida, P. aeruginosa and B. subtilis. These organisms have previously been linked to the degradation of hydrocarbons. In a study by Prakash et al. [13], a total of 59 bacteria were isolated from soil contaminated with hydrocarbons, out of which only Bacillus sp., Pseudomonas sp., and Micrococcus species could grow in 1% of diesel oil which indicated their capability to utilize and degrade diesel as a carbon source. In a different study, Titah et al. [14] isolated Staphylococcus and Micrococcus species capable of degrading diesel oil from the ship dismantling facility at Tanjungjati, Madura, Indonesia. Geetha et al. [15] used the hole-plate diffusion assay technique and found that out of 14 bacterial isolates, 9 were able to grow around the holes and used diesel as a carbon and energy source.

In the present study, the three bacterial species (P. putida, P. aeruginosa and B. subtilis) isolated were further screened for their potential to degrade diesel oil. The results revealed that P. putida had the highest degradation potential followed by P. aeruginosa and B. subtilis with the absorbance of 2.27±0.03, 2.23±0.03 and 2.00±0.00 after 120 h of incubation respectively. P. putida had been previously reported to degrade various fractions of hydrocarbon [8]. Similarly, Kaczorek and Olszanowski [16] reported that an increase in biodegradation of diesel oil by P. putida was observed after the addition of saponins to the system (76%) and that even without the addition of biosurfactants, its degradation was comparable to the system with the added biosurfactants. In a different study, Shankar et al. [17] reported that out of the 49 isolated bacteria isolated from contaminated soil, 32 of them accounting for 65.30 % were able to degrade the various hydrocarbon fractions (including diesel) as the sole carbon source. Kaczorek and Olszanowski [16], on the
other hand, reported an increase in biodegradation of diesel oil by *P. putida* following the addition of saponins to the system (76%). In another study, Titah et al [14] screened thirteen (13) bacterial isolates for the potential to utilize diesel oil in different concentrations ranging from 0 to 15% (v/v). It was revealed that out of the 13 isolates screened, only two belonging to *Staphylococcus* and *Micrococcus* species were found to degrade the diesel compared to other bacteria isolated in the study. However, in a different study, Piroillo et al [18] demonstrated the ability of *P. aeruginosa* to degrade as high as 20% diesel concentration after 144 h of incubation. These findings were attributed to the production of rhamnolipid produced by the isolates.

To determine the effect of diesel concentration on the degradation, the best candidate in this study, *P. putida* was subjected to biodegradation at different diesel concentrations (1-3% (v/v)). The results revealed that the degradation of the isolate was observed at 1% (v/v) diesel concentration followed by 2% with 2.40±0.00 and 2.27±0.03 respectively, while the least degradation was recorded at 3% (v/v) after 120 h of incubation. Our findings corroborate the fact that diesel concentration in the medium increases, bacterial growth decreases, and vice versa [14]. This might be because of the toxic effects of high diesel concentrations on the growth of bacteria in the medium, which causes stress and shock [11].

Similar to the findings of the present study, Farag et al [19] investigated the effect of crude oil on the degradation potential of *Pseudomonas* species at 0.2%, 0.5%, 1%, and 1.5% concentrations and found the greatest degradation potential for oil removal when at 0.5 percent (v/v) of crude oil. On the other hand, Palanisamy et al [11] observed the highest degradation of diesel by *Acinetobacter baumannii* at 4% (v/v) but could not tolerate 5% (v/v) of the oil while minimal degradation was recorded at 1% (v/v). However, in a different study, biodegradation of up to 6% diesel has been reported to be aided by the addition of 0.2% w/v glucose and 0.1% w/v yeast extract [20]. Furthermore, Nicolau et al [21] reported the biodegradation of 2-ethylhexyl nitrate which was employed as an additive of diesel fuel, by *M. austroafricanum*, though the results indicated that metabolites accumulated when biodegradation was taking place in the medium.

**CONCLUSION**

In this study, three (3) diesel degrading bacterial species were isolated from contaminated soil and all have shown various degradation capacities. Th three bacterial species included *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Bacillus subtilis*. *P. putida* was found to be the best candidate based on the screening result and was used in the determination of the effect of diesel concentration on its degradation. The maximum degradation was observed at 1% concentration while the least was recorded at 3% (v/v) suggesting the influence of diesel concentration on its degradation.

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- 74 -
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