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Characterization and Potential of Plant Growth-Promoting Rhizobacteria (PGPR) Isolates Capacity Correlating with Their Hydrocarbon Biodegradation Capability

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ABSTRACT

The aim of this research is to find the characteristics of three PGPR isolates—*Azospirillum* sp., *Bacillus* sp., and *Pseudomonas* sp.: First, by profiling their characteristics in a liquid bioremediation system and secondly by measuring their performance as a bioagent in a soil phytoremediation system using ramie plant (*Boehmia niviea* L.). A Randomized Block Design in triplicate is used: (1) a Nitrogen-free medium with mineral media containing 1% (wt/v) petroleum hydrocarbons; and (2) 1% (wt/v) glucose medium as control. We tested their petroleum-degrading capacity, nitrogenase activity, phytohormones production, and ramie plant growth. The results showed that both *Pseudomonas* sp. (98.7%, 81.78% degradation efficiency) and *Azospirillum* sp. (93.80%, 83.70%) were the superior candidate in both systems. They both show reduced but adequate phytohormone production, managing to improve ramie plant growth. Both also showed reduced but sufficient nitrogen fixing capabilities to improve hydrocarbon degradation activity effectively. Meanwhile, *Bacillus sp.* has the lowest biodegradation capabilities (84.07%; 78.6%) and lowest nitrogenase activity, while failing to improve plant growth. Therefore *Bacillus sp.* would be more beneficial in a bacterial consortium where its characteristics (high IAA production) can be coupled with other isolates that can offset its lack of phytohormone or nitrogenase activity.

INTRODUCTION

According to the BPS (2020), the waste generated from oil spills and petroleum production deposits reached 37.38% of the total petroleum production in 2019 (where total production was 259,246.8 [in thousand] barrels). As such, the government requires the oil and gas industry to rehabilitate oil-contaminated land in waste disposal areas in several petroleum industries following the Decree of the State Minister for the Environment No.128 (Keputusan Menteri, 2003). Therefore, the discovery of technology to remove petroleum waste contamination is very important. One promising technology is a phytoremediation system using Plant Growth Promoting Rhizobacteria (PGPR).

Plant Growth Promoting Rhizobacteria (PGPR) are a bacterial group that assists with plant nutrients through many mechanisms (de-Bashan, Hernandez, & Bashan, 2012). Several comprehensive and critical reviews describing operational tools in PGPB (Plant Growth Promoting Bacteria) or PGPR (Plant Growth Promoting Rhizobacteria) were published in recent years (Hansda, Kumar, Anshumali, & Usmani, 2014; Zhuang, Chen, Shim, & Bai, 2007), promote plant growth (Backer et al., 2018); initiate quorum sensing to conjunct action to achieve a particular population density (Khan, Afzal, Iqbal, & Khan, 2013) and increases plant tolerance toward drought and metal contaminants, hydrocarbon and salinity

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stress (Ponmurugan & Gopi, 2006) and solubilize-bonded phosphates in soil (Zaidi, Khan, Ahemad, & Oves, 2009), improved plant growth parameters increase in root length (Bhatt & Vyas, 2014) and produce phytohormones (IAA and gibberellic acid) which supports better plant growth (Ponmurugan & Gopi, 2006).

Another use of PGPR that is worth investigating is the use in a plant-assisted hydrocarbon biodegradation system (phytoremediation) by helping plant growth while simultaneously assisting biodegradation removal. Therefore, the selection and characterisation of PGPR isolate as a phytoremediation bioagent must be done to find the PGPR isolates that exhibit two capabilities: a biofertilizer and a hydrocarbon biodegrader.

Those phytohormones play a vital role in regulating plant growth (Iqbal et al., 2019). It also improves stress tolerances and resistance to pathogens (Bashan, Puente, de-Bashan, & Hernandez, 2008; Egamberdieva, Wirth, Alqarawi, Abd-Allah, & Hashem, 2017; Iqbal et al., 2019). Three crucial traits that significantly affect how effectively a PGPR isolate works in a phytoremediation system are its capacity to create phytohormones, fix nitrogen, and biodegrade hydrocarbons. Furthermore, the synergistic interaction between microbes and plants in the rhizosphere plays an essential role in improving the efficacy performance of phytoremediation (de-Bashan, Hernandez, & Bashan, 2012; Glick, 2003; Huang, El-Alawi, Penrose, Glick, & Greenberg, 2004; Sinha & Mukherjee, 2008).

Many studies have been conducted with *Pseudomonas* sp. and *Bacillus* sp. Alotaibi, St-Arnaud, & Hijri (2022), Babalola & Akindolire (2011), and Sayyed & Patel (2011) reported that *Pseudomonas* sp. and *Bacillus* sp. are Plant Growth Promoting Rhizobacteria (PGPR), with a predominant rhizobacteria population in the rhizosphere of many plants. Furthermore, Kundan, Pant, Jadon, & Agrawal (2015)OMSW have fertilizer characteristics, which make it a potential source for organic fertilization. Composting of OMSW treatment process was conducted in this study to eliminate the phytotoxicity and solve the environmental impact of this waste. Recycling of OMSW was carried out via composting of six batches of trials using equal proportions of OMSW, cow manure (C reported that *Pseudomonas* sp. plays an important role when it acts as an efficient PGPR and increases the plant's yield. Alotaibi, St-Arnaud, & Hijri (2022) investigated *P. plecoglossicida* ET27 as a PGPR isolate that showed highest growth under 3% *n*-hexadecane.

Other studies have also reported that many PGPR inoculants can produce phytohormones in varied capacities, such as *Pseudomonas* spp., *Bacillus* spp., and *Azotobacter* spp., which are predominant in soil (Iqbal et al., 2019)toluene, and xylene. The *Bacillus* sp. PG-8 showed capability to survive under the abiotic stress conditions (Gohil, Raval, Panchal, & Rajput, 2022). A study of *Azospirillum brasilense* strain SR80 found that the capability to degrade 56.5% of crude oil and indole-3-acetic acid was not inhibited by existing oil in the medium (Muratova et al., 2005); however, that researches still did not wholly report the characteristic of their N-fixation capacity or many other phytohormones produced in a petroleum-contaminated medium. Cruz-Hernández, Mendoza-Herrera, Bocanegra-García, & Rivera (2022) reported that *Azospirillum* strains can degrade xenobiotics and show tolerance to different heavy metals, and suggested that the importance of continuing studies aimed at their use individually or in bacterial consortium.

The petroleum hydrocarbon degradation mechanism is mediated by specific enzyme systems, especially oxigenase (Das & Chandran, 2011). The Initial attack of hydrocarbon cleavage is conducted through varying means (Saeki, Sasaki, Komatsu, Miura, & Matsuda, 2009; Varjani & Upasani, 2016). Microorganisms enzymes for hydrocarbon biodegradation are encoded on plasmids located on chromosomes (Salleh, Ghazali, Abd Rahman, & Basri, 2003). Whyte et al. (1998) reported that the Q15 plasmid plays a role in hydrocarbon degradation. Various plasmids such as Q15, OCT, TOL, NAH7, pND140 and pND160 with the presence of genes alkA, alkM, alkB, theA, LadA, assA1 and assA2 and nahA-M also play a role in petroleum hydrocarbon pollutant degradation (Abbasian, Lockington, Mallavarapu, & Naidu, 2015; Wilkes, Buckel, Golding, & Rabus, 2016).

The different PGPR isolate with their own characteristics can potentially be used to enhance the performance of a Phytoremediation management system (Alotaibi, St-Arnaud, & Hijri, 2022). Therefore, a study to determine the profile of the isolate characteristic as PGPB/PGPR isolates and degrading bacteria is precisely the right strategy

to obtain superior performing phytoremediation system.

The aim of this research is to find the characteristics of three PGPR isolates and compare their performance and potential uses. First by profiling their characteristics in a liquid bioremediation system and secondly by measuring their performance as a bioagent in a soil phytoremediation system using ramie plant. We therefore tested and compared three isolates *Azospirillum* sp., *Bacillus* sp., and *Pseudomonas* sp. in a 1% (10,000 mg/kg) petroleum hydrocarbon-Nitrogen free medium to assess their nitrogenase enzyme activity and plant growth-promoting traits (plant-hormone production capacity (IAA, gibberellin, zeatin, and kinetin) while using petroleum hydrocarbon as their carbon source in condition without nitrogen source, and phytoremediation performance in soil systems using ramie plants (*Boehmia niviea* L.).

MATERIALS AND METHODS

This research was carried out in the Soil Biology Laboratory, Department of Soil Science and Land Resources, Faculty of Agriculture, and at the Central Laboratory of Universitas Padjadjaran, from May to November 2020. During the research phase of the efficacy test of isolates in the hydrocarbon phytoremediation soil system was carried out in a greenhouse.

Isolates Selected and Petroleum Hydrocarbon Source

Azospirillum sp., *Pseudomonas* sp., and *Bacillus* sp. cultures were isolated from the rhizosphere of maize (*Zea mays*) in Inceptisols of an experimental field area Jatinangor District, West Java, Indonesia. The sample soil from *Zea mays* rhizosphere area was 25 g of sample soil from five points, then mixed homogeneously. Moreover, 10 g of composite soil sample was diluted by sterile NaCl physiologic solution, and then serial dilutions were carried out 1:10 up to a $1:10^4$ dilution. From the sample soil solution was taken 1 ml and put into a 1% petroleum crude liquid medium (100 ml volumes). The preparation solution soil sample was incubated with shaking at 100 rpm for 14 days in 30°C temperature conditions. Finally, the soil bacteria culture hydrocarbon acclimated was received. Furthermore, the sample culture was inoculated into the solid petroleum hydrocarbon media and incubated for seven days. Each colony growth in tangible petroleum media was isolated using Ose and inoculated onto nutrient agar media as a pure stock culture. Three isolates that showed the best increase were identified using the Bergeys' manual method.

Petroleum crude oil was obtained from a storage tank at PT Pertamina (Indonesia Oil and Gas Mining Corporation) in Balongan, Indramayu, West Java, Indonesia. Two litres of petroleum samples were directly collected from a 100 API gravity separator and mixed to obtain composite samples. The samples were then stored at 24°C in a room without direct sunlight.

Medium Preparation of Hydrocarbon degradation capacity assessment

We used two different mediums for the growth of the bacterial isolates: (1) A mineral medium containing 1% TPH (total petroleum hydrocarbon) nitrogen-free and prepared in the following manner: 1000 ml liquid mineral media (1 l distilled water, 0.5 g KH_2PO_4, 1.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$) was mixed with 10 ml of trace-elements (consisting of 12 g $Na_2EDTA.2H_2O$, 2 g $FeSO_4.7H_2O$, 1 g $CaCl_2$, 0.4 g $ZnSO_4.7H_2O$, 10 g $NaSO_4$, 0.4 g $MnSO_4.4H_2O$, 0.1 g $CuSO_4.5H_2O$ and 0.5 g $Na_2MoO_4.2H_2O$) inside a conical flask by adding 1% TPH (10,000 ppm TPH) of crude petroleum oil as the carbon source for the isolates. Moreover, (2) An uncontaminated mineral media used as a control treatment was added with 1% (wt/v) glucose as the carbon source.

Set-Up of Biodegradation Petroleum Hydrocarbon Investigation in Liquid System

The study was conducted on a laboratory scale. The research design was a Randomized Block Design (RBD) with three replicates. The first treatment factor was two types of media (1% petroleum mineral media and 1% glucose mineral media). The second treatment factor was three isolates (*Azospirillium* sp., *Bacillus* sp. and *Pseudomonas* sp.). A 1,000 ml Erlenmeyer flask was used as a liquid batch reactor system. It was filled with 500 ml of a mineral-Nitrogen free medium containing 1% TPH of petroleum crude oil and control treatment. Then 5 ml (10^7) CFU per ml cell density) of each isolate was inoculated onto a prepared petroleum-contaminated medium and an uncontaminated medium. The pH of each medium was adjusted to between 6.8 – 7.0. The prepared experiment cultures were incubated and shaken in a rotary shaker incubator at 100 rpm, and the incubation temperature was set at 28°C for 30

days. A sample culture was taken at the end of the petroleum hydrocarbon biodegradation process.

Total Petroleum Hydrocarbon Residues Determination

A hydrocarbon degradation efficiency analysis for determining the types of residual hydrocarbon was conducted using the total petroleum hydrocarbon (TPH) gravimetric method, based on the EPA Solid Waste Evaluation Test Method at 354°C. First, the prepared mixture sample was extracted and dried using a rotary evaporator to remove all the remaining solvents. The dried residues were then gravimetrically weighed and analysed using a Gas Chromatography-Mass Spectra (Shimazu QP-5050 series Ultra 2010) using the Split method was retention time (rt) × 5 ms a nonpolar column and a 30 m length of time. The injector temperature was 250°C, and the retention time (rt) was set up for 30 minutes.

Hydrocarbon Biodegradation Efficiency Calculation

The resulting TPH data, expressed as the petroleum hydrocarbon's degradation-efficiency percentage degree (DE), is calculated by Eq. 1. (Baldera-Moreno et al., 2022).

$$DE\ (\%) = \left|\frac{C0 - Ct}{C0}\right| \times 100\% \quad \text{.....1)}$$

Where: *C0* is the initial TPH concentration of the petroleum crude oil, and *Ct* is the TPH concentration after incubation (i.e., the degradation process) at time *t*.

Assessment of Phytohormone Quantities

The method (Patel & Saraf, 2017) assessed phytohormones production. First, the bacterial isolate culture sample was obtained from the batch culture of each treatment after incubating for 30 days at temperature conditions of 28 ± 2°C. Then, the sample cultures were further assessed to produce various phytohormones, such as IAA, zeatin, kinetin, and gibberellic acid.

Nitrogenase Activity Assessment

The nitrogenase activity was assessed using the acetylene reduction assay (ARA) method (Herridge & Peoples, 1990). In addition, a chemical analysis of nitrogenase enzyme was carried out using a Shimadzu QP 5050 (Shimadzu, Japan) mass spectrometer coupled with a Hitachi 263-50 (Hitachi, Tokyo, Japan) gas chromatograph; helium was used as the carrier gas at the constant-flow mode (Herridge & Peoples, 1990).

Efficacy Assessment of Isolates in the Hydrocarbon Phytoremediation in Soil System by Using Ramie Plant

Isolates Propagation

The culture of each isolate was propagated by growing in nutrient broth medium for *Bacillus* sp. and *Pseudomonas* sp., and Okon broth medium for *Azospirillum* sp. in erlenmeyer volume of 250 ml containing 150 ml of media. Each isolate was inoculated into the media and shaken at a speed of 100 rpm for 48 hours for *Bacillus* sp and *Pseudomonas* sp. and 72 hours for *Azospirillum* sp.

Set Up Soil Contaminated by Petroleum Hydrocarbons for Phytoremediation Preparation in a Soil System

The soil as a medium used in this research is the order Inceptisols Jatinangor. Each treatment was set in experimental containing 10 kg of soil contaminated by crude oil concentration of 5% TPH, was stirred homogeneously, and added with 1% organic soil (wt/wt petroleum). Each pot of plant media was inoculated by 2% (v/wt) of each culture isolate per weight of petroleum crude oil. The mixture of contaminated soil and isolate culture was stirred until homogeneous. Furthermore, 2-month-old cuttings of ramie seedlings were implanted into contaminated soil media that had been prepared according to treatment, and each treatment was repeated three times.

Statistical Analysis

The experiment data were analyzed using Statistical Product and Service Solutions (SPSS) version 15.0. Analysis of variance (ANOVA) was performed and significant differences were assessed at 5% significance level ($p < 0.05$). Analyzed the correlation and regression using the SPSS program ver. 15.0. Anova Correlation-Regression was used to analyze the correlation between degradation efficiency and nitrogenase isolate activity. The difference between treatments in the average treatment value is based on the Independent-Samples Student t-test at a 5% significance level ($p < 0.05$). Statistical analysis used the SPSS 15.0 software.

RESULTS AND DISCUSSION

Hydrocarbon Biodegradation Capabilities of Isolates in a Liquid System

The results for the hydrocarbon-degrading capability of isolates can be seen in Table 1. Three isolates demonstrated the ability to degrade complex petroleum hydrocarbon components into shorter aliphatic and cyclo-hydrocarbon chain compounds. The hydrocarbon biodegradation capability of *Pseudomonas* sp. was a (98.70 ± 0.61)% degradation efficiency (%DE), *Azospirillum* sp. was (93.80 ± 1.06)% (%DE). The hydrocarbon degradation level of *Pseudomonas* sp. was significantly (p < 0.05) higher than *Azospirillum* sp. and *Bacillus* sp. Moreover, *Bacillus* sp. showed (84.07 ± 10.00)% degradation efficiency, the lowest significantly (p < 0.05) hydrocarbon degradation capability compared to the other.

Table 1. Hydrocarbon-degrading capability (percentage ± SD) after 30 days incubation (each data point is derived from three replicates of the batch reactor system)

Treatment	Degradation Efficiency of Petroleum Hydrocarbon (%) (Mean±SD)
Azospirillum sp.	93.80 ± 1.06
Bacillus sp.	84.07 ± 10.00
Pseudomonas sp.	98.70 ± 0.61

Remarks:* Mean of three replicates in batch reactor condition. There is a significant difference in the average treatment value based on the Independent-samples Student's t-test at a 5% significance level (p < 0.05)

This study revealed that *Azospirillum* sp. showed higher hydrocarbon biodegradation efficiency than Azospirillum *brasilense* strain SR80 which showed only 56.5% crude oil biodegradation (Muratova et al. 2005). In line with Viñas, Sabaté, Espuny, & Solanas (2005), *Azospirillum* sp. proved to be highly effective at degrading petroleum hydrocarbon. Furthermore, this study revealed that *Azospirillum* sp. showed metabolic adaptation by enacting flocculation-cell clumping by excreting biomolecule extracellular in unfavourable or stressful conditions, such as the presence of hydrocarbons. As reported by Bible et al. (2015), *Azospirillum brasiliense* has the formation of an "envelope-remodelling" mechanism for protecting its cell through excreting Lipopolysaccharides (LPS) molecules surrounding its cell. Therefore, it can be reasonably assumed to be the reason that *Azospirillum* sp. in this research can adapt to petroleum hydrocarbon contaminated media and are even capable of effectively consuming hydrocarbon. Furthermore, Burdman, Okon, & Jurkevitch (2000), Lerner et al. (2009), and Vanbleu, Choudhury, Carlson, & Vanderleyden (2005) reported that the LPS component of *Azospirillum brasilense* is fucose, and mannose is protecting material for *Azospirillum* spp.

Bacillus sp. showed the lowest hydrocarbon-degrading capability out of the three. Our result differs from Bano, Shahzad, & Siddiqui (2015) who found that *Bacillus altitudinis* can degrade more than 80% of oil sludge after five days of incubation. In contrast, *Bacillus* sp. in this study showed a slower biodegradation capacity, requiring 30 days to reach the efficiency biodegradation level at 84.07%. Meanwhile, Al-Dhabaan (2019) reported that *Bacillus* sp. could only degrade petroleum hydrocarbons with a 70–75% efficiency in a liquid polluted soil during a 28-day incubation. *Bacillus megaterium* non-nitrogenase activity demonstrated oil-degrading capabilities, while *Bacillus subtilis* and *Bacillus* sp. as nitrogen fixers revealed strong abilities to degrade hydrocarbons (Essien, Udoukpo, Etesin, & Etuk, 2013). *Bacillus* sp. is in a bacterial group that forms endospores, especially in unfavourable conditions (such as the presence of toxic hydrocarbons). This could be explained by the fact that the hydrocarbon metabolism pathway of *Bacillus* sp. may require a longer time because it is prioritizing the the germination proces and formation of endospores first rather than using its energy in breaking up petroleum hydrocarbons. However, this hypothesis still needs to be proven through another intrinsic tracing of the metabolism of this isolate.

Pseudomonas sp. is also a good candidate for a dual role in hydrocarbon degradation. Its superior hydrocarbon-degrading capabilities show favorable characteristics: good phytohormones production and a good nitrogenase activity that can cover its hydrocarbon degradation activities. This research found that *Pseudomonas* sp. was able to remove as many as 40 hydrocarbon compounds species contained in petroleum oil with a (C11 – C18) chain during a 30-day incubation period and to release such as hydrocarbon residues in the (C6 – C7) chain: pentane 2-methyl, hexyl hydride, cyclopentane methyl, pentane 2.3-dimethyl, and hexane 3-methyl. Whereas Al-Mailem, Kansour, & Radwan (2019), Babalola & Akindolire (2011), and Sayyed & Patel (2011) found that *Pseudomonas aeruginosa* can utilize pure hydrocarbons dissolved in alkanes with a C9 – C40 chain length and

aromatic hydrocarbons, such as benzene, biphenyl, naphthalene, phenanthrene, and toluene, as the carbon source. The investigation conducted by Yateem, Al-Sharrah, & Bin-Haji (2007)respectively, under aerobic conditions. The isolated HDMs were identified using 16S rRNA gene sequencing and fatty acid methyl ester (FAME reported that *Pseudomonas boreopolis* is the most-effective isolate related to the capability to degrade hydrocarbon compared with *Bacillus mojavensis* and *B. licheniformis.*

Liu et al. (2013) reported that *Pseudomonas* sp. SB exhibited a high degree of hydrocarbon degradation by its ability to degrade oil sludges. Moreover, Zhang et al. (2012) also found that *Pseudomonas aeruginosa* and *Pseudomonas* sp. can effectively degrade hydrocarbons because the *Pseudomonas* isolate was capable of producing rhamnolipids as a bio-surfactant. It is worth noting that bio-surfactants produced by *Pseudomonas* sp. will improve the effectivity of the hydrocarbon absorption process as a substrate for immediate catalyzing to generate energy for activity and growth.

Nevertheless, all of the research on its biodegradation abilities were reported in condition of adequate nitrogen availabilty. Sufficient nitrogen availability in the process of hydrocarbon degradation has been reported as to be the main factor in bioremediation success. According to Beškoski et al. (2011), Gkorezis et al. (2016)given that many PHCs are biodegradable, bio- and phyto-remediation are often viable approaches for active and passive remediation. This review focuses on phytoremediation with particular interest on the interactions between and use of plant-associated bacteria to restore PHC polluted sites. Plant-associated bacteria include endophytic, phyllospheric, and rhizospheric bacteria, and cooperation between these bacteria and their host plants allows for greater plant survivability and treatment outcomes in contaminated sites. Bacterially driven PHC bioremediation is attributed to the presence of diverse suites of metabolic genes for aliphatic and aromatic hydrocarbons, along with a broader suite of physiological properties including biosurfactant production, biofilm formation, chemotaxis to hydrocarbons, and flexibility in cell-surface hydrophobicity. In soils impacted by PHC contamination, microbial bioremediation generally relies on the addition of high-energy electron acceptors (e.g., oxygen, and Zhao et al. (2011), microbial degrader activity to remove hydrocarbons depends on adequate nutrients supply, especially nitrogen and phosphorous. An optimal C:N:P:K ratio is approximately 100:10:1:0.1.

This research found that even without extra nitrogen input*, Azospirillum sp.* and *Pseudomonas sp. in a liquid bioremediation system* manages to degrade petroleum hydrocarbon effectively. This shows that *Azospirillum* sp. and *Pseudomonas* sp. can fulfill their Nitrogen needs because of their Nitrogen fixing capabilitites.

Hydrocarbon Residue

Forty-five hydrocarbon compounds were detected in the initial petroleum crude oil sample, composed of hydrocarbon chains of various lengths, ranging from C11 to C18, dominated by a polycyclic aromatic hydrocarbon (PAH) chain with varying forms of PAH groups (Fig. 1 and Table 2).

After 30 days of incubation, all isolates demonstrated hydrocarbon degradation capabilities using petroleum hydrocarbon as a carbon source, transforming polycyclic aromatic hydrocarbon (PAH) chain with varying PAH groups into shorter aliphatic and cyclo-hydrocarbon hydrocarbon chains we refer to as its hydrocarbon residue. *Azospirillum* sp. left six, *Bacillus* sp. left twelve, and *Pseudomonas* sp. left five hydrocarbon residue compounds (Table 3).

The six hydrocarbon residues detected in the reactor of *Azospirillum* sp. culture consist of hexyl hydride (c_6h14), cyclopentane methyl (c_6h_{12}), pentane 2.3-dimethyl (c_7h_{16}), pentane 3-ethyl (c_7h_{14}), n-heptane (c_7h_{14}), cyclohexane-methyl (c_7h_{14}). In the reactor of *Bacillus* sp. culture detected C6 to C16 of hydrocarbon. Moreover, *Pseudomonas* sp. could cleave hydrocarbon aromatic rings to form aliphatic chains in various carbon chains and break hydrocarbon longer chains into shorter chains (C6 and C7). It also was resting five hydrocarbons detected, thereof were pentane 2 methyl (c_6h_{14}), hexyl hydride (c_6h_{14}), cyclopentane methyl (c_6h_{12}), pentane 2.3-dimethyl (c_7h_{16}), hexane 3-methyl (c_7h_{16}), see Table 3.

This research found that *Pseudomonas* sp. was able to remove as many as 40 hydrocarbon compounds contained in petroleum oil with a (C11 – C18) chain during a 30-day incubation period and to release hydrocarbon residues in the (C6 – C7) chain range: pentane 2-methyl, hexyl hydride, cyclopentane methyl, pentane 2.3-dimethyl, and hexane 3-methyl. Whereas Al-Mailem, Kansour, & Radwan (2019), Babalola & Akindolire (2011), and Sayyed & Patel (2011) found that *Pseudomonas aeruginosa* can utilize pure hydrocarbons dissolved in alkanes with a C9 – C40 chain length and aromatic hydrocarbons, such as benzene, biphenyl, naphthalene, phenanthrene, and toluene.

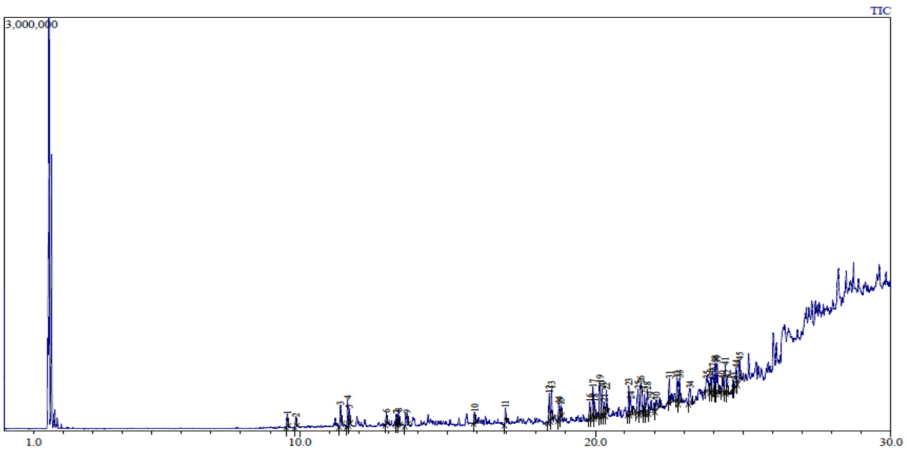

Fig. 1. Chromatogram of initial petroleum hydrocarbon compounds

Table 2. Hydrocarbon compounds detected in the Petroleum initial sample before degradation

Peak No	Name of HC Residue	Peak No	Name of HC Residue	Peak No	Name of HC Residue	Peak No	Name of HC Residue
1	Naphthalene 2-methyl ($C_{11}H_{10}$)	13	Phenanthrene 1-methyl ($C_{15}H_{12}$)	25	Phenanthrene 2.3.5-trimethyl ($C_{17}H_{16}$)	37	Pyrene 1.3-dymethyl ($C_{18}H_{14}$)
2	Naphthalene 2-methyl ($C_{11}H_{10}$)	14	Dihydrocyclopropa phenanthrene 1-methyl ($C_{15}H_{12}$)	26	Phenanthrene 2.3.5-trimethyl ($C_{17}H_{16}$)	38	Pyrene 1.3-dymethyl ($C_{18}H_{14}$)
3	Naphthalene 1.5-dimethyl ($C_{12}H_{12}$)	15	Dihydrocyclopropa phenanthrene 1-methyl ($C_{15}H_{12}$)	27	Phenanthrene 2.3.5-trimethyl ($C_{17}H_{16}$)	39	Pyrene 1.3-dymethyl ($C_{18}H_{14}$)
4	Naphthalene 1.4-dimethyl ($C_{12}H_{12}$)	16	Phenanthrene 2.5-dimethyl ($C_{16}H_{14}$)	28	Phenanthrene 2.3.5-trimethyl ($C_{17}H_{16}$)	40	Pyrene 1.3-dymethyl ($C_{18}H_{14}$)
5	Naphthalene 1.7-dimethyl ($C_{12}H_{12}$)	17	Phenanthrene 3.6-dimethyl ($C_{16}H_{14}$)	29	Phenanthrene 2.3.5-trimethyl ($C_{17}H_{16}$)	41	Pyrene 1.3-dymethyl ($C_{18}H_{14}$)
6	Naphthalene 2-(1-methylethyl) ($C_{13}H_{14}$)	18	Phenanthrene 3.6-dimethyl ($C_{16}H_{14}$)	30	Phenanthrene 2.3.5-trimethyl ($C_{17}H_{16}$)	42	1.1':2'.1''-Terphenyl ($C_{18}H_{14}$)
7	Naphthalene 2.3.6-trimethyl ($C_{13}H_{14}$)	19	Anthracene 1.4-dimethyl ($C_{16}H_{14}$)	31	Pyrene 1-methyl ($C_{17}H_{12}$)	43	1.1':2'.1''-Terphenyl ($C_{18}H_{14}$)
8	Naphtalen 2.4.6-Trimetyl ($C_{13}H_{14}$)	20	Anthracene 9.10-dimethyl ($C_{16}H_{14}$)	32	Pyrene 4-methyl ($C_{17}H_{12}$)	44	Benzo(a) phenanthrene ($C_{18}H_{12}$)
9	Naphthalene 2.3.6-trimethyl ($C_{13}H_{14}$)	21	Phenanthrene 2.7-dimethyl ($C_{16}H_{14}$)	33	Pyrene 1-methyl ($C_{17}H_{12}$)	45	Triphenylene ($C_{18}H_{12}$)
10	Antrachene ($C_{14}H_{14}$)	22	Anthracene 1.4-dimethyl ($C_{16}H_{14}$)	34	Phenanthrene 2.4.5.7-tetra-Methyl ($C_{18}H_{18}$)	—	—
11	Benz(a) azulene ($C_{14}H_{10}$)	23	Beta-pyrene ($C_{16}H_{14}$)	35	Pyrene 1.3-dimethyl ($C_{18}H_{14}$)	—	—
12	Phenanthrene 1-methyl ($C_{15}H_{12}$)	24	Anthracene ($C_{17}H_{16}$)	36	Azulene 2.6-dimethyl-4-phenyl ($C_{18}H_{16}$)	—	—

Nitrogenase Activity Profile and Its Correlation with Degradation Efficiency Degree of Isolates in a Liquid System

This study showed that *Azospirillum* sp. and *Pseudomonas* sp. showed a higher nitrogenase activity profile than *Bacillus* sp. Both isolates showed high hydrocarbon biodegradation performance. The activity profile of the nitrogenase enzyme in each treatment media is shown in Table 4. The nitrogenase enzyme activity of *Azospirillum* sp. does not show a significant ($p > 0.05$) difference between control and hydrocarbon media (a Nitrogen free environment with a presence of 1% hydrocarbon). Between *Azospirillum* sp. and *Pseudomonas* sp. It also shows no significant ($p > 0.05$) difference, but Azospirillum sp. shows a higher significant ($p < 0.05$) difference with *Bacillus* sp. *Bacillus* sp. showed the lowest nitrogenase activity among the isolates that we tested in this study. Moreover, the nitrogenase activity of *Bacillus* sp. also decreased by 41.98% ($p < 0.0$) in hydrocarbon media (Table 4).

Pseudomonas sp. showed ($p < 0.05$) the highest nitrogenase activity out of the three isolates in 1% glucose media. However, it decreased significantly by 74.29% ($p < 0.05$) in hydrocarbon media conditions.

The ability of *Azospirillum* sp. to adapt to toxic hydrocarbon conditions by sustaining its Nitrogenase activity was an interesting result. This study found that *Azospirillum* sp. showed relatively stable nitrogenase activity when grown in a Nitrogen free environment with a presence of 1% hydrocarbon. It indicates that *Azospirillium* sp. manages to independently receive adequate Nitrogen for its metabolic process of petroleum hydrocarbon.

Table 3. The Hydrocarbon (HC) Residue after degradation by the isolates

Peak No	HC Residue Detected in *Azospirillum* sp. culture	HC Residue Detected in *Bacillus* sp. culture	HC Residue Detected in *Pseudomonas* sp. culture
1	Hexyl hydride (C_6H_{14})	Hexyl hydride (C_6H_{14})	Pentane 2-methyl (C_6H_{14})
2	Cyclopentane methyl (C_6H_{12})	Methylcyclopentane (C_6H_{12})	Hexyl hydride (C_6H_{14})
3	Pentane 2.3-dimethyl (C_7H_{16})	Pentane 2.3-dimethyl (C_7H_{16})	Cyclopentane methyl (C_6H_{12})
4	Pentane 3-ethyl (C_7H_{14})	Pentane 2.3.4-trimethyl (C_8H_{18})	Pentane 2.3-dimethyl (C_7H_{16})
5	n-Heptane (C_7H_{14})	n-Heptane (C_7H_{16})	Hexane 3-methyl (C_7H_{16})
6	Cyclohexane-methyl (C_7H_{14})	Cyclohexane-methyl (C_7H_{14})	—
7	—	Cyclopentane-ethyl (C_7H_{14})	—
8	—	Heptane 2.34-trimethyl ($C_{10}H_{22}$)	—
9	—	Heptane 3-methyl (C_8H_{18})	—
10	—	Octane (C_8H_{18})	—
11	—	n-Hexadecane ($C_{16}H_{34}$)	—
12	—	Nonane 3-methyl-5-propyl ($C_{13}H_{28}$)	—

Table 4. Nitrogenase activity of isolates (each data point is derived from three replicates of the batch reactors system)

Bacterial Isolate	Growth Media	Nitrogenase Activity (µmol/h)	Change (Δ%)
Azospirillum sp.	Glucose 1%	0.0086 ^{b}	- 2.12
	HC 1%- N-free	0.0084 ^{b}	
Bacillus sp.	Glucose 1%	0.0081 ^{b}	- 41.98
	HC 1%- N-free	0.0047 ^{a}	
Pseudomonas sp.	Glucose 1%	0.0307 ^{c}	- 74.29
	HC 1% - N-free	0.0079 ^{b}	

Remarks : Mean of three replicates in batch reactor condition; HC = Hydrocarbon. N-Free = nitrogen-free. Numbers followed by the same letter notation were not significantly different based on Duncan's test at a 5% significance level ($p < 0.05$)

We hypothetize that that this phenomenon can be explained by *Azospirillum* sp., ability to produce extracellular material in the form of slime that attaches and surrounds the cell when they are exposed to stressful conditions. It has been reported by Bahat-Samet, Castro-Sowinski, & Okon (2004) that *Azospirillum* sp. has the ability to produce exopolysaccharides (EPS), which play a role in protecting against unfavorable, toxic, or extreme environmental conditions. EPS was produced by *Azospirillum* sp. and served as a barrier to protect nitrogenase activity against substance disturbances. It can be reasonably assumed that the same phenomenon served as the mechanism that protects *Azospirillum* sp. nitrogenase activity in high oxygen exposure. Bible et al. (2015) also reported that *Azospirillum brasilense* exposed to oxygen stress would form flocculation cell that clumps to protect against the stresses of the environment.

Meanwhile, the nitrogenase activity of *Bacillus* sp. was among the lowest and correlates well with its also low hydrocarbon biodegradation ability. Existing hydrocarbons inhibited the nitrogenase enzyme activity of *Bacillus* sp.

Anova Correlation - Regression analysis (Table 5 and Table 6) shows a significant correlation between hydrocarbon degradation efficiency and nitrogenase activity. The correlation-regression analysis (ANOVA) graph can be seen in Fig. 2. The regression equation are as follows: Y = 3222.143X + 69.598, where Y = Hydrocarbon Degradation Efficiency (%), and X = Nitrogenase Activity in 1% HC-N Free Media (mol/h). The regression equation means that increasing every unit of nitrogenase activity will significantly increase 3222.143 units of Hydrocarbon Degradation Efficiency ($p< 0.05$).

This study revealed that the isolates' hydrocarbon degradation capacity correlates positively with an increase of nitrogenase activity. Proving that the higher the isolates ability of independenly fixing nitrogen, the more efficiently it can degrade hydrocarbons regardless of its environment's lack of Nitrogen supply.

Table 5. Analysis of variance for the regression equation that correlates nitrogenase activity in 1% HC-N free media with hydrocarbon degradation efficiency

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	258.194	1	258.194	23.262	.002
	Residual	77.695	7	11.099		
	Total	335.889	8			

Remarks: The regression equation has a significant effect in correlating the independent variable (nitrogenase activity in 1% HC-N free media) with the dependent variable (hydrocarbon degradation efficiency) if the probability value (Sig.) <0.05

Table 6. The regression equation correlates the nitrogenase activity in 1% HC-N free media with the hydrocarbon degradation efficiency

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	69.598	4.814		14.458	.000
	Nitrogenase Activity in 1% HC-N Free Media (µmol/h)	3222.143	668.066	.877	4.823	.002

Remark: Regression equation: $y = 3222.143x + 69.598$, where y = hydrocarbon degradation efficiency (%), x = nitrogenase activity in 1% HC-N free media (µmol/h)

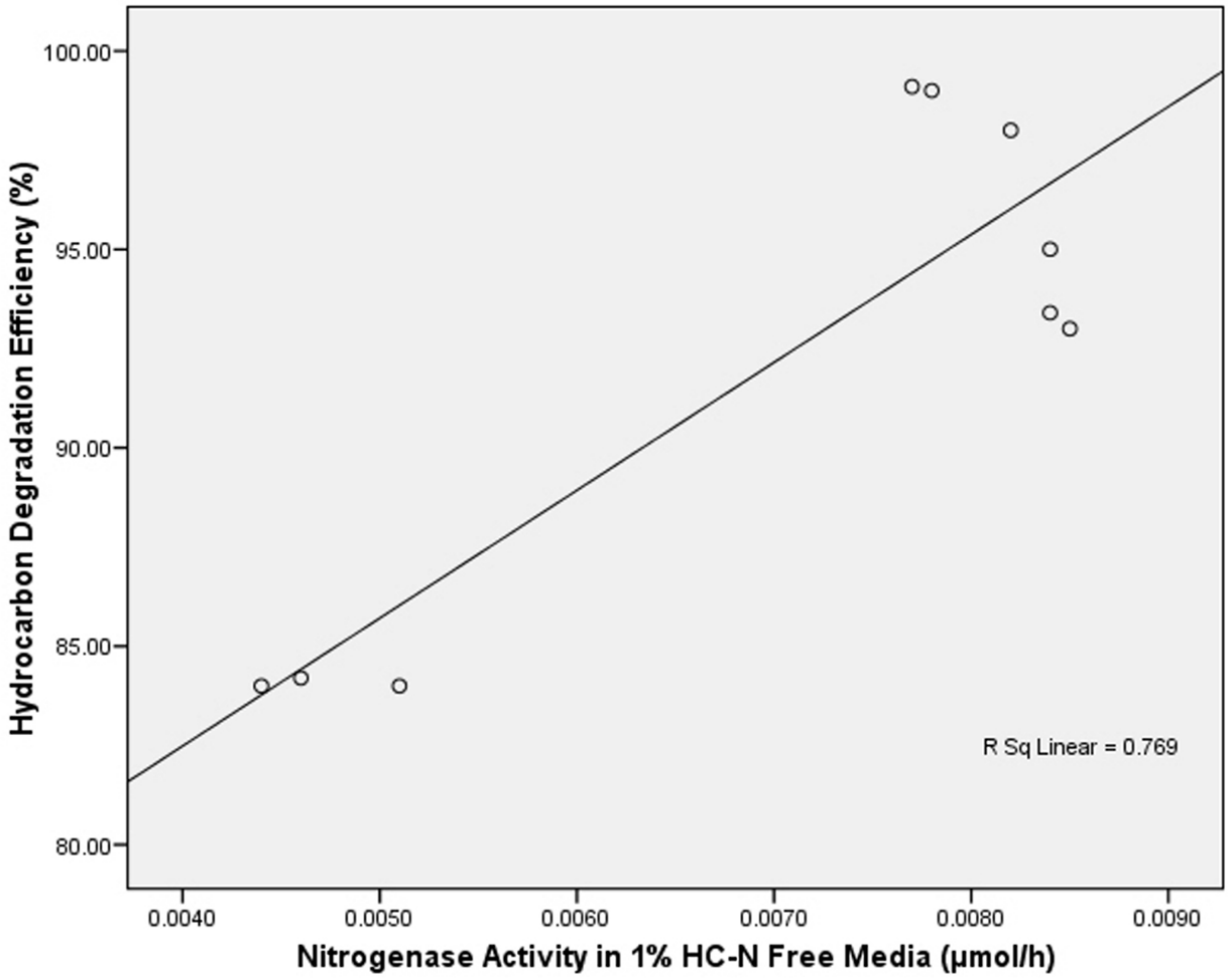

Fig. 2. The Correlation – Regression between hydrocarbon degradation degree and nitrogenase activity

Table 7. Phytohormone production comparison between an uncontaminated (Glucose 1%) and hydrocarbon contaminated medium (HC 1%-Nfree)

Treatment	Phytohormone production profile (mg/l)							
	IAA	Δ%	Kinetin	Δ%	Zeatin	Δ%	GA	Δ%
Azospirillum sp. in 1% glucose	0.122 ^{ab}	-44.51	0.126 ^{a}	-51.47	0.033 ^{a}	47.40	3.490 ^{e}	-78.82
Azospirillum sp. in 1% HC-N free	0.068 ^{a}		0.061 ^{a}		0.048 ^{a}		0.739 ^{b}	
Bacillus sp. in 1% glucose	0.6260 ^{c}	-73.91	nd	nd	0.9333 ^{c}	-56.98	1,253 ^{c}	-74.62
Bacillus sp. in 1% HC-N free	0.1633 ^{b}		nd		0.4015 ^{b}		0,318 ^{a}	
Pseudomonas sp. in 1% glucose	0.637 ^{c}	-81.58	0.147 ^{a}	-32.74	0.979 ^{c}	-65.52	1.448 ^{d}	-68.33
Pseudomonas sp. in 1% HC-N free	0.117 ^{ab}		0.098 a		0.337 ^{b}		0.458 ^{a}	

Remarks : * Each data presented is a mean of three replicates on butch reactor condition. Nfree = nitrogen-free; Δ% = value change; GA = Gibberellin; nd = non detected. Numbers followed by the same letter notation were not significantly different based on Duncan's test at a 5% significance level ($p < 0.05$)

The Phytohormones Production Profile of Isolates in a Liquid System

The phytohormones production profile of isolates in each treatment of the liquid system is shown in Table 7.

The result showed that *Azospirillum* sp. in a 1% contaminated hydrocarbon and a nitrogen-free medium decreased many of its phytohormones productions: IAA production decreased by 44.51% ($p < 0.05$), gibberellin production decreased by 78.82% ($p < 0.05$), and kinetin production decreased by 51.45% ($p < 0.05$) from 0.126 mg/l to 0.061 mg/l. The production of zeatin, however, increased by 47.40% ($p < 0.05$) from 0.033 mg/l to 0.048 mg/l. *Azospirillum* sp. showed the lowest IAA, kinetin, and zeatin production, with concentrations below 0.1 mg/l. However, it produced the highest gibberellin out of the three isolates, about 1.6 x as much as *Pseudomonas* sp.

Bacillus sp. phytohormone production: The IAA produced by *Bacillus* sp. decreased by 73.91% ($p < 0.05$) from 0.6260 to 0.1633 mg/l in the hydrocarbon-contaminated nitrogen-free medium. In the same conditions, gibberellin production decreased by 74.62% ($p < 0.05$). Kinetin was not detected. Zeatin production decreased by 56.98% ($p < 0.05$) from 0.9333 mg/l to 0.4015 mg/l. *Bacillus* sp. did not produce any kinetin, had the highest ($p < 0.05$) zeatin production at 0.4015 mg/l, showed a decrease of 74.62% ($p < 0.05$)of giberellin production, and the highest ($p < 0.05$) IAA production (0.1633 mg/l) out of the three isolates.

Phytohormones produced by *Pseudomonas* sp. in the hydrocarbon contaminated nitrogen-free medium: IAA by 81.58% ($p < 0.05$) from 0.637 to 0.117 mg/l, gibberellin decreased by 68.33% ($p < 0.05$) from 1.448 to 0.458 mg/l, kinetin decreased by 32.74% ($p < 0.05$) from 0.147 to 0.098 mg/l, and zeatin decreased by 65.52% ($p < 0.05$) from 0.979 to 0.337 mg/l. *Pseudomonas* sp. has the second highest($p < 0.05$) gibberellin production at 0.458 mg/l; the second highest ($p < 0.05$) zeatin production at 0.337 mg/l, the second highest ($p < 0.05$) IAA production at 0.1173 mg/l, and the highest ($p < 0.05$) amount of kinetin at 0.098 mg/l.

Pseudomonas sp. showed the highest decrease in IAA and zeatin production in hydrocarbon media, and *Azospirillum* sp. showed the highest decrease in kinetin and gibberellin. The IAA, Gibberellin, zeatin, and kinetin produced in *Azospirillum* sp., *Bacillus* sp., and *Pseudomonas* sp. were inhibited by petroleum hydrocarbons as a carbon source.

The reasons for the declines are still unclear. However, it is known that petroleum hydrocarbon is a toxic compound that can inhibit bacterial growth that leads to decreasing phytohormone synthesis by isolates. This is In line with Bakaeva et al. (2020) results that showed crude oil as inhibiting the production of IAA, cytokinin, and abscisic acid. But their results for zeatin production was not reported.

Our study found that Zeatin produced by *Azospirillum s*p. increased significantly in the hydrocarbon contaminated nitrogen-free medium. This interesting phenomenon may be attributed to *Azospirillum* sp.›s protection mechanism, whereby it maintains some enzymatic systems by excreting some amount of exopolysaccharides. Such protection mechanism has been reported by Bahat-Samet, Castro-Sowinski, & Okon (2004).

Meanwhile, the other phytohormones, such as IAA, kinetin, and gibberellin, were inhibited by the existance of hydrocarbons. We hypothetized that the decline in adaptation capability of the isolates were happening due to the still toxic nature of the new intermediate hydrocarbon compounds even after each stage of the hydrocarbon chain breakdown has happened.

Efficacy of Isolates to Petroleum Phytoremediation Performance in a Soil System Using Ramie Plant

The results of the effectiveness of the three isolates in degrading hydrocarbons in a solid phytoremediation system using ramie plants are shown in Table 8.

The results of the investigation revealed that the hydrocarbon efficiency degradation of *Azospirillum* sp. and *Pseudomonas* sp. are around the same (no significant difference) at 83.70% ($p < 0.05$) and 81.78% ($p < 0.05$), respectively. Meanwhile, the ability of *Bacillus* sp. to degrade hydrocarbons was increasing but not significantly different compared control treatment, which was 78.6% ($p < 0.05$) and was considerably lower than *Azospirillum* sp. and *Pseudomonas* sp. application. The three isolates showed an increase in degradation efficiency compared to control, by 11.90%, 5.10%, and 9.33%, respectively, for *Azospirillum* sp, *Bacillus* sp., and *Pseudomonas* sp. The three isolates showed that they can degrade hydrocarbons in the soil system at a hydrocarbon concentration level of 5% TPH by using ramie plant.

The hydrocarbon degradation efficiency of *Azospirillum* sp. was the highest among the isolates. This phenomenon indicates that the nitrogenase

enzyme activity of *Azospirillum* sp. is relatively more capable of supplying sufficient nitrogen requirements during hydrocarbon biodegradation. *Azospirillum* sp. has relatively more capabilities than the other isolates in maintaining their nitrogenase enzyme activity without being inhibited by high hydrocarbon concentrations. This is in line with Beškoski et al. (2011) and Zhao et al. (2011) who reported that sufficient nitrogen availability during hydrocarbon degradation is a crucial factor in a bioremediation soil system. Furthermore, it is found that *Azospirillum* sp. tended to increase plant growth. So, *Azospirillum* sp. has shown to be a good candidate for a dual role in a phytoremediation system: good hydrocarbon degradation capabilities with adequate nitrogen supply and tends to promote the growth of ramie plants. *Pseudomonas* sp. seems to have decreased its nitrogenase activity due to exposure to high hydrocarbon concentrations. Nevertheless, *Pseudomonas* sp. showed better plant growth promoting characteristics with a significantly higher ramie plant growth. This isolate is also a good candidate for the dual function that we're after. This is in line with Backer et al. (2018)in combination with the plant form the holobiont. Plants regulate the composition and activity of their associated bacterial community carefully. These microbes provide a wide range of services and benefits to the plant; in return, the plant provides the microbial community with reduced carbon and other metabolites. Soils are generally a moist environment, rich in reduced carbon which supports extensive soil microbial communities. The rhizomicrobiome is of great importance to agriculture owing to the rich diversity of root exudates and plant cell debris that attract diverse and unique patterns of microbial colonization. Microbes of the rhizomicrobiome play key roles in nutrient acquisition and assimilation, improved soil texture, secreting, and modulating extracellular molecules such as hormones, secondary metabolites, antibiotics, and various signal compounds, all leading to enhancement of plant growth. The microbes and compounds they secrete constitute valuable biostimulants and play pivotal roles in modulating plant stress responses. Research has demonstrated that inoculating plants with plant-growth promoting rhizobacteria (PGPR who reported that PGPR can indeed promotes plant growth; and increases plant tolerance toward hydrocarbon stress (Ponmurugan & Gopi, 2006; Gohil, Raval, Panchal, & Rajput, 2022).

Bacillus sp. on the other hand was not capable of increasing its hydrocarbon degradation efficiency and also fails at improving the growth of the ramie plant compared to control. The PGPR properties of *Bacillus* sp. have not been able to significantly act as a growth promoter for ramie plant. This phenomenon probably occurred because the production of phytohormones from *Bacillus* sp. decreased significantly in the presence of hydrocarbons. This low level of phytohormone production is not sufficient in increasing the growth of ramie plants during the hydrocarbon degradation process. We suggests that the application of *Bacillus* sp. in a phytoremediation sytem would be better suited in a bacterial consortium that combines the strengths and weaknesses of the various isolates. For example other isolates that exhibit a higher level of nitrogen fixation ability in a hydrocarbon environment could be used to provide the consortium with its much needed nutrients so that the phytoremediation system can perform efficiently. The consortium could also benefit from Bacillus sp.'s characteristics (e.g., IAA) in some phytohormone production (wheather a higher level of that hormone production can be beneficial or hinder plant growth is another question that needs to be investigated).

Table 8. Effectivity of isolates to petroleum phytoremediation performance using ramie plant in the soil system

Treatment (2% v/ wt) doses of Isolates	HC Degradation efficiency in soil (%)	Increasing of HC Degradation Efficiency in soil (%)	Ramie plant height during phytoremediation (cm)
Control (without isolate)	74.80 [a]	-	62.08 [a]
Azospirillum sp.	83.70 [b]	11.90 [b]	65.61 [ab]
Bacillus sp.	78.60 a	5.10 [a]	62.51 [a]
Pseudomonas sp.	81.78 b	9.33 [b]	70.90 [b]

Remarks: * Mean of three replicates in soil system phytoremediation condition; HC = Hydrocarbon. Numbers followed by the same letter notation were not significantly different based on Duncan's test at a 5% level of significance ($p < 0.05$) at the age of ramie plants 70 days

We hypothesized that the higher degree of hydrocarbon degradation efficiency of *Azospirillum* sp. and *Pseudomonas* sp. has something to do with the synergistic interaction between the two isolates and the sorghum, resulting in a higher biodegradation performance than *Bacillus* sp. The synergistic interaction factor between microbial-biostimulant and phytoremediator plants plays an essential role in the success of the hydrocarbon degradation process in the phytoremediation system. The synergistic interaction between microbes and plants in the rhizosphere plays an important role in improving the efficacy and performance of a phytoremediation system (Glick, 2003; 2012; Huang, El-Alawi, Penrose, Glick, & Greenberg, 2004; Sinha & Mukherjee, 2008). Root exudates can also stimulate the PGPR growth and facilitates the uptake of nutrient by degrading soil contaminants (Hontzeas, Zoidakis, Glick, & Abu-Omar, 2004).

CONCLUSION

This research has revealed that *Azospirillium* sp. and *Pseudomonas* sp. are two promising bacterial isolates that can function for a dual role as a plant growth promoter and hydrocarbon degrader. They have good hydrocarbon degradation capabilities of 93.4% and 98.7% with adequate phytohormone production and sufficient nitrogen-fixing in a liquid system. They also show hydrocarbon degradation capabilities of 83.70% and 81.78% in a phytoremediation soil system using ramie plant. The increasing nitrogenase enzyme activity of those two isolate significantly improved petroleum hydrocarbon degradation efficiency. Meanwhile, *Bacillus sp.* has the lowest nitrogenase activity and hydrocarbon-degrading capability with a 84.07% hydrocarbon biodegradation in a liquid system and 78.60% in a phytoremediation soil system using ramie plant. We are suggesting that *Bacillus sp.* can be more beneficial in a bacterial consortium where other bacterial isolates can offset its lack of nitrogenase activity. Therefore, *Pseudomonas* sp. and *Azospirillum* sp. are both two promising bioagents for use in a liquid bioremediation sytem and a solid phytoremediation soil system.

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