BIODIVERSITAS
Volume 23, Number 8, August 2022
Pages: 4138-4143

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230835

Short Communication: Determination of crude oil degradation efficiency of biofilm producing bacteria isolated from oil contaminated site

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Manuscript received: 2 June 2022. Revision accepted: 27 July 2022.

Abstract. *Khoo DH, Shivanand P, Taha H. 2022. Short Communication: Determination of crude oil degradation efficiency of biofilm producing bacteria isolated from oil contaminated site. Biodiversitas 23: 4138-4143.* Microbes have gained much attention for their application in the bioremediation of petroleum contaminated areas, and biofilm producing bacteria are considered potential candidates for this purpose. The aim of this study was to screen six oil degrading bacterial strains for their ability to form biofilm, and to measure the crude oil degradation efficiency of selected strains. It was found that only two strains, *Micrococcus* sp. UBDBH15 and *Pseudomonas* sp. UBDBH26 were categorized as strong biofilm producers, with the latter showing the highest amount of biofilm formed. Under the conditions tested, *Pseudomonas* sp. UBDBH26 was also found to have the highest degradation rate (0.0047 g/day) with a significant degradation of 7.07 ± 3.02 and 11.38 ± 2.93 % of crude oil after 7 and 14 days respectively. *Micrococcus* sp. UBDBH15 had a lower rate of degradation (0.0033 g/day), resulting in a non-significant degradation. However, in comparison with a non-biofilm producer, *Enterobacter* sp. UBDBH06, this study suggested that biofilm might enhance the degradation of oil, but further studies are needed to confirm this assumption.

Keywords: Bacteria, biodegradation, biofilm, bioremediation, petroleum

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of petroleum chemicals comprising approximately 10,000 toxic and ubiquitous environmental pollutants (Ukiwe et al. 2013). These chemicals can be exposed through direct contact, vapors, or secondary contamination of water supplies (Thapa et al. 2012). PAHs are very dangerous for all living things, one of them for humans (Mohanty and Das 2018). Cellular functions can be affected by the cytotoxicity, carcinogenicity and mutagenicity of the chemicals, leading to various human diseases (Das et al. 2014; Hamoudi-Belarbi et al. 2018). Thus, great attention has been given to microbial bioremediation of crude oil from contaminated areas (Meliani and Bensoltane 2014).

Bacteria are known to be the primary and most active hydrocarbon degraders (Lima et al. 2019). Many bacteria can utilize hydrocarbons as a carbon source by utilizing enzymes such as oxygenases and peroxidases to degrade the chemical compounds (Bisht 2015). The harmful hydrocarbons can be transformed into harmless products such as carbon dioxide and water (Das and Chandran 2011). Most bacteria exist as sessile communities in the form of biofilm instead of in planktonic form (Davies et al. 1998). The biofilm can consist of mono- or multi-species community (Edwards and Kjellerup 2013), resulting in a variety of complex relationships involving inter- and/or intra-species interaction (Simões et al. 2009). The bacteria are typically found enclosed in self-secreted slimy and glue-like substances called extracellular polymeric substances which aid the anchorage of the microbial cells on surfaces (Costerton et al. 1999; Wimpenny et al. 2000; Singh et al. 2006). Biofilm can form on various surfaces, including living tissues, medical devices, water systems and aquatic ecosystems (Prakash et al. 2003).

Biofilm is known to have many properties which enable the microbes to adapt better to the environment when compared to their planktonic forms (Simões et al. 2009; Sharma et al. 2019). This is through interaction and communication within the community, gene transfer, nutrient availability, and protection from environmental stressors such as temperature, UV radiation, desiccation and toxic contaminants (Costerton et al. 1999; Edwards and Kjellerup 2013). Thus, biofilm producing bacteria are suitable candidates to be used as a tool in the bioremediation of PAHs. In this study, six petroleum degrading bacterial strains that were previously isolated from oil-contaminated sites (Taha et al. 2020) were screened for their ability to form biofilm. The strong biofilm producers were then selected for the determination of their crude oil degradation efficiency.

MATERIALS AND METHODS

Petroleum degrading bacteria

Six strains of culturable petroleum degrading bacteria were used in this study. These strains were previously isolated and identified through DNA barcoding by our

laboratory from petroleum-contaminated sites in Brunei Darussalam, and were shown to be able in utilizing petroleum as their carbon source (Taha et al. 2020). These six strains were *Bacillus* sp. UBDBH12, *Enterobacter* sp. UBDBH06, *Micrococcus* sp. UBDBH15, *Pseudoalteromonas* sp. UBDBH01, *Pseudomonas* sp. UBDBH26 and *Pseudomonas* sp. UBDBH29.

Prior to this study, we re-confirmed that the six strains had the ability to degrade petroleum. This was carried out by streaking a single bacterial colony using an inoculating loop onto three different growth media: nutrient agar (NA) supplemented with 1% petroleum, Bushnell Haas agar (BHA), and BHA supplemented with 1% petroleum. The petroleum used was crude oil C0280 (PASCO Scientific, USA). The agar plates were incubated at 37°C for 5 days. The six isolates were observed to grow well on the NA, and BHA plates supplemented with petroleum but not on the BHA plate without petroleum supplementation, confirming these bacterial strains could utilize petroleum.

Quantification of biofilm formation

Biofilm production was determined quantitatively for all six bacterial strains by using microtiter plate assay (O'Toole 2011). Each strain was cultured for 24-48 hours in nutrient broth (NB). The culture was diluted with phosphate-buffered saline (PBS) to obtain an absorbance of 0.5 with a spectrophotometer (Thermo, USA) at 600 nm. The standardized culture (100 µL) was then transferred into a 96-well microtiter plate in triplicate, and incubated at 37°C for 24 hours. The plate was rinsed by immersion into a tub of distilled water for three times to remove any free-floating planktonic bacteria. After staining with crystal violet dye for 10-15 minutes, the plate was rinsed three times again with distilled water, and dried overnight. The dye in the well was solubilized with 30% acetic acid, and incubated at room temperature for 10-15 minutes. The absorbance or optical density (OD) was measured with a microplate reader (BioTek Instruments, USA) at 550 nm. For negative control, uninoculated well containing 30% acetic acid was used.

Following Kirmusaoglu (2019), the OD cut-off (ODc) was determined as follows: average OD of the negative control + (3 x standard deviation of negative control), and the bacterial strains were categorized into the following: non-biofilm producer ($OD \leq ODc$), weak biofilm producer ($ODc < OD \leq 2 \times ODc$), moderate biofilm producer ($2 \times ODc < OD \leq 4 \times ODc$) and strong biofilm producer ($4 \times ODc < OD$).

Microscopic observation of biofilm formation

A number of small glass slides were immersed into a Falcon tube filled with 15 mL of Bushnell Haas (BH) medium and supplemented with 1% petroleum (crude oil C0280; PASCO Scientific, USA) as the sole source of carbon. Each selected bacterial strain was inoculated into the Falcon tube, and incubated at 37°C for 48 hours. However, at different time intervals, some of the glass slides were collected, washed thoroughly with 1% saline solution to remove any free planktonic cells, and Gram stained. Any biofilm formation on the glass slides was observed under 100x oil immersion OLYMPUS microscope.

Microbial petroleum degradation

To determine the efficiency of petroleum degradation, each selected bacterial strain was cultured in NB for 24 - 48 hours. The culture was first standardized to an absorbance of 0.5 and 1 mL was then transferred into three 250 mL Erlenmeyer flasks (representing Day 0, Day 7, and Day 14) containing 100 mL BH medium and 1 % petroleum (crude oil C0280; PASCO Scientific, USA). The flasks labeled Day 7 and Day 14 were incubated at 37°C in an incubator shaker (Jeio Tech, Korea) at 150 rpm for 7 and 14 days, respectively, whereas the flask labeled Day 0 was not incubated. For control, three flasks (representing Day 0, Day 7, and Day 14) were similarly prepared and tested but without the inoculation of any bacterial strain. The experiment was conducted in 5-7 replicates.

Gravimetric analysis was used to measure the amount of petroleum degraded, following Latha and Kalaivani (2012). A volume of n-hexane (5 mL) was added into the flasks, and the mixture was transferred to a separating funnel, where it was further mixed and allowed to settle into two layers. The n-hexane layer containing crude oil was collected, and the remaining aqueous layer was similarly extracted again with n-hexane to ensure complete oil recovery. The collected extract was treated with 0.4 g of anhydrous sodium sulfate to absorb any moisture, and then decanted into a new beaker leaving behind the anhydrous sodium sulfate. The n-hexane solvent was evaporated in an oven (Astell Scientific, England) at 60°C overnight, leaving behind crude oil to be weighed.

The percentage of degraded crude oil was calculated as follows: [(average weight of crude oil at Day 0 - average weight of crude oil at Day 7 or 14)/ average weight of crude oil at Day 0] x 100%. To measure the rate of oil degradation, a plot of weight of crude oil against time was first plotted, and then, the gradient of its regression line was determined.

Statistical analysis

Data are presented as mean value ± standard deviation. Shapiro-Wilk test and Levene's test showed the data were normally distributed and with equal variances. One-way ANOVA was conducted to test any significant difference among the mean values with the significance level set at $p < 0.05$. Tukey HSD post hoc test was used to determine which mean values differed.

RESULTS AND DISCUSSION

This study initially screened six strains of culturable petroleum degrading bacteria for their ability to form biofilm. A microtiter plate assay revealed that among the six strains, *Pseudomonas* sp. UBDBH26 showed the highest OD of 0.868 ± 0.025, followed by *Micrococcus* sp. UBDBH15 with an OD of 0.511 ± 0.024, while the other four strains showed much lower values of less than 0.2. This means that *Pseudomonas* sp. UBDBH26 produced the

highest amount of biofilm compared to the other strains. Following Kirmusaoglu (2019), two strains (*Pseudomonas* sp. UBDBH26 and *Micrococcus* sp. UBDBH15) were classified as strong biofilm producers, one strain (*Pseudoalteromonas* sp. UBDBH01) as weak biofilm producer and the other three strains (*Pseudomonas* sp. UBDBH29, *Bacillus* sp. UBDBH12 and *Enterobacter* sp. UBDBH06) as non-biofilm producers (Table 1).

Based on the screening, we selected the two strong biofilm producers, *Pseudomonas* sp. UBDBH26 and *Micrococcus* sp. UBDBH15 for further analyses. In addition, we also selected one non-biofilm producer, *Enterobacter* sp. UBDBH06 for comparison.

Biofilm formation was further validated by observing the three selected strains under a microscope after 6, 18, 24 and 48 hours of incubation in BH medium supplemented with 1 % petroleum. According to Khoo et al. (2021), the process of biofilm formation follows a sequence of events: attachment of cells to a surface, formation of colony, growth and maturation, and finally cell detachment from the surface. In Figure 1, initial cell adhesion could be observed for all three strains after 6 hours. After 18 hours, new cell clusters could be observed on the glass slides from the biofilm producing strains, *Micrococcus* sp. UBDBH15 and *Pseudomonas* sp. UBDBH26. This seemed to be followed by bacterial growth and maturation after 24 hours for *Pseudomonas* sp. UBDBH26 and 48 hours for *Micrococcus* sp. UBDBH15. This suggested that the growth and maturation for *Pseudomonas* sp. UBDBH26 was faster than *Micrococcus* sp. UBDBH15, causing more biofilm formation by the former than the latter. Less cells were observed for *Pseudomonas* sp. UBDBH26 after 48 hours, which suggested that the detachment of cells from the glass slide had occurred after 24 hours. In comparison, *Enterobacter* sp. UBDBH06 only showed tiny cell clusters after 48 hours, indicating no substantial amount of biofilm was formed.

Table 1. Quantification of biofilm formation

Species	Optical density (OD)	Biofilm classification
Pseudoalteromonas sp. UBDBH01	0.181 ± 0.027	Weak producer
Enterobacter sp. UBDBH06	0.060 ± 0.006	Non producer
Bacillus sp. UBDBH12	0.095 ± 0.014	Non producer
Micrococcus sp. UBDBH15	0.511 ± 0.024	Strong producer
Pseudomonas sp. UBDBH26	0.868 ± 0.025	Strong producer
Pseudomonas sp. UBDBH29	0.114 ± 0.008	Non producer

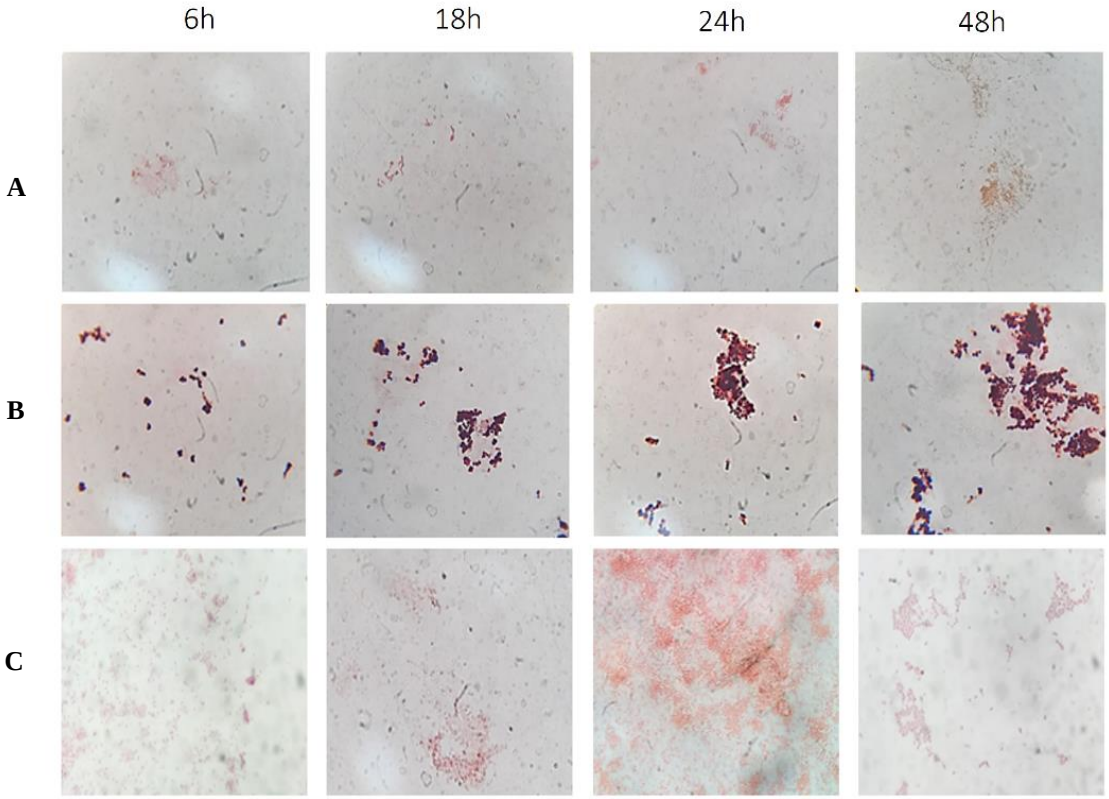

Figure 1. Microscopic observation of biofilm formation. Three bacterial strains, *Enterobacter* sp. UBDBH06 (A), *Micrococcus* sp. UBDBH15 (B) and *Pseudomonas* sp. UBDBH26 (C) were observed after 6, 18, 24 and 48 hours of incubation

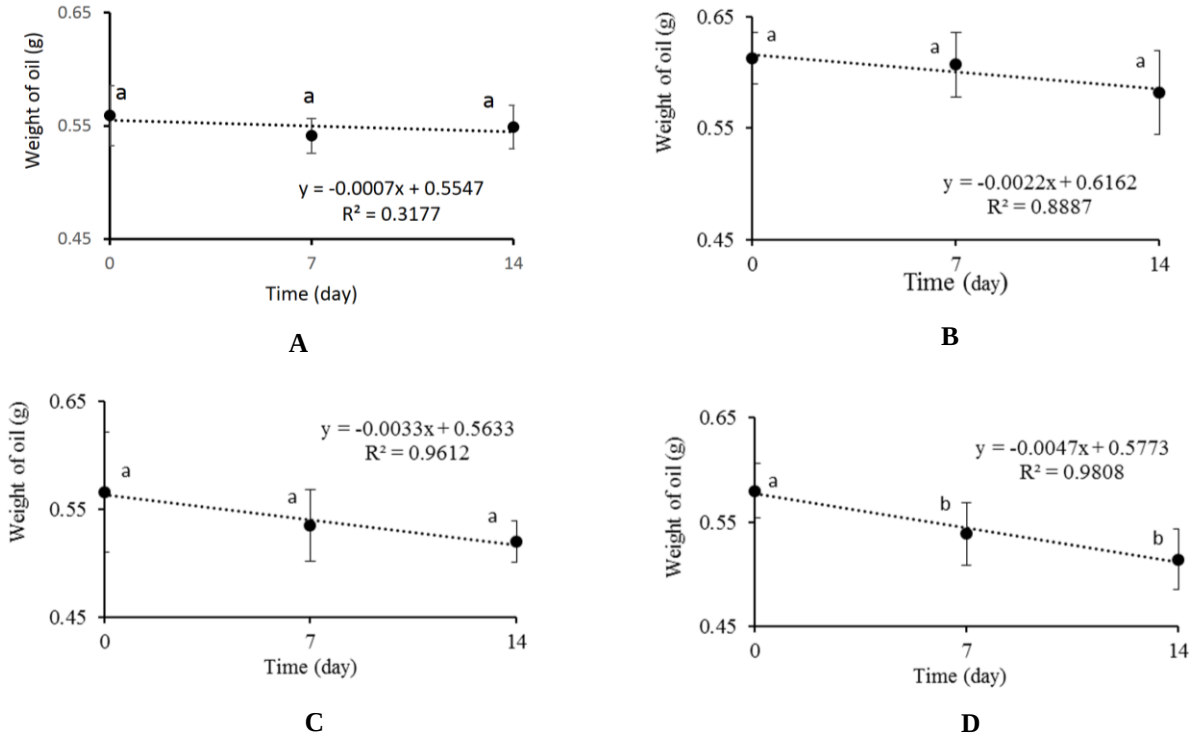

Figure 2. Biodegradation of petroleum by control (A), *Enterobacter* sp. UBDBH06 (B), *Micrococcus* sp. UBDBH15 (C) and *Pseudomonas* sp. UBDBH26 (D). Equation and R^2 value of the regression line are shown. For mean values with the same letter, the difference is not statistically significant, whereas with different letters, they are significantly different.

Table 2. Microbial efficiency of petroleum degradation

Species	Amount of oil degraded (%)		Rate of oil degradation (g/day)
	Day 7	Day 14	
Enterobacter sp. UBDBH06	2.12 ± 2.87	5.06 ± 3.87	0.0022
Micrococcus sp. UBDBH15	5.43 ± 3.27	8.13 ± 1.87	0.0033
Pseudomonas sp. UBDBH26	7.07 ± 3.02	11.38 ± 2.93	0.0047

The three selected petroleum degrading strains were analyzed for their efficiency in degrading petroleum. Figure 2 shows the amount of crude oil present in the growth medium, which was measured before and after 7 and 14 days of incubation. There was a decrease in the average amount of crude oil present in the growth medium with time. However, statistically, only *Pseudomonas* sp. UBDBH26 showed a significant decrease on Day 7 ($p < 0.05$) and Day 14 ($p < 0.01$) when compared to Day 0, while for the other two strains and control (no microbial inoculation), the decrease was not considered as statistically significant.

Table 2 summarizes the efficiency of the three strains in degrading crude oil. *Pseudomonas* sp. UBDBH26 showed the highest rate of degradation with a statistically significant degradation of 7.07 ± 3.02 and 11.38 ± 2.93 % of crude oil after 7 and 14 days of incubation, respectively. *Micrococcus* sp. UBDBH15 and *Enterobacter* sp. UBDBH06 had lower and lowest rates of degradation, respectively, with the amounts of crude oil degraded were not statistically significant under the stated conditions.

Several studies have reported that *Pseudomonas* species is a dominant species when isolated from hydrocarbon contaminated areas, and it has the ability to utilize and degrade hydrocarbons (Subathra et al. 2013; Ra et al. 2019). A study on *Pseudomonas* isolates showed that the capacity to degrade hydrocarbon by twenty isolates ranged from 6.2 to 88.1 % in 120 hours (Rado et al. 2017). In this study, the percentages of crude oil degradation by *Pseudomonas* sp. UBDBH26 are within the stated range, although lower compared to some of the isolates. In this study, no significant difference on crude oil degradation by *Pseudomonas* sp. UBDBH26 was observed between Day 7 and Day 14. This could be due to the depletion of nutrients from the growth medium, and hence, slowing down the bacterial growth and degradation of crude oil by Day 14. For further degradation, adding appropriate nutrients and microbes might be helpful.

It has also been reported that *Micrococcus* sp. is an efficient crude oil degrader, and thus, a potential species for bioremediation (Kawo and Bacha 2016). A previous study showed that *Micrococcus* sp. could degrade hydrocarbons by 27 % at optimal conditions (Kumar et al. 2013). However, this present study did not observe any significant oil degradation by *Micrococcus* sp. UBDBH15 at the specified conditions. However, oil degradation would likely be affected by the conditions tested, and hence, efforts should be made to determine the optimal conditions for biodegradation.

Comparison between the strains showed a statistically significant difference in the oil degradation between *Micrococcus* sp. UBDBH15 and *Enterobacter* sp. UBDBH06 for Day 7 ($p < 0.01$) and Day 14 ($p < 0.05$), as well as between *Pseudomonas* sp. UBDBH26 and *Enterobacter* sp. UBDBH06 for Day 7 ($p < 0.01$) and Day 14 ($p < 0.01$). This suggested that the biofilm producers were relatively better than the non-biofilm producer in degrading crude oil, although further studies are necessary to confirm this. Many bacterial strains are capable of oil degradation as the hydrocarbons can be utilized as a carbon source (Bisht 2015). However, in their planktonic form, the ability to degrade oil can be affected by the varieties of environmental stresses and harmful chemicals (Sharma et al. 2019). The presence of biofilm can protect bacteria by providing stability and resilience from any harmful chemicals, allowing the bacteria to continue their activities under stressful conditions (Koo et al. 2017). As the biofilm producing bacteria are able to continue their activities, this would not hinder the biodegradation of crude oil. According to Xu et al. (2018), several factors can limit biodegradation efficiency, which include the bioavailability of hydrocarbons to bacteria, and the high concentration of hydrocarbons that can inhibit bacterial growth, leading to low efficiency. Biofilm could provide protection to the microbes. In addition, bacteria living within a biofilm are known to differ from the planktonic ones in different ways such as better growth rate and gene expression since the biofilm provides a different microenvironment and allows cell to cell communication (Sharma et al. 2019). Thus, biofilm producing bacteria are potential candidates for bioremediation.

When comparing different bacterial species, apart from the ability to form biofilm, it is also important to note that the hydrocarbon degradation pathways may differ due to the fact that different bacteria may produce different enzymes. For example, some oil degrading bacteria have the ability to metabolize specific alkanes, while other bacterial species can break down aromatic or resin parts of hydrocarbons (Xu et al. 2018). It is rare to find bacteria that have the ability to degrade both aliphatic and aromatic hydrocarbons effectively (Olowomofe et al. 2019). Thus, this could also explain the difference in the degradation rates observed in the three strains.

In conclusion, this study found that different bacterial strains had different ability in forming biofilm. Two strains, *Pseudomonas* sp. UBDBH26 and *Micrococcus* sp. UBDBH15 was shown to produce higher levels of biofilm. The strongest biofilm producer, *Pseudomonas* sp. UBDBH26 was shown to significantly degrade crude oil, and thus, it has the potential to be considered for bioremediation. However, optimal conditions for biodegradation should be further investigated. In addition, this study also suggested that a biofilm producer might be a better candidate for petroleum degradation compared to a non-biofilm producer, and therefore, further studies should be conducted.

ACKNOWLEDGEMENTS

This research was supported by Universiti Brunei Darussalam (grant no. UBD/OAVCRI/CRGWG(010)/170501).

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