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The Potential of *Serratia marcescens* in Bioremediation of Crude-oil Polluted Soil

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Abstract

Bioremediation, which involves the use of microorganisms, is an environmentally friendly approach in restoring crude-oil polluted environments. The use of *Serratia marcescens* (SM) in bioremediation of crude-oil polluted soil was investigated in this study. Soil from a farm was inoculated in nutrient broth for bacterial enrichment. The broth culture was inoculated on nutrient agar and incubated at ambient temperatures (27 - 32 °C). Bacterial isolates with red colonies were selected and identified. Identified SM was used in preparing bacterial-mineral-salts suspension. The bioremediation experiment consisted of two setups: Control (CT) and setup treated with SM (TSM). Both setups contained crude-oil polluted soil. Mineral-salt solution and the bacterial-mineral-salt suspension were added to setup CT and TSM, respectively. Total hydrocarbon concentration (THC), total heterotrophic bacteria (THB), hydrocarbon utilizing bacteria (HUB), total fungi (TF), and hydrocarbon utilizing fungi (HUF) in the setups were determined at various times. About 38.3 % and 46.5 % THC reduction were achieved in setup CT and TSM, respectively. The percentage of THB that are HUB was higher in setup TSM on day 7 (8.8 %) and day 28 (4.4 %) but higher in setup CT on day 14 (1.7 %) and day 21 (6.6 %). The percentage of TF that are HUF was higher in setup TSM on day 14 (98.0 %) and on day 28 (41.6 %) but higher in setup CT on day 21 (85.3 %). It is concluded that SM has a potential for use in bioremediation of crude-oil polluted soil.

Keywords: Bioremediation, Crude-oil polluted soil, Hydrocarbon-degradation potential, Red colonies, *Serratia marcescens*

INTRODUCTION

Pollution of the terrestrial environment with crude oil and its products is an inevitable consequence of oil exploration, extraction, and transportation.

Crude-oil-producing communities thus often experience devastating consequences of crude oil spills, which include economic loss due to loss of farmlands and wildlife, shortage of drinking water due to surface and groundwater contamination, and health issues resulting from inhalation of fumes and ingestion of contaminated water or food (Chukwuka *et al.*, 2018; Ojimba, 2011; Ordinioha & Brisibe, 2013). Therefore, It is necessary to employ countermeasures to return polluted environments to their natural state. One of the promising countermeasures against crude oil pollution is bioremediation. Bioremediation involves using biodegradation agents, mainly bacteria and fungi, to break down or degrade pollutants (Bala *et al.*, 2022). An advantage of bioremediation is that the biodegradation agents are fairly ubiquitous in natural environments. However, the hydrocarbon-degrading potential of a prospective bioremediation agent should be

known before such an agent is deployed for full-scale bioremediation work. Also, the prospective biodegradation agent should have the ability to tolerate high concentrations of the pollutant, effectively/efficiently degrade the pollutant, be isolated and cultivated easily, grow relatively fast, and survive in a wide range of environmental stress (Hossain *et al.*, 2022; Huang *et al.*, 2022; Tahri *et al.*, 2013).

Serratia marcescens have been implicated in some bioremediation studies as hydrocarbon degrading/utilizing microorganisms (Agbor & Antai, 2019; Akoachere *et al.*, 2008; Bidja-Abena, 2020). It is commonly found in moist soil, water, and some plants (Gillen & Gibbs, 2012). *Serratia marcescens* is a rod-shaped Gram-negative motile bacterium known for its distinct red pigment called prodigiosin (Hejazi & Falkiner, 1997). This pigment makes the bacterium easily identifiable on nutrient agar plates. However, researchers have shown that the pigment is not produced at temperatures above 32 ° C (Haddix & Shanks, 2018; Romanowski *et al.*, 2019).

A wide range of carbon sources including glucose, maltose, starch, glycerol, and chitin (a polymer of N-acetyl-D-glucosamine), can be metabolized by *Serratia marcescens* (Bhargavi *et al.*, 2012; Yan *et al.*, 2019). This is due to *Serratia marcescens* being able to produce various enzymes. These enzymes include amylase, chitinase, lipase, and protease (Sharma & Tiwari, 2005), contributing to its ability to break down complex organic compounds. *Serratia marcescens* can adapt to harsh environmental conditions such as elevated salt concentrations and heavy metal contamination (Adedoyin *et al.*, 2023; Ketola & Hiltunen, 2014). Some strains of *Serratia marcescens* are been explored for their potential in bio-pesticide production (Konecka *et al.*, 2019; Fu *et al.*, 2021; Tao *et al.*, 2022). Due to its ability to metabolize various carbon sources and adapt to harsh environmental conditions, *Serratia marcescens* could be a prospective biodegradation agent. The aim of this study was thus to determine the potential of using *Serratia marcescens* in the bioremediation of crude oil-polluted soil.

MATERIALS AND METHODS

Sample Collection

A large quantity of soil of about 3 kg was collected from the demonstration farm () of the Rivers State University (RSU), Nigeria, located within the University's main campus, and transported to the Microbiology laboratory, RSU, for bacterial isolation and bioremediation experimentation.

Isolation of *Serratia marcescens*

A quantity (10 g) of the soil was placed in a conical flask containing 100 ml sterile nutrient broth, and the inoculated flask was incubated at ambient temperatures (27 - 32 °C) for 24 hours for microbial enrichment. After incubation, ten-fold serial dilution was carried out to a dilution of 10^{-5} using sterile normal saline. Aliquot of 0.1 ml of the different dilutions were then spread inoculated in duplicates on nutrient agar (NA) plates. Inoculated NA plates were incubated at ambient temperatures for 48 hours. After incubation, the plates were checked for the presence of red colonies. Such colonies were subcultured onto fresh NA plates and slants to obtain pure isolates and stock cultures. The isolates were subjected to Gram staining and microscopic examination and the following physicochemical and biochemical tests: catalase, oxidase, motility, citrate utilization, indole production, Methyl-Red, Vogues-Proskauer, salt tolerance (7 % NaCl tolerance), and fermentation tests using

glucose, lactose, mannitol, sucrose, and xylose. These tests were carried out as described by Peekate (2022).

Preparation of *Serratia marcescens* isolates for Bioremediation Experiment.

A broth culture was prepared from a stock culture of identified *Serratia marcescens*. The broth culture was used to prepare plate cultures of *Serratia marcescens*; a sterile swab stick was dipped into the broth culture and used to inoculate the entire surface of sterile NA plates. Inoculation was done in quadruplicate. The inoculated plates were incubated at ambient temperatures (27 - 32 °C) for 24 hours. After incubation, the ensuing colonial growth was scooped using a sterile spatula and transferred into 200 ml sterile mineral salt solution (MSS). The composition (g/L) of the MSS was as follows: MgSO₄.7H₂O - 0.42, KH₂PO₄ - 0.83, NaCl - 10.0, KCl - 0.29, Na₂HPO₄ - 1.25, NaNO₃ - 0.42 (Source: Odokuma & Dickson, 2003). The resulting bacterial-mineral-salt suspension was used in the bioremediation experiment.

Bioremediation Experiment and Monitoring

About 1 kg of soil was placed in 2 glass troughs and polluted with 200 ml crude oil. The artificially polluted soils were allowed undisturbed in the laboratory for 1 week. After this period, the total hydrocarbon concentration (THC) and populations of total heterotrophic bacteria (THB), hydrocarbon-utilizing bacteria (HUB), total fungi (TF), and hydrocarbon-utilizing fungi (HUF) in the soils were determined. Then, about 100 ml of the bacterial-mineral-salts suspension earlier described was added to the polluted soil in one of the troughs, and the trough was labeled TSM; 100 ml sterile MSS was added to the polluted soil in the other trough, and the trough was labeled control (CT). The setups were maintained for 28 days at ambient temperatures (27 - 32 °C); the relative humidity of the atmosphere during the 28 days was 58 - 61 %. The soil in the setups was tilled once every week, and the moisture content was maintained at 10-20 % using non-sterile MSS. The THC in the setups was determined at day 28, while THB, HUB, TF, and HUF populations were determined at weekly intervals for the 28-days.

Determination of THC

The THC in the soil samples was determined using the spectrophotometric method described by Peekate *et al.* (2023). In the method, a 5 g soil sample was placed in a 150 ml beaker, and 10 ml hexane was added.

The mixture was stirred for about 30 seconds and then filtered through Whatman No. 1 filter paper held in a glass funnel into a 150 ml conical flask. The absorbance of the filtrate at 420 nm was then measured using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China). Absorbance reading was then used to determine THC through extrapolation from a previously obtained plots of concentrations of crude oil in hexane against absorbance at 420 nm.

Determination of Population of THB, HUB, TF, and HUF

The population of THB, HUB, TF, and HUF in the soil samples was determined by subjecting 1 g of the soil samples through a ten-fold serial dilution process to obtain 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ dilutions. Sterile normal saline was used in the dilution process. Aliquots of 0.1 ml of the 10⁻³, 10⁻⁴, and 10⁻⁵ dilutions were spread inoculated in duplicates on nutrient agar (NA) plates; 0.1 ml of 10⁻², 10⁻³, and 10⁻⁴ dilutions were spread inoculated in duplicates on mineral-salts agar (MSA) plates containing 100 mg/ml ketoconazole, MSA plates containing tetracycline (100 µg/ml), and potato dextrose agar (PDA) plates. Inoculated sets of MSA plates were supplied with petroleum hydrocarbons using the vapor phase transfer technique (Ebuehi et al., 2005). Inoculated NA plates were incubated at 35 °C for 24 hours, while inoculated sets of MSA and PDA plates were incubated at ambient temperatures (27 - 32 °C) for 5 days. After incubation, ensuing colonies on NA plates, MSA plates containing ketoconazole, MSA plates containing tetracycline, and PDA plates were

counted and used to calculate the THB, HUB, HUF, and TF populations.

Statistical Analysis

The total hydrocarbon concentrations obtained on day 28 in the control and treatment setups were analyzed for significant difference using the Analysis of Variance (ANOVA). The confidence interval was set at 95 % (α = 0.05). ANOVA was calculated using a data analysis tool in Microsoft Excel® 2007.

RESULTS

Morphology, physicochemical, and biochemical characteristics of the bacteria isolates

Of the bacterial isolates with red colonies, three were randomly selected. The isolates were all motile Gram-negative rods; they were all positive to catalase, citrate utilization, Methyl-Red, Vogues-Proskauer, and salt tolerance tests; they were all negative to oxidase and indole production tests; they all produced acid and gas during glucose fermentation, but produced only acid during mannitol and sucrose fermentations; and they did not ferment lactose and xylose.

Hydrocarbon Concentration in the Setups

The total hydrocarbon concentration (THC) in the polluted soil before the treatments was 84182 ± 4866 mg/kg; by day 28, the THC in the experimental setups had reduced below the initial THC value (Table 1). About 38.3 % reduction in THCs was achieved in the control setup (CT), and a 46.5 % reduction in the setup (TSM) treated with *Serratia marcescens*. However, the THCs in both setups on day 28 were not significantly different (P > 0.05).

Table 1: Hydrocarbon Concentrations in the Setups

Day		THC (mg/kg)		Mean ± SD	EHD (%)
		R1	R2		
1	PSbT	87 623	80 741	84 182 ± 4 866	-
28	CT	52 258	51 575	51 917 ± 483	38.3
	TSM	47 856	42 239	45 048 ± 3 972	46.5

THC: Total hydrocarbon concentration, EHD: Extent of Hydrocarbon Degradation (EHD (%) = $\frac{THC(day\ 1) - THC(day\ 28)}{THC(day\ 1)} \times 100$), R: Replicate, SD: Standard deviation, PSbT: Polluted Soil before treatment, CT: control setup, TSM: Setup treated with *Serratia marcescens*

Population of THB and HUB in the Setups

The populations of total heterotrophic bacteria (THB) and hydrocarbon-utilizing bacteria (HUB) in the polluted soil before the treatments were 2.5±0.3 × 10⁶ CFU/g and 4.3±1.4 × 10⁴ CFU/g,

respectively. During the experiment, from day 7 to 28, the population of THB in CT ranged from 1.9±1.1 × 10⁷ to 6.1±2.7 × 10⁷ CFU/g, while the population of HUB ranged from 2.1±1.3 × 10⁵ to 4.0±2.9 × 10⁶ CFU/g.

In setup TSM, the population of THB ranged from $2.6 \pm 1.9 \times 10^6$ to $7.1 \pm 4.0 \times 10^7$ CFU/g, while the population of HUB ranged from $2.3 \pm 1.7 \times 10^5$ to $1.7 \pm 1.0 \times 10^6$ CFU/g. In comparison, the mean THB population in setup CT was higher than the mean THB population in setup TSM on day 7, slightly higher on days 14 and 28, but the same on day 21, as shown in Figure 1. However, ANOVA of THB populations in both setups on days 7, 14, and 28 showed that the THB populations were not significantly different ($P > 0.05$). On the other hand, the mean HUB population in setup CT was slightly higher than the mean HUB

population in setup TSM from day 14 to 28. Statistical analysis of HUB populations in both setups on days 14, 21, and 28 showed that the HUB populations were significantly different ($P < 0.05$) on day 14 but were not significantly different ($P > 0.05$) on days 21 and 28. The percentage of THB that are HUB was higher in setup TSM on day 7 (8.8 %) and day 28 (4.4 %), as shown in Figure 2. Though the percentage of THB that are HUB was higher in setup CT on day 14 (1.7 %) and on day 21 (6.6 %), it was not as high as that observed in setup TSM on days 7 and 28.

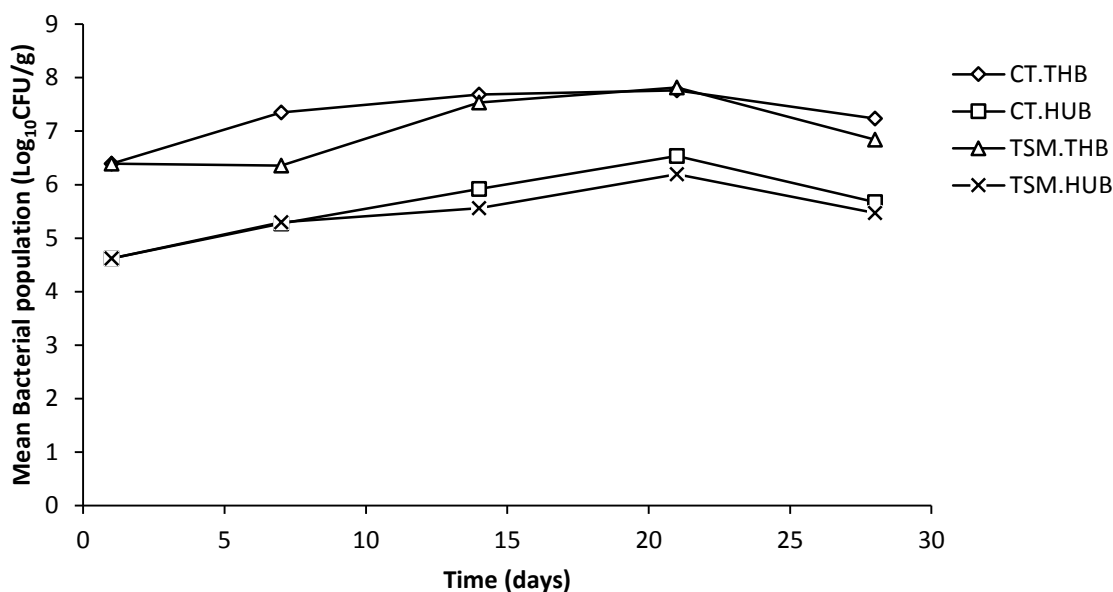


Figure 1: Total heterotrophic and hydrocarbon utilizing bacterial populations in the setups.

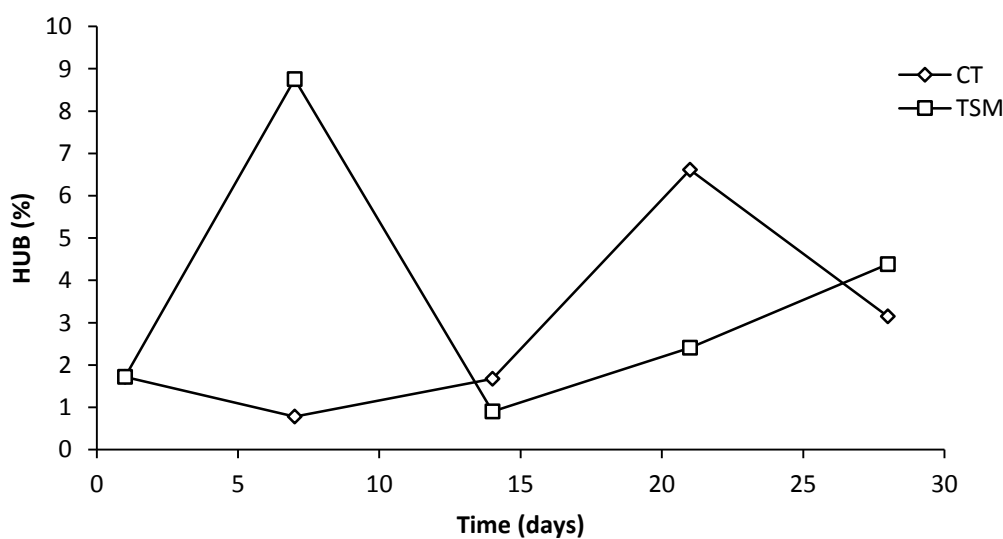


Figure 2: Percentage of hydrocarbon utilizing bacteria in the setups.

Population of TF and HUF in the Setups

The population of total fungi (TF) and hydrocarbon-utilizing fungi (HUF) in the polluted soil before the experiment was $4.0 \pm 3.7 \times 10^5$ CFU/g and $2.5 \pm 0.3 \times 10^5$ CFU/g, respectively. During the experiment, from day 7 to 28, the population of TF in CT ranged from $1.3 \pm 0.1 \times 10^6$ to $7.3 \pm 1.0 \times 10^6$ CFU/g, while the population of HUF ranged from $3.1 \pm 2.4 \times 10^5$ to $5.2 \pm 1.6 \times 10^6$ CFU/g. In setup TSM, the population of TF ranged from $6.1 \pm 5.5 \times 10^5$ to $1.4 \pm 0.4 \times 10^7$ CFU/g, while the population of HUF ranged from $1.6 \pm 0.8 \times 10^5$ to $2.9 \pm 1.8 \times 10^6$ CFU/g. There were no clear-cut differences in the populations of TF and HUF in both setups for the duration of the

experiment, as shown in Figure 3; Populations of TF and HUF appeared to occupy a band between 5.2 to 7.2 Log₁₀ CFU/g. However, TF populations in setup CT stood out as the highest on day 7, TF populations in setup TSM stood out as the highest on day 21, and HUF population in setup CT stood out as the least on day 28. Statistical analysis of the fungal populations in both setups on days 7, 21, and 28 showed that the fungal populations were not significantly different ($P > 0.05$). The percentage of TF that are HUF was higher in setup TSM on day 14 (98.0%) and on day 28 (41.6%), as shown in Figure 4; on day 21, it was higher (85.3%) in setup CT.

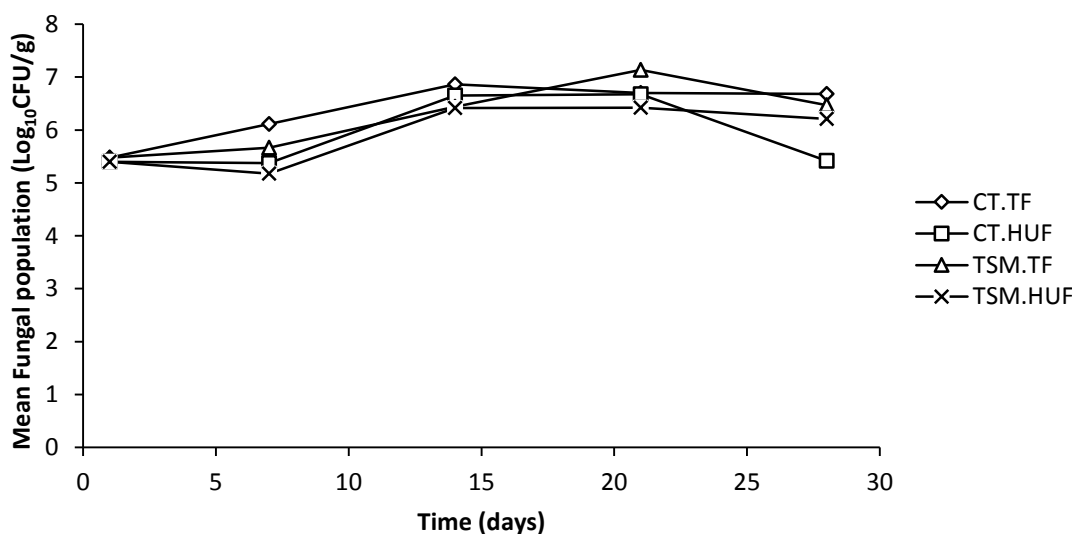


Figure 3: Total and hydrocarbon utilizing fungal populations in the setups.

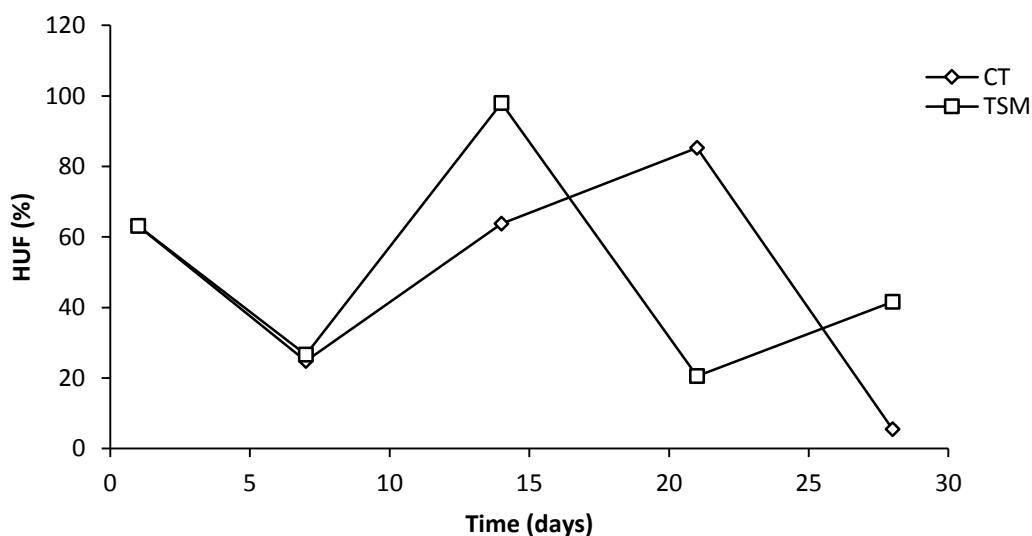


Figure 4: Percentage of hydrocarbon utilizing fungi in the setups.

DISCUSSION

The addition of microorganisms during the remediation of crude oil-polluted environments can enhance the rate of degradation of the hydrocarbon pollutants. Such microorganisms should be hydrocarbon degraders able to withstand the stress brought about by the toxic nature of hydrocarbons and variations in environmental conditions. *Serratia marcescens* can adapt to harsh environmental conditions (Adedoyin *et al.*, 2023; Ketola & Hiltunen, 2014) and has been implicated as a hydrocarbon-degrading microorganism (Agbor & Antai, 2019). *Serratia marcescens* can be identified on nutrient agar plates as red colonies due to its red pigment, which it produces at temperatures below 32 °C. In this study, the red colonies on nutrient agar plate cultures incubated at ambient temperatures (27 - 32 °C) were shown to possess similar physicochemical and biochemical characteristics as attributed to *Serratia marcescens* in other related works (Agbodjato *et al.*, 2015; Othman *et al.*, 2019; Patel *et al.*, 2016). Also, the use of “Advanced Bacterial Identification Software” (https://www.tgw1916.net/bacteria_Enterobacteriales_input.php) revealed the identity of the bacterial isolates with red colonies as *Serratia marcescens* with 87.5 % similarity.

The extent of hydrocarbon degradation in crude-oil polluted soil treated with *S. marcescens* and nutrients in mineral salts after 28 days was 46.5 %. In the control, with nutrients but not the bacterium, the extent of hydrocarbon degradation was 38.3 %. The degradation as a result of the addition of the bacterium was not significantly different ($P > 0.05$) from that achieved in the control setup. This indicates that the nutrients added to both setups played a major role in enhancing hydrocarbon biodegradation. Also, the fairly narrow gap in the extent of hydrocarbon degradation between the control and treatment could result from the bacterium not previously exposed to hydrocarbons. In some related works (Bidja-Abena *et al.*, 2020; Obiajulu *et al.*, 2022; Olajide & Adeloye, 2023), the addition of *S. marcescens* to hydrocarbon polluted medium resulted in a higher extent of hydrocarbon degradation. In Olajide & Adeloye (2023), hydrocarbon degradation of 54.7 - 86.9 % was achieved after 7 days of shaker incubation of different setups of water contaminated with separate fractions of crude-oil at 1 % (v/v) concentration inoculated with *S. marcescens*; in Obiajulu *et al* (2022), 78.7 - 79.3 % was achieved after 14 days of incubation with intermittent shaking of a mineral-salts solution contaminated with crude-oil at 1 % (v/v) concentration. In Bidja-Abena *et*

al. (2020), the addition of *S. marcescens* to a mineral-salts solution contaminated with crude oil at about 0.4 % (w/v) concentration resulted in 65.5 % hydrocarbon degradation after 20 days of shaker incubation. The higher extent of hydrocarbon degradation obtained in the aforementioned related works could be attributed to their use of single bacterium, strains of *S. marcescens* from hydrocarbon-polluted environments, lower pollutant concentration (0.4 and 1 %), fractions of crude oil, and aqueous solutions or water as the polluted medium. Water and aqueous solutions are homogenous mediums, and under a shaker, incubation allows for even distribution of microorganisms, nutrients, and aeration; this could not be achievable in soil, a heterogeneous medium. *Serratia marcescens* used in this study were isolated from unpolluted soil and may not have fully developed hydrocarbon degrading capabilities as those resident in hydrocarbon-polluted environments for a long time.

Furthermore the bacterium was added into polluted soil, having an adequate population of other microorganisms. Some antagonism can occur in the resulting cultures, limiting hydrocarbon degrading activity. On the other hand, the estimated pollutant (crude oil) concentration used in this study is calculated to be about 20 % (v/w). This is much greater than the 0.4 and 1 % used in the other studies.

The total heterotrophic bacterial population (THBP) was greater than the hydrocarbon-utilizing bacterial population (HUBP) in both setups during the experiment. This has been observed in some bioremediation setups in some studies (Chikere *et al.*, 2012; Odokuma & Dickson, 2003; Peekate *et al.*, 2018) and indicates that probably not all bacteria in hydrocarbon-polluted soils are hydrocarbon degraders. It may also indicate the likely presence of organic substrates other than hydrocarbons in hydrocarbon-polluted soils. On the other hand, there was a lag phase in THBP from day 1 to 7 in the setup treated with *Serratia marcescens*. This could indicate a period of adaptation of the indigenous bacteria to the presence of the introduced bacteria (*S. marcescens*). The percent HUBP was, for the most part, higher in the setup treated with *S. marcescens* (setup TSM) than in the control setup. This indicates a higher level of hydrocarbon bio-utilization in the setup TSM compared to the control setup. The higher level of bio-utilization supports the higher extent of hydrocarbon degradation observed in setup TSM. As with HUBP, the percent HUFPP was, for the most part, higher in the setup treated with *S. marcescens* than in the control setup.

This reinforces the suggestion that there was a higher hydrocarbon bio-utilization in the setup TSM compared to the control setup. There was not much difference in the total fungal population (TFP) and the hydrocarbon-utilizing fungal population (HUFP) in both setups during the experiment. This could indicate that all fungi in hydrocarbon-polluted soils are hydrocarbon degraders. *Serratia marcescens* has been considered an opportunistic pathogen (Khanna *et al.*, 2013; Murdoch *et al.*, 2011). However, it is not responsible for a particular infection in humans, animals, or plants. Being opportunistic, it will only cause infection in immunocompromised persons. *Serratia marcescens* may thus be a potential candidate for bioremediation of polluted environments.

CONCLUSION

Serratia marcescens, a bacterium implicated as a hydrocarbon degrader, can easily be identified

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