Physical Sciences, Life Science and Engineering Volume: 2, Number 2, 2025, *Page*: 1-10

Bioremediation of Petrol Engine Oil Contaminated Soils With Pyoverdine From *Pseudomonas Putida*

Sata Kathum Ahmed Ajjam

Department of Chemical Engineering University of Babylon, Hillah, Iraq

DOI:
https://doi.org/10.47134/pslse.v2i2.334
*Correspondence: Sata Kathum Ahmed Ajjam
Email: satajam58@yahoo.com

Received: 20-01-2025
Accepted: 20-02-2025
Published: 21-03-2025

Abstract: *The poisoning of aquatic and subterranean habitats by petroleum and its compounds is one of the most alarming environmental issues. This work proposes the use of Pseudomonas spp. for the degrading treatment of petroleum engine oil. An isolated bacterial strain from soils affected by engine oil, Pseudomonas putida, was able to manufacture pyoverdine with a higher productivity level of 18.8%. With the use of Sepharose 4B activated with epichlorhydrin the pyoverdine was isolated. It eluted as two isoforms in two peaks. The degradation of gasoline engine oil with purified pyoverdine increased over time, reaching its maximum level after nine days. As time increased, the degradation level decreased, suggesting that using microbial products as biological alternatives could be a more economical and effective way to reduce pollutants and conserve natural resources.*

Keywords: *Pyoverdine, Pseudomonas Putida, Engine Oil*

Introduction

Petroleum hydrocarbon-contaminated soil is a major environmental issue that has threatened both ecological security and human health (Chen et al, 2015). Bioremediation techniques are thought to be the most economical and environmentally friendly options for soil remediation when compared to chemical and physical remediation technologies (Wu, 2018). Even if humanity is working to improve the usage of renewable energy, there is still a huge need for petroleum and fuels today. Petroleum hydrocarbon transportation can lead to unintentional spills that seriously harm the environment, particularly marine ecosystems. Hydrocarbons can absorb by soils and organisms or evaporate or move following unintentional spills (Das et al, 2022). Fuels are the petroleum products that are most commonly released into the environment. Fuels are produced using most of the crude oil that is processed, with the largest amount coming from those that are used to move goods

or power automobiles (Bala et al, 2022). Because of their toxicity and detrimental effects on living things, spills of fuels and petroleum derivatives must be cleaned up. Physical, chemical, and biological treatments are some of the remediation strategies used to clean up the environment following hydrocarbon pollution in water. Barriers are one of the most used physical techniques for separating the contaminants in water (Bala et al, 2022). Chemical treatments include the use of emulsifiers, which reduce the interfacial area by forming an oil-water emulsion, and absorption materials, such as activated carbons, which can immobilize and remove hydrocarbons (Salari et al, 2022).

The biological treatments that depend on can consuming the hydrocarbons that were used as a source of energy and carbon and by microorganisms often known as bioremediation (Goveas et al, 2022). Changing them into harmless and less hazardous substances (Rehman et al, 2018). Numerous bacteria, including *Rhodococcus* sp., *Pseudomonas* sp., *Acinetobacter* sp., and *Bacillus* sp., have been shown to be able to break down diesel (Chai et al, 2021((Khalid et al, 2021).

Nearly 40 structurally distinct pyoverdines have been identified as the primary pigments of luminous *Pseudomonas* strains (Akbar et al, 2021). Fluorescent isolates of *Ps. putida*, *P. syringae*, and P. *aeruginosa* produce pyrodine, a significant class of siderophores (Barrientos et al, 2019). Pyoverdines are water-soluble, fluorescent, yellow-green pigments that are mostly produced by *P. fluorescens*, however other species, such as *P. aeruginosa*, also produce them. They are the main and most well-known siderophores and include a variety of chromopeptides with hydroxamate and catechol groups (Dell'Anno, 2022) (Saha et al, 2016). The purpose of this study is to identify and screen for strong biosurfactant-producing *Pseudomonas* species that have the ability to degrade engine oil.

Methodology

Isolation of *Pseudomonas* spp. From Polluted Soils

In order to complete this work, 15 samples of soils contaminated by engine oil were gathered at stations in Baghdad and sealed in sterile bags of plastic until reaching to the laboratory. Distilled water was mixed with one gram of each soil sample, stirred for an hour, and then left to settle for half an hour. Serial dilutions in the range of 10^{-2} to 10^{-8} were made with distilled water. MacConkey agar, *Pseudomonas* agar, and Baird Parker agar plates were inoculated with one loopful of each sample, and subsequently the plates were incubated at 30°C for 24 hours. The developing colonies were subjected to biochemical and physiological tests (Su et al, 2018). The Vitek 2 mechanism also protects these isolates.

Detection of pyoverdine productivity

1. Primary screening

Pyoverdine synthesis by *Pseudomonas* spp. was detected using chrome azurol S (CAS) agar plates. When pyoverdine is present, the medium's color shifts from greenish blue to orange due to its strong affinity for chelated iron. For twenty-four hours, Pseudomonas spp. isolates were grown in minimal media 9 (MM9) Salt Solution Stock and succinic medium that contained four grams of succinic acid, six grams of KH2PO4, four grams of KH2HPO4,

0.2 grams of MgSO47H2O, one gram of (NH4)2SO4, and ddH2O at 30 °C (Schywn, 2009). Following ten minutes of centrifugation, the supernatant was filtered and placed on chrome azurol S (CAS) agar plates. After 24 at 30°C, pyoverdine was identified visually by a change in the medium's color.

2. Secondary screening

The producer isolates were cultivated in a succinic medium and minimal media 9 (MM9) salt solution stock for twenty-four hours at 200 rpm and 30°C. Following growth, the culture medium was centrifuged for five minutes at 6000 rpm. Using the CAS assay method as outlined by (Xiao et al, 2012) (Meyer et al, 2017), the quantity of pyoverdine was ascertained as follows: After combining 5 ml of the filtrate with 1 ml of CAS reagent and 5 ml of Shuttle solution, the mixture was allowed to sit for a short period. The existence of pyoverdine is associated with the transformation of blue to orange. Pyoverdine level % = Ar − As/Ar × 100 was the formula used to determine the amount of pyoverdine after reading the absorbancy at 630 nm. Ar represents an absorption of medium with CAS reagent and shuttle solution, while As represents an absorption of culture supernatant CAS reagent and shuttle solution.

Purification of pyoverdine

After being cultivated for 24 hours at 30°C in a minimal media 9 (MM9) salt solution stock and succinic medium, the selected bacterial isolate was centrifuged for five minutes at 8000 rpm. Purification was carried out in accordance with (Jankiewicz et al, 2009), a crude extract was obtained after the centrifugation process. The supernatant was membrane filtered to produce the cell-free crude pyoverdine solution and put into Sepharose 4B chelating column activated with epichlorhydrin and diiminoacetic acid that chelates Cu2+ ions as a ligand. The column was equilibrated with 10 mM HEPES buffer (pH 7.0), containing 50 mM NaCl. The elution step was with 10 mM acetate buffer (pH 5.5) containing 50 mM NaCl. About 1.5 ml fractions were gathered. After combining and lyophilizing the pyoverdine-containing fractions, they were stored until they were needed.

Efficiency of pyoverdine in removing of engine oil contaminated from contaminated soils

The engine Sterilized plastic bags were used to collect soil tainted with oil from a gas station. 50 ml of purified pyoverdine (100 µg/ml) was mixed with roughly 25 g of polluted soil, then incubated for 24 hours at 30°C. As a control, distilled water was used in place of the pyoverdine with the same conditions. The liquid solution that was produced from the soil after the centrifugation for 15 minutes. Every three days until 21 days, the same amounts of toluene and treated soil sample were combined to measure the amount of residual hydrocarbons in the test and control. Following centrifugation, the amount of dissolved hydrocarbon in toluene was detected at 410 nm according to (Gamalero, 2003). Hydrocarbon degradation efficiency % = (absorbency of control- absorbency of test)/ absorbency of control absorbency of control x 100.

Result and Dicscussion

Isolation and *Pseudomonas* spp. from engine oil contaminated soils

Following the culture of engine oil-contaminated soil samples on MacConkey agar medium, *Pseudomonas* agar, and Baird Parker agar plates medium, several *Pseudomonas* species were discovered, including three (23%) isolates of *P. stutzeri*, four (31%), and six (46%) isolates of *P. putida* (figure 1).

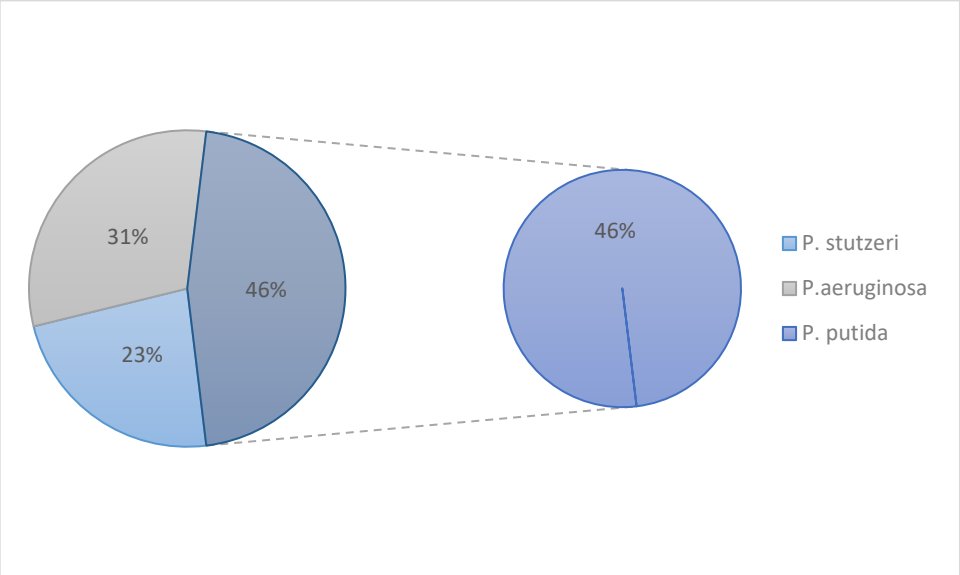

Figure 1. percentages for *Pseudomonas* spp. isolation from engine oil contaminated soils

Pyoverdines are synthesized in the cytoplasm and then released into the extracellular media from the periplasm. Non-ribosomal peptide synthetases are among the many distinct enzymes that are involved (Dell'Anno et al, 2022). CAS agar plates are useful for detecting siderophores in Gram-negative bacteria, but large dosages of HDTMA are detrimental to Gram-positive bacteria and fungi (Louden et all, 2011). In spite of their structure, siderophores can be identified using the universal colorimetric method known as the chrome azurol S assay. In this assay, iron is scavenged by siderophores from a Fe-CAS-hexadecyl trimethyl ammonium bromide complex, and the color changes from blue to orange as the CAS dye is released (Barrientos et al, 2019).

Detection of pyoverdine productivity

Primary screening

By measuring the orange zone's diameter, which varied between 2.1 and 3.7 mm, the synthesis of pyoverdine was evaluated in thirteen isolates of Pseudomonas species. *Pseudomonas aeruginosa* produced the lowest quantities of pyoverdine, while *Pseudomonas putida* produced the highest levels (figure 2).

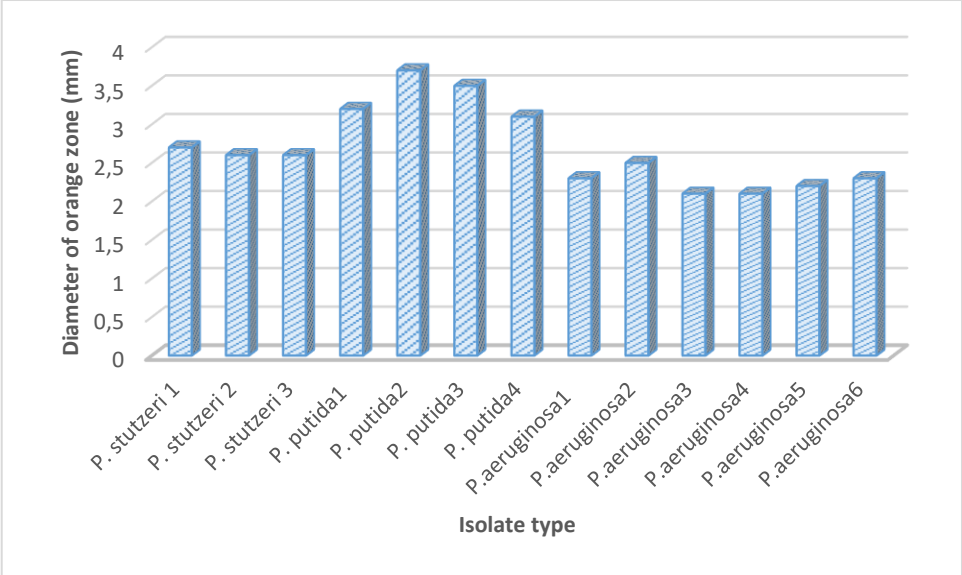

Figure 2. Detection of pyoverdine productivity depending on the orange zone's diameter

Secondary screening

Secondary screening was performed on *Pseudomonas putida* isolates that demonstrated greater levels of pyoverdine productivity, which ranged from 12.6 to 18.8% (figure 3).

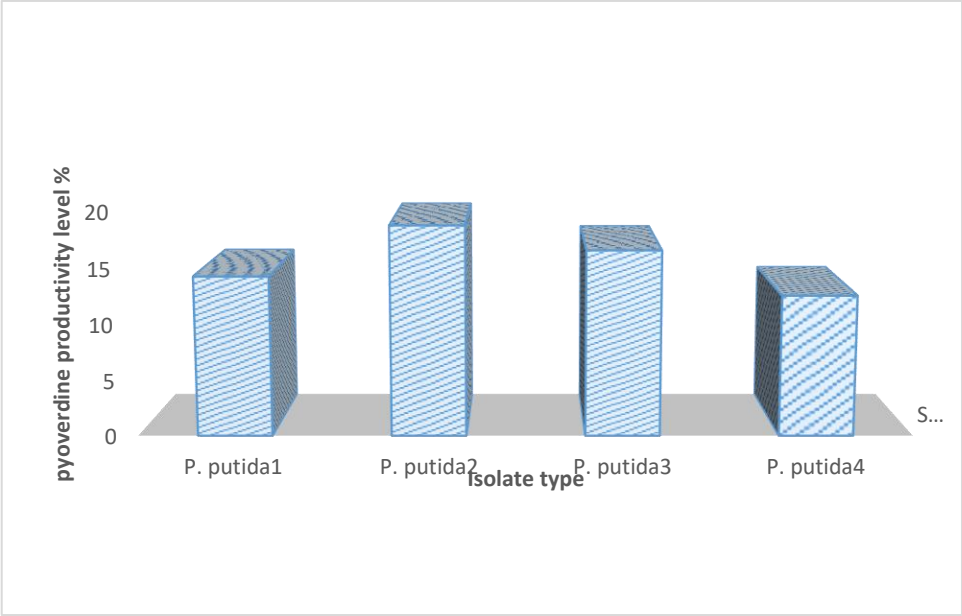

Figure 3. Detection of pyoverdine productivity depending on the pyoverdine level

Under iron-deficient growth conditions, *P. putida* and *P. aeruginosa* create pyrodine, a luminous siderophore with a strong affinity for iron. (Meyer et al, 2017). It was shown that soil-isolated Pyoverdine, which was isolated and filtered from every stock and organism, was produced by Pseudomonas species (Santos et al, 2018).

Purification of pyoverdine

Pyoverdine productivity was induced by *P. putida*2 growing in succinic medium and minimal media 9 (MM9). Pyoverdine was purified by Sepharose 4B chelating column activated with epichlorhydrin and diiminoacetic acid that chelates Cu2+ ions as a ligand. The pyoverdine was then rinsed by running it through an acetate buffer that contained NaCl. As shown in figure (Bala, 2022), two peaks emerged following the elution step and the 400 nm reading. Two isoforms of pyoverdine were detected, with pyoverdine productivity levels for the collected fractions in each peak being 29% and 32%, respectively. 11.45 mg of pyoverdine was obtained by combining and lyophilizing the fractions.

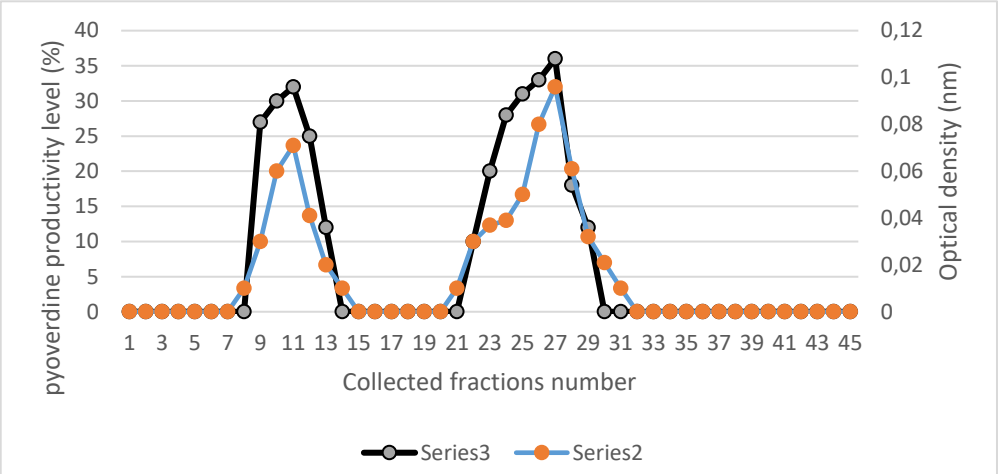

Figure 4. Pyoverdine purification from *P. putida*2 by activating Sepharose 4B

Pseudomonas fluorescens 2-79 was used to isolate three different forms of pyoverdines using copper-chelate Sepharose and Sephadex G-15 columns (Xiao et al, 2012). A strain of *P. fluorescens* was utilized to purify pyoverdine using chelating chromatography, yielding three distinct isoforms of the drug (Xiao et al, 1995). Additionally, (Gamalero, 2003) purified siderophores produced by two distinct *P. fluorescens* strains using chelating chromatography. *P. fluorescens* produced a single peak with at 400 nm, but other isolate of *P. fluorescens* produced two peaks, suggesting the presence of two pyoverdine isoforms with different affinities for the copper ion-saturated chelating substrate. Acetate buffer mixed with NaCl was used in both instances to elute siderophores from the column.

Efficiency of pyoverdine in removing of engine oil contaminated from contaminated soils

The findings showed that after 24 hours of incubation the contaminated soil with pyoverdine, the efficiency of engine oil degradation in the contaminated soils increased with longer incubation times, reaching a maximum level of 67% after 9 days. Following this, the efficiency of degradation decreased with longer incubation times, as shown in figure (Salari et al, 2022). Because of this, an alternative method for cleaning up places affected by oil is bioremediation. Microbial colonies and their metabolites are essential to this process

because they can either directly destroy pollutants or interact with other microorganisms that have been added.

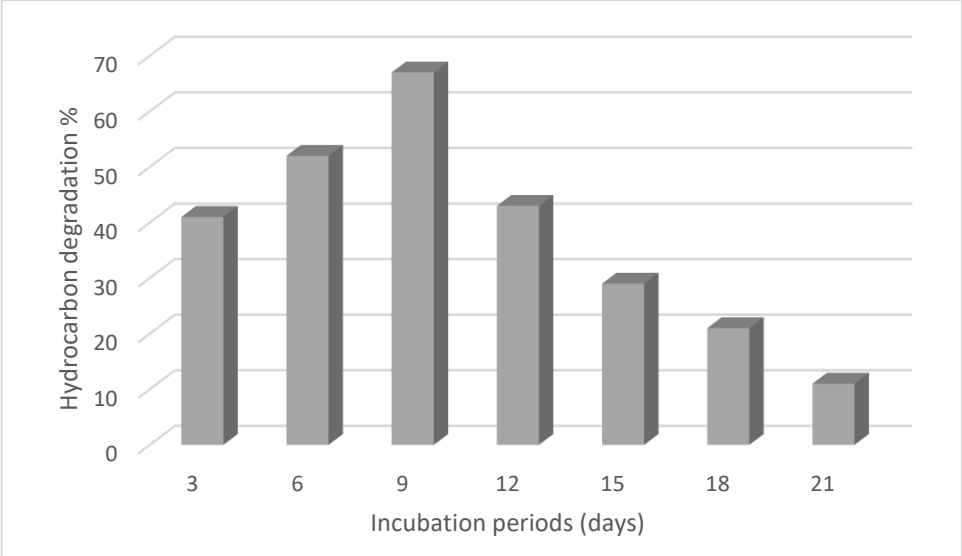

Figure 5. Detection an efficiency of the efficiency of engine oil degradation with pyoverdine at different incubation periods

In order to facilitate the bioremediation process, the bacteria generate biosurfactants that make diesel more soluble in water (Khalid et al, 2021). A bright yellow pigment was created in the presence of biodiesel; this was separated, refined, and identified as a pyoverdine by the use of UV-vis and fluorescence spectroscopy. These findings imply that *Marinomonas* sp. ef1 can be employed in the conversion of these contaminants into compounds of interest and in hydrocarbon bioremediation (Zannotti et al, 2023).

Conclusion

According to this study, *P. putida* may use the hydrocarbons found in engine oil as a carbon source, enabling them to proliferate and develop under these harsh conditions. *P. putida* revealed higher levels in pyoverdine productivity compared with other *Pseudomonas* species. The pyoverdine was used successfully for engine oil degradation with increasing the exposure period to pyoverdine with maximum removal activity after 9 days then the activity of pyoverdine decreased with increasing the time of exposure.

References

Akbar, S. and Stevens, D.C. (2021). Functional genomics study of *Pseudomonas putida* to determine traits associated with avoidance of a myxobacterial predator. Sci Rep., 11:16445.

Bala, S., Garg, D., Thirumalesh, B. V., Sharma, M., Sridhar, K., Inbaraj, B. S. and Tripathi, M. (2022). Recent Strategies for Bioremediation of Emerging Pollutants: A Review for a Green and Sustainable Environment, Toxics, 10:484-489.

Barrientos-Moreno, L., Molina-Henares, M.A., Pastor-García, M., Ramos-González, M.I. and Espinosa-Urgel, M. (2019). Arginine Biosynthesis Modulates Pyoverdine Production and Release in *Pseudomonas putida* as Part of the Mechanism of Adaptation to Oxidative Stress. J. Bacteriol., 201:10.

Cai , Y. Y., Wang, R. K., Rao, P. H., Wu, B. C., Yan, L. L., Hu, L. J., Park, S., Ryu, M. and Zhou, X. Y. (2021). Bioremediation of Petroleum Hydrocarbons Using *Acinetobacter* sp. SCYY-5 Isolated from Contaminated Oil Sludge: Strategy and Effectiveness Study. Int. J. Environ. Res. Public Health, 18:819.

Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D. and Zhang, J. (2015). Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: applications, microbes and future research needs. Biotechnol. Adv.,33 (6 Pt 1):745-55.

Das, D. N. and Ravi, N. (2022). Influences of polycyclic aromatic hydrocarbon on the epigenome toxicity and its applicability in human health risk assessment, Environ. Res., 3: 12-17.

Dell'Anno, F.; Vitale, G.A.; Buonocore, C.; Vitale, L.; Palma Esposito, F.; Coppola, D.; Della Sala, G.; Tedesco, P.; de Pascale, D. (202). Novel Insights on Pyoverdine: From Biosynthesis to Biotechnological Application. *Int. J. Mol. Sci.*, *23*:11507.

Gamalero, E., Fracchia, L., Cavaletto, M., Garbaye, J., Frey-Klett, P., Varese, G.C. and Martinotti, M.G. (2003). Characterization of functional traits of two fluorescent Pseudomonads isolated from basidiomes of ectomycorrhizal fungi. Soil Biolog. Biochem. 35:55-65.

Gamalero, E., Fracchia, L., Cavaletto, M., Garbaye, J., Frey-Klett, P., Varese, G.C. and Martinotti, M.G. (2003). Characterization of functional traits of two fluorescent Pseudomonads isolated from basidiomes of ectomycorrhizal fungi. Soil Biolog. Biochem. 35:55-65.

Goveas, L. C., Nayak, S. and Selvaraj, R.(2022). Concise review on bacterial degradation of petroleum hydrocarbons: Emphasis on Indian marine environment, Bioresour. Technol. Rep., 19:101136.

Goveas, L. C., Nayak, S. and Selvaraj, R.(2022). Concise review on bacterial degradation of petroleum hydrocarbons: Emphasis on Indian marine environment, Bioresour. Technol. Rep., 19:101136.

Himps, S.D., Mobley, H.L.T. (2019). Siderophore Detection Using Chrome Azurol S and Cross-Feeding Assays. Methods Mol Biol.;2021: 97-108.

Jankiewicz, U. and Kuzawińska, O. (2009). Purification and Partial Characterization of Pyoverdine synthesized By *Pseudomonas putida*, EJPAU, 12(1):11-17.

Khalid, F. E., Lim, Z. S., Sabri, S., Gomez-Fuentes, C., Zulkharnain, A. and Ahmad, S. A. (2021). Bioremediation of Diesel Contaminated Marine Water by Bacteria: A Review and Bibliometric Analysis, J. Mar. Sci. Eng., 9:155.

Khalid, F. E., Lim, Z. S., Sabri, S. Gomez-Fuentes, C., Zulkharnain, A. and Ahmad, S. A. (2021). Bioremediation of Diesel Contaminated Marine Water by Bacteria: A Review and Bibliometric Analysis, J. Mar. Sci. Eng., 9:155.

Louden, B.C.; Haarmann, D. and Lynne, A.M. (2011). Use of blue agar CAS Assay for siderophore Detection. Journal of Microbiol. & Biology Edu., 3:51-53.

Meyer, J.M. and Abdallah, M.A. (2007). The fluorescent pigment of *Pseudomonas fluorescens*: Biosynthesis, purification and physicochemical properties. J. Gen. Microbiol., 107: 319–32.

Rehman, K., Imran, A., Amin, I. and Afzal, M. (2018). Inoculation with bacteria in floating treatment wetlands positively modulates the phytoremediation of oil field wastewater, J. Hazard. Mater., 349:242 -251.

Salari, M., Rahmanian, V., Hashemi, S. A., Chiang, W. H., Lai, C. W., Mousavi, S. M. and Gholami, A.(2022). Bioremediation Treatment of Polyaromatic Hydrocarbons for Environmental Sustainability. Water; 14(23):3980.

Salari, M., Rahmanian, V., Hashemi, S. A., Chiang, W. H., Lai, C. W., Mousavi, S. M. and Gholami, A.(2022). Bioremediation Treatment of Polyaromatic Hydrocarbons for Environmental Sustainability. Water; 14(23):3980.

Saha, M., Sarkar, S., Sarkar, B., Sharma, B. K., Bhattacharjee, S. and Tribedi, P. (2016). Microbial siderophores and their potential applications: a review, Environ. Sci. Pollut. Res., 23: 3984 -3999.

Santos, J.V.O.; Ferreira, Y.L.A.; Silva, L.L.S.; de Lyra, I.M.; Palácio, S.B.; Cavalcanti, I.M.F. (2018). Use of bioremediation for the removal of petroleum hydrocarbons from the soil: an overview. International Journal of Environment, Agriculture and Biotechnology (IJEAB), 3(5):201-209.

Su, S.S.; Lae, K.Z. and Ngwe, H. (2018). Isolation and Identification of *Pseudomonas aeruginosa* from the Clinical Soil. University of Yangon Research J., 8:271-275.

Schywn, B.; Neilands, J.B. (2009).Universal chemical assay for the detection and determination of siderophores. Anal. Biochem., 160:47–56.

Xiao, G.R. and Kisaalita, W.S. (2012). Purification of Pyoverdines of *Pseudomonas fluorescens* 2-79 by Copper-Chelate Chromatography. Applied and Environmental Microbiology, 61(11):33-38.

Xiao, R. and Kisaalita, W.S. (1995). Purification of pyoverdines of *Pseudomonas fluorescens* 2-79 by Copper-Chelate Chromatography. Appl. Environ. Microbiol. 61:3769-3774.

Xie, W., Yao, Z., Yang, H., Sun, C. and Li, X. (2018). *Pseudomonas aeruginosa* L10: A Hydrocarbon-Degrading, Biosurfactant-Producing, and Plant-Growth-Promoting

Endophytic Bacterium Isolated From a Reed (*Phragmites australis*). Front Microbiol., 25(9):1087.

Zannotti, M., Ramasamy, K.P., Loggi, V., Vassallo, A., Pucciarelli, S. and Giovannetti, R. (2023). Hydrocarbon degradation strategy and pyoverdine production using the salt tolerant Antarctic bacterium *Marinomonas* sp. ef1. RSC Adv.;13(28):19276-19285.