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Biodegradation of Diesel by *Acinetobacter lwoffii* and *Vibrio alginolyticus* Isolated from Ship Dismantling Facility in Tanjungjati Coast, Madura, Indonesia

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Abstract

Presence of diesel in environment will cause serious effects to ecosystembecause the components of diesel are potentially carcinogenic and toxic for environment. Diesel contaminated seawater and soil samples were collected from ship dismantling facility in Tanjungjati coast, Madura, Indonesia.Isolation of diesel degrading bacteria was performed using serial dilution method. Preliminary and secondary screening of diesel degrading bacteria were carried out by using nutrient agar medium and Mineral Salt Medium (MSM) containing diesel (0; 5; 10; and 15% (v/v))as a carbon source.The diesel degradation was conducted by inoculating bacteria that potentially degrade diesel into MSM with optimum concentration and diesel degradation percentage was detected by GC-MS analysis.In this study, thirteen bacteria strains were isolated which had the potential to degrade diesel. Based on preliminary and secondary screening, the optimum concentration was found to be 10% (v/v) of diesel and bacteria coded AT and EL have highest growth with 8.01 and 8.13 log CFU/mL for 7 days incubation. Bacterial code of AT and EL were identified as *Acinetobacter lwoffii* and *Vibrio alginolyticus*. The result showed that *V. alginolyticus* and *A. lwoffii* were able to degrade diesel about 26.78% and 1.85% for 14 days of incubation at initial diesel of 10% (v/v). In conclusion, *V. alginolyticus* may has a potential as an degrader for the bioremediation of marine environments polluted by dieselwith further investigation through biostimulation and bioaugmentation technique.

Keywords: *Acinetobacter lwoffii*, Biodegradation, Diesel, Ship dismantling facility, *Vibrio alginolyticus*

INTRODUCTION

Pollution due to petroleum hydrocarbons, like diesel fuel, has caused ecosystem damage in terrestrial and aquatic habitat [11]. It has been recognized as one of the most hazardous wastes [10]. Ship dismantling activity in Indonesia, located in Tanjungjati coast Madura, is one source of diesel pollution in coastal area. It has caused seawater and soil contamination indicated by the changing of seawater and soil color to become muddy and black. It has spilled diesel about 1500 tons/year to seawater.

Diesel, as fuel engine, is a product from crued oil destillation with temperature between 200°C and 350°C at atmospheric pressure [16]. It contains rich in light weight hydrocarbons with the range of C_8-C_{26} and polyaromatic hydrocarbons (PAHs) [21]. Diesel was reported as seawater and soil contaminant [17]. About 1.7 – 8.8 million metric tons of diesel were released to aquatic environment and soil[5]. Presence of diesel in seawater and soil will be cause serious effects to marine ecosystem [20] because the components of diesel are potentially carcinogenic and toxic for environment [21]. A recent research has shown that biological process is the best practice for removing diesel spills in seawater and soil.

Bioremediation is one of the technology that utilizes the metabolism of microorganism to remove pollutants and detoxify the hazardous nature of the compounds. Indigenous microorganisms isolated from diesel contaminated area have more efficient capability in degrading diesel [20]. Many isolated bacterias from diesel contaminated area, like *Acinetobacter* sp, *Vibrio* sp, *Moraxella* sp, and *Bacillus* sp [3] have been found to degrade diesel. Isiodu et al. [11] reported that *Vibrio alginolyticus* G19 could be isolated from crude oil contaminated brackish water and have ability to degrade diesel with consotium bacteria up to 90%. Hamzah et al.[8] also reported that *Acinetobacter lwoffii* was isolated from soil contaminated with oil and also can degrade TPH up to 60%. Some bacteria isolated from diesel contaminated area have ability to producing enzyme to degrade and utilize diesel as carbon and energy source [18]. Furthermore, microorganism has capability to produce biosurfactant in order to increase the solubility of diesel.Based on our previous study showed that *Acinetobacter lwoffii* and *Vibrio alginolyticus* could be isolated from diesel contaminated coastal area due to the ship dismantling activities [24].

The aim of the present study was to determine the ability of diesel degradation abitily on two potential bacteria that isolated from ship dismantling area in Tanjungjati coast, Madura, Indonesia.

MATERIALS and METHODS

Site description

The aerobic bacteria used in this study were isolated from ship dismantling facility. It is located in Tanjungjati coast, Madura, Indonesia and showed in Figure 1.

Sample collection

Diesel contaminated seawater and soil samples were aseptically collected below surface at a depth of 20 cm [3]

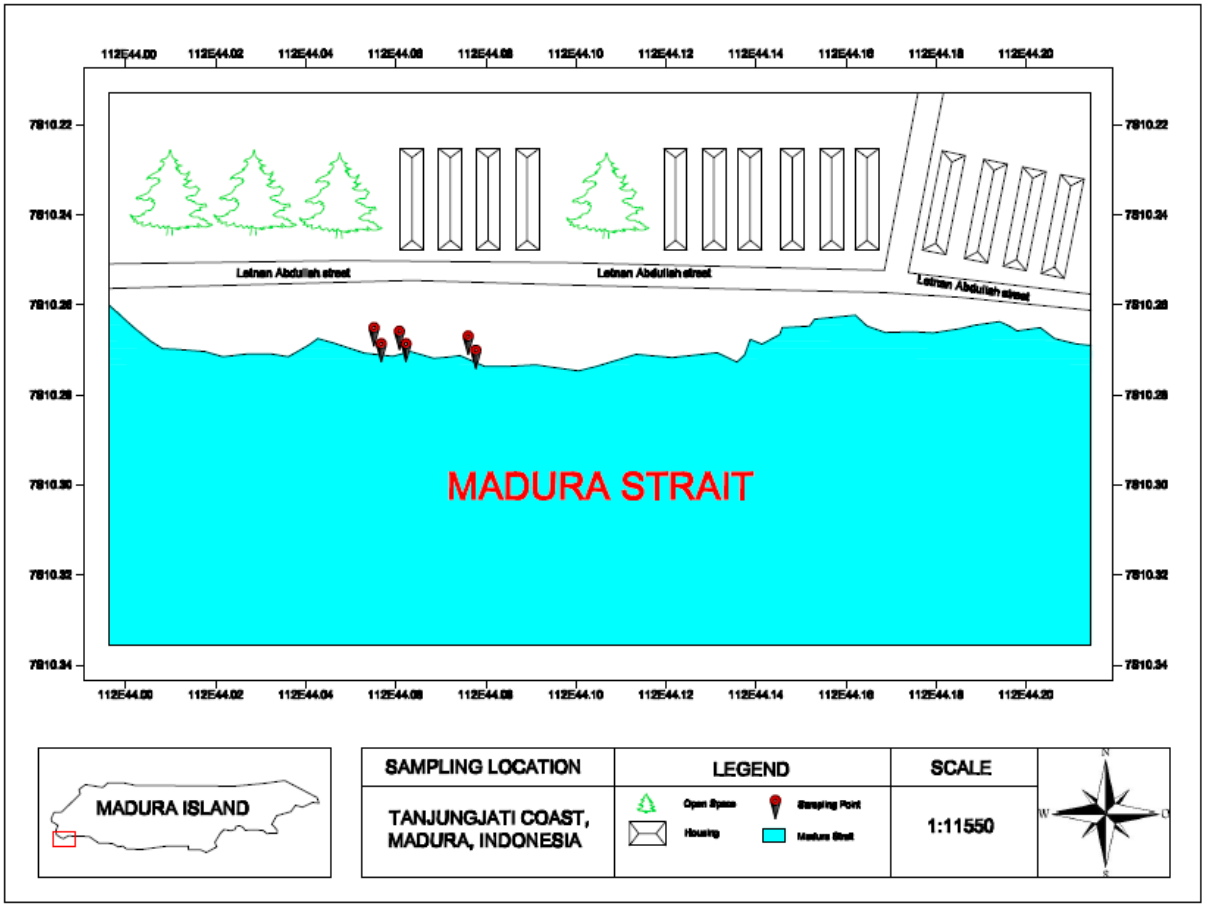


Figure 1. Sampling Location

and randomly selected in three different points. The seawater samples were collected into sterilized bottles and the soil samples were collected into sterilized plastic bags. All collected samples stored in ice box at 4°C temperature [2] and then transferred to environmental remediation laboratory of Institut Teknologi Sepuluh Nopember (ITS) for further analysis.

Physicochemical analysis

The pH and temperature of seawater and soil samples were measured by pH meter (Cyberscan pH 510, Singapore) and thermometer (OneMed, Indonesia). The seawater salinity measured by salinity meter (pH Onlab, USA). The total hydrocarbon contents of the seawater and soil were extracted using n – hexane (Fulltime, USA) followed by sample clean up and separation [17]. The sample extracts were analyzed by GCMS QP2010S (Shimadzu, Japan).

Isolation of diesel degrading bacteria

The isolation of bacteria was conducted based on our previous study [24]. The procedure was about 100 mL of each seawater samples were mixed into 500 mL sterilized Schott bottle (Pyrex, Germany). 10 grams of each soil samples were suspended in 100 mL of 0.85% NaCl into sterilized 250 mL Erlenmeyer (Pyrex, Germany) [20]. After that, all samples shaken by rotary shaker (Memmert, Germany) at 150 rpm for 1 hour [19]. 1 mL of each samples was transferred into tube containing 9 mL of 0.85% NaCl and followed by serial dilutions up to 10^{-8}. 0.1 mL of 10^{-6}, 10^{-7}, and 10^{-8} dilutions were spreaded on the surface of nutrient agar plate and then incubated in Incubator (Ogawa Seiki, Japan) at 37°C for 24 hours [17]. After the incubation period, different colonies appeared on agar surface were counted by bacteria colony counter (Topac, USA) and streaked into nutrient agar plates for purification and identification. After that, the isolated colonies were transferred into nutrient agar slants and stored in refrigerator at 4°C for further analysis [4].

Preliminary and secondary screening of diesel degrading bacteria

Preliminary screening of diesel degrading bacteria were carried out by using streak plate method [14]. Nutrient agar medium containing various concentration of diesel were streaked with isolated bacteria onto the surface of nutrient agar medium. Diesel concentrations used for screening of diesel degrading bacteria were 0%, 5%, 10%, and 15% (v/v) [2, 17]. Streaked diesel contaminated nutrient agar then incubated in incubator at 37°C for 24 – 48 hours [14].

The result of preliminary screening is determined by qualitative and quantitative analysis [19]. The qualitative analysis was done based on physical characteristic appearance of isolated bacteria. The quantitative analysis was done based on the surface area percentage of bacterial growth in various diesel concentration contaminated nutrient agar medium that compared to control by using bacteria colony counter. Bacterial isolates grown at high concentrations will be further analyzed at secondary screening.

Secondary screening of diesel degrading bacteria is carried out in Mineral Salt Medium (MSM) containing diesel as a carbon source. The purpose of secondary screening is to

determine the optimum concentration and select two bacterial isolates that potentially reduce the diesel. MSM consists of (NH4) 2SO4 - 1 g / L; KH2PO4 - 1 g / L; K2HPO4 - 1 g / L; MgSO4.7H2O - 0.2 g / L; FeCl3 - 0.05 g / L; CaCl2 - 0.02 g / L; and NaCl - 16.53 g / L [21]. 10% (v/v) of bacterial isolates with OD_{600} = 1 were cultured in MSM containing diesel with concentrations of 0, 5, 10, and 15% (v/v) and incubated for 7 days at 150 rpm agitation. Secondary screening results are determined based on the number of bacterial colonies. The optimum concentration and two bacterial isolates with the highest number of colonies will be used in diesel biodegradation test.

Identification of diesel degrading bacteria

Two bacterial isolates were identified based on morphological characterization and biochemical test according to Bergey's Manual of Determination Bacteriology.The method for identification of bacteria was conducted based on our previous study [24].

Biodegradation test

The biodegradation test was conducted based on method from Palanisamy et al.[17].The biodegradation testing was carried out in 250 mL Erlenmeyer flask (Pyrex, Germany) containing with 160 mL of MSM and diesel as the carbon source and energy with bacteria. About 10% (v/v) of inoculum bacteria (OD_{600} = 1) were added into MSM containing diesel. The diesel cultures were incubated for 14 days with agitation of 150 rpm on rotary shaker (Memmert, Germany). The salinity used in biodegradation test was 16.53‰ based on salinity in the existing condition in Tanjungjati coast, Madura, Indonesia. Some parameters that monitoredwereoptical density (OD), pH, and temperatureevery 24 hours. The collected samples were extracted and analyzed to check the total petroleum hydrocarbon degradation by gas chromatography.The assessments were carried out in duplicate.

Gas Chromatography – Mass Spectrometry (GC-MS) analysis

The determination of diesel degradation by isolated bacteria was carried out using GC-MS. After biodegardation testing period, samples were extracted based on based on modified method [17]. About 5 mL of the diesel cultures were extracted with twice 20 mL of n – hexane (Fulltime, USA) as a solvent by using separating funnels (Pyrex, Germany) to remove cellular material [17]. After that, The diesel residues were transferred into vial and incubated in Waterbath (Memmert, Germany) with 55°C for 3 days to remove n – hexane.

The diesel residues were analyzed by GCMS QP2010S (Shimadzu, Japan). A capillary column Rxi-5Sil MS (30 m × 0.25 mm × 0.25 µm) was used to separate hydrocarbons. The operation conditions were sample size (0.8 µL). Helium was used as the carrier gas. The injector and detector temperatures were maintained at 310°C.The oven was programmed at an initial temperature of 50°C and this was held for 5 min. Then ramped at 5°C/min to 300°C and held for 15 min.The electron impact ionization was tuned at 70 eV. The degradation rate of diesel oil was determined according to the following equation:

$$Rd = \frac{Ac - As}{Ac} \times 100\%$$

where Rd, Ac and As are the degradation rate of diesel oil, the amount of diesel oil remaining in the control culture, and the amount of diesel oil remaining in the sample culture [27].

RESULTS and DISCUSSION

Physicochemical analysis of diesel contaminated seawater and soil

The physicochemical analysis of seawater and soil samples were performed to determine the physical factorsthat could indicate the types of microorganisms recovered from diesel contaminated seawater and soil.The pH of three soil samples werestrong acid and the pH of three seawater samples were neutral. The physicochemical analysis of diesel contaminated seawater and soil were shown in Table 1. The average of salinity in Tanjungjati coast was 16.53‰. It mean that the water type in Tanjungjati coast was brackish water. The salinity range of brackish water is 5‰ - 30‰. Different with Maharani et al. [13] the salinity in Probolinggo coast, Indonesia, near Java sea, was about 28.9 – 32.95‰. It mean that the water type in Probolinggo coast was saline water where the salinity was more than > 30‰.

Table 1. Physicochemical properties of diesel contaminated seawater and soil

Physicochemical Properties	Seawater samples			Soil Samples		
	1	2	3	1	2	3
pH	8.1	7.3	7.6	< 3	3.2	3
Temperature (°C)	< 32	< 32	< 32	33.7	38.6	36.8
Salinity (‰)	15.8	16	17.8	-	-	-
Soil Colour	-	-	-	Thick brown	Thick brown	Thick brown

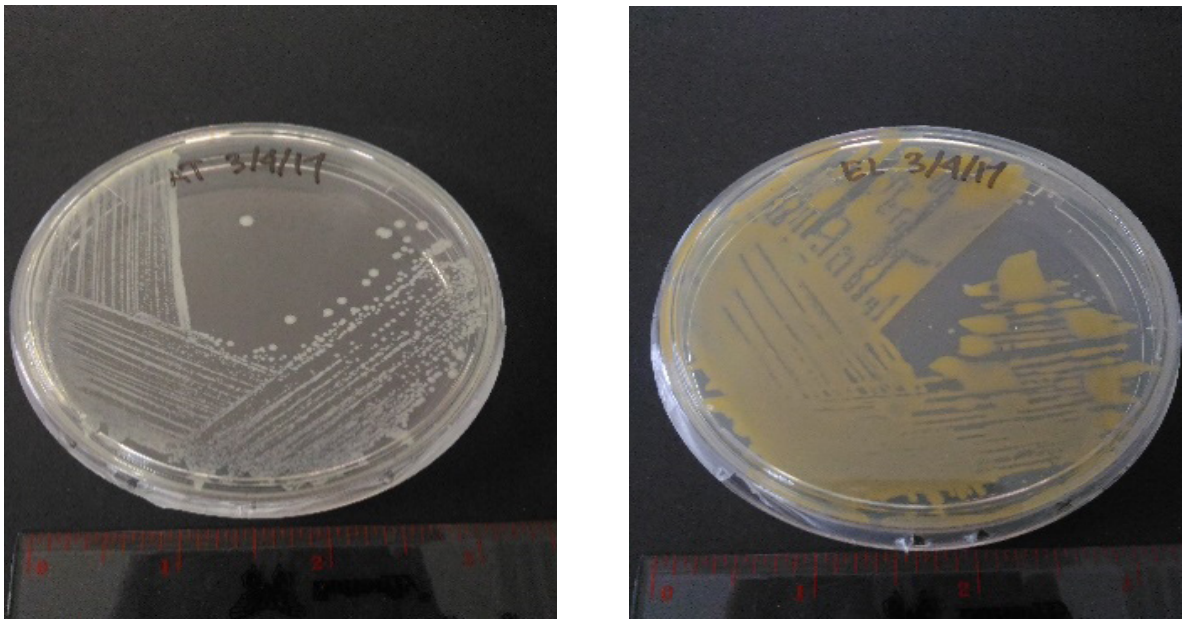
Isolation of diesel degrading bacteria

Based on our previous study [24], fifteenbacterialstrains were isolated from diesel contaminated seawater and soil by using serial dilution method, although only thirteen bacteria strain were used for further investigation due to two of bacterial strain could not be re-grown.Six colonies of seawater bacteria, namely with code of AL, BL, CL, DL, EL, FL and seven colonies of soil bacteria, namely with code of AT, BT, CT, ET, FT, GT, HT were obtained from the samples. Figure 2depicted the bacterial strain with code of AT and EL that re-growth on NA media.

Preliminary screening of diesel degrading bacteria

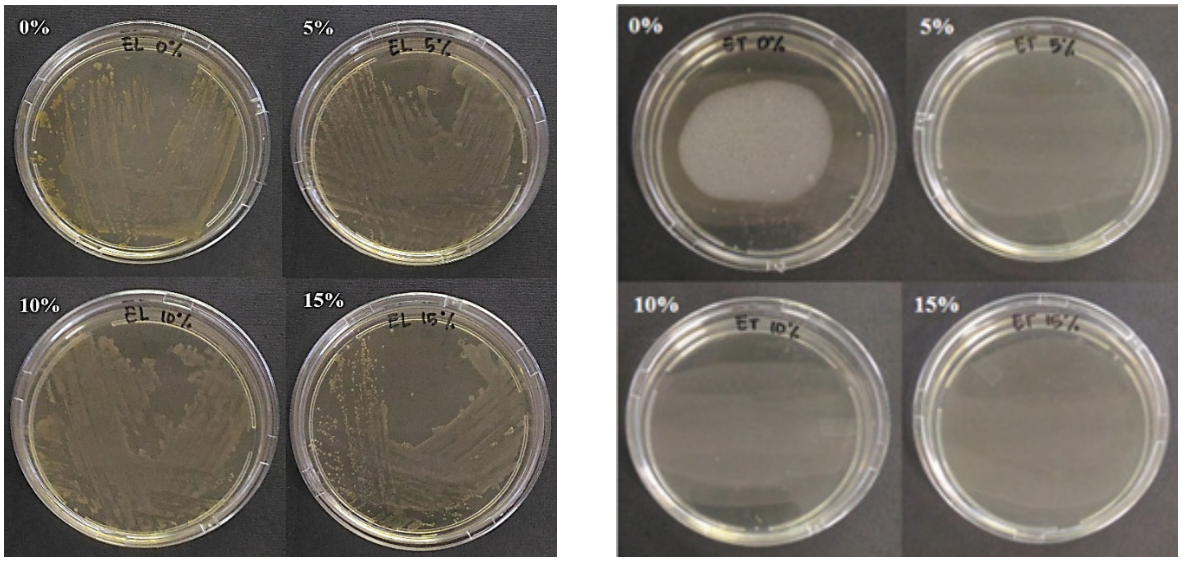
Thirteen of isolated bacteria were capable of utilizing diesel in various concentration as carbon source and energy. The abilitiy of isolatedbacteria to be grown in various diesel concentration wereshown in Table 2. Itsuggest that isolates AT, CT, GT, AL, CL, and EL had the broader ranges of diesel utilization ability. Those bacterial isolates that were grown at high concentrations were be used for further analyzed at secondary screening.

According to Table 2, the result of preliminary screening test showed thatsix bacteria have high growth in diesel contaminated medium. Growth percentage of each bacterial code ofAT, CT, GT, AL, CL, and EL were about 76.6; 80.85; 65.96; 68.09; 80.85; and 87.23%, respectively in 15% (v/v) of diesel contaminated medium. It was indicated that the capability of bacterial isolates utilizing diesel were different one another. Figure 3 described the appearance of bacterial



(a) (b)

Figure 2. (a) Bacteria coded AT and (b) Bacteria coded EL



(a) (b)

Figure 3. Result of preliminary screening for bacterial code of EL (a) and ET (b) in various diesel concentration

code of EL and ET on NA media. Figure 3 (a) showed that bacterial code of EL could grow well, meanwhile the bacterial code of ET could not grow on those media.

Table 2. Bacteria growth in various diesel concentration after 48 hours

Bacteria code	Diesel Concentration (%)			
	0	5	10	15
Soil Samples				
AT	+++++	+++++	++++	++++
BT	+++++	++	-	-
CT	+++++	+++++	+++++	+++++
ET	+++++	-	-	-
FT	+++++	+++	++	++
GT	+++++	+++++	+++	++++
HT	+++++	+	+	+
Seawater Samples				
AL	+++++	++++	+++++	++++
BL	+++++	+	+	++
CL	+++++	++++	+++++	+++++
DL	+++++	+++	++	++
EL	+++++	+++++	+++++	+++++
FL	+++++	+	+	-

Table Notes:
+++++ : 81 – 100% growth without color changing
++++ : 61 – 80% growth without color changing or 81 – 100 % growth with color changing
+++ : 41 – 60% growth without color changing or 61 – 80% growth with color changing
++ : 21 – 40% growth without color changing or 41 – 60% growth with color changing
\+ : ≤ 20% growth without/with color changing or 21 – 40% growth with color changing
\- : No growth

Secondary screening of diesel degrading bacteria

Initial diesel concentration of 0; 5; 10 and 15% (v/v) were used for secondary screening with six isolate bacteria in MSM medium. Results showed that the optimum growth of all bacteriain MSM medium with containing 10% of diesel oil, as shown in Figure 4. Bacterial code of EL showed

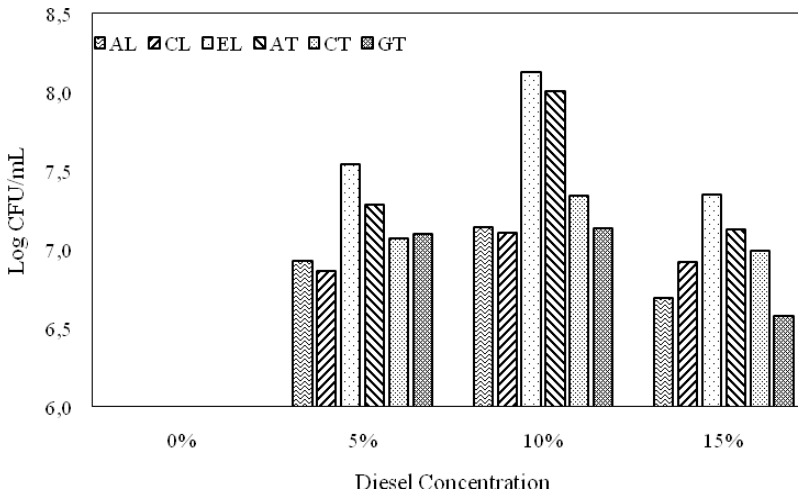


Figure 4. The result of secondary screening of diesel degrading bacteria after 7 days

the highest growth of 8.13 log CFU/mL and bacterial code of AT showed the next highest growth with 8.01 log CFU/mL in 10% of diesel.

Minimum growth of isolated bacteria were observed in 0% (v/v) of initial diesel concentration. Luo et al. [12]reported that at high diesel concentration, diesel would provide a better as carbon source for growth of bacteria. The growth of bacteria were incraesed with increase in diesel concentration [17]. All of bacteria did not possed higher growth at above 10% (v/v) diesel concentration. The reason for decreased consumption of diesel oil at high concentration may be attributed due to stress of diesel on bacteria [17].

Identification of diesel degrading bacteria

Based on our previous study [24], the idenfification of two of bacterial strains that have a high growth at secondary screening were identified as *Acinetobacter lwoffii* for bacterial code of AT and *Vibrio alginolyticus* for bacterial code of EL. Similary with Bhasheer et al. [3] reported that diesel degrading bacteria species *Acinetobacter sp* and *Vibrio sp* were isolated from diesel contaminated site. Isiodu et al. [11] also reported that *V. alginolyticus* was isolated from brackish water contaminated with crude oil. In commonly, *Acinetobacteria sp*. could be isolated from diesel contaminated soil [3, 17]. Deepika and Bramhachari [6] and Hamzah et al. [8] also reported that *A. lwoffii* was isolated from diesel contaminated mangrove sediment and soil.

Based on morphological and biochemical characteritation, *V.alginolyticus* is Gram-negative short rod, motile, and catalase positive while *A. lwoffii* is Gram-negative coccus, catalse positive, and non motile. Isiodu et al.[11] and Hamzah et al.[8] reported that *V. alginolyticus* and *A. lwoffii* was able to grow in diesel contaminated medium. Those bacteria commonly had isolated from tropical climate and were capable to degrade of hydrocarbon compound [11]. Bhasheer et al.[3] reported that *Acinetobacter* sp and *Vibrio* sp could be isolated from diesel contaminated area.

Biodegradation of diesel

V. alginolyticus and *A. lwoffii* were incubated in the MSM supplied with 10% (v/v) of diesel for 14 days. Figure 5of bacterial growth showed that lag period for growth of *V. alginolyticus* was shorter than*A. lwoffii*and subsequently resulted in higher growth that indicated higher diesel degradation.

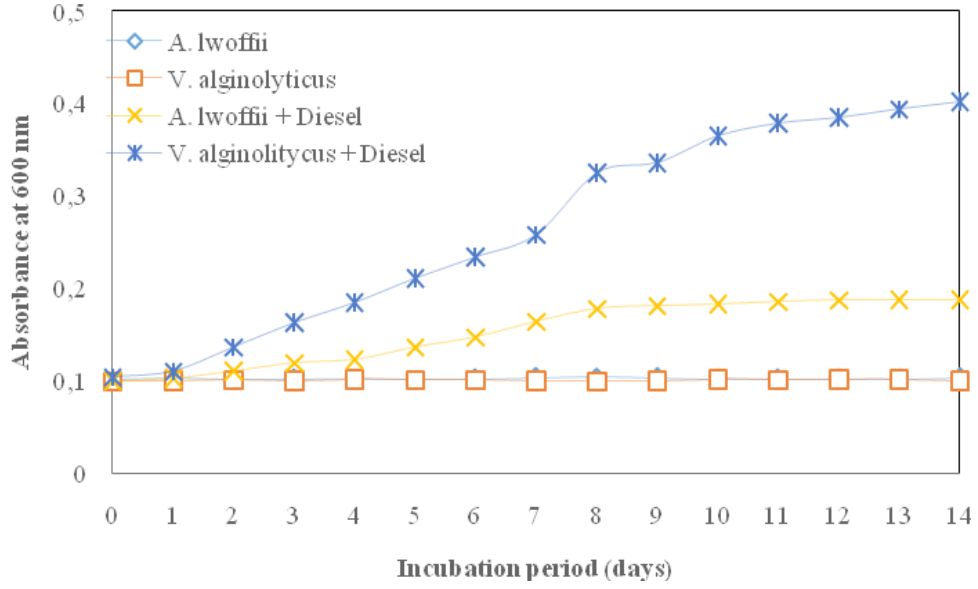


Figure 5. Bacterial growth in MSM containing 10% (v/v) of diesel

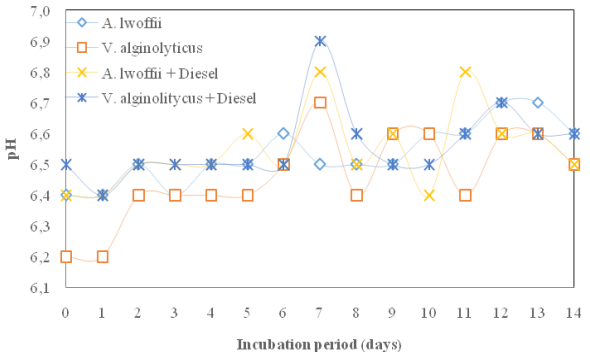


Figure 6. pH of isolated bacteria in MSM containing 10% (v/v) of diesel

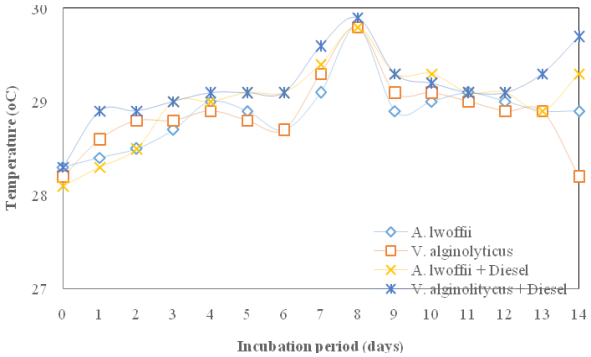


Figure 7. Temperature of isolated bacteria in MSM containing 10% (v/v) of diesel

pH and temperature are an important factor that affects the diesel degradation by bacteria [17]. In this study, pH and temperature were measured to know that bacteria were still growth in range of pH and temperatur for bacteria growth and showed in Figure 6 and Figure 7.

Figure 6 showed that pH were measured between 6.2 – 7. According to Ibrahim [10] the maximum rate of biodegradation process was achieved when the pH was neutral.In other studies showed that optimum pH for growth of *A. lwoffii* was 6.5 [8] while *V. Alginolyticus* was 7.5 [11]. According to Whang et al. [25]microbial growth and diesel biodegradation was found at pH 7.2, while decreasing or increasing the pH reduced the degradation efficiency considerably. Sathishkumar et al. [22] also reported that the optimum pH for the degradation of crude oil was found at pH 7.Hence, the optimization of pH is veryimportant for the enhanced growth of bacteria and also for selection of effective bioremediation strategy[17].

Maintenance of temperature is as important as pH which strongly affects bacterial growth.It greatly affects all the metabolic processes of bacteria [10]. Figure 7 showed that temperature of all bacteria were measured and showed between 28.9 – 29.9°C. According to Palanisamy et al. [17]the degradation efficiency decreased greatly with the increase of temperature. Deng et al. [7] also reported that the decreasing in temperature could lower the degradation because it can lower the fluidity of its cell membrane, and further restrain its exchange between intracellular and extracellular masses.

The biodegradation of diesel was investigated by GC-MS analysis. GC-MS was showed reduction in the intensity of diesel peaks after it was degraded by*A. lwoffii*and *V. alginolyticus* when compared with control (Figure 8 and Figure 9). The result was showed that *V. alginolyticus*and *A. lwoffii* could be degrade up to 26.78% and 1.85% for 14 days of incubation.

Based on Figure 7 showed that *A. lwoffii* was able to degrade short chain ($C_{12} – C_{18}$) n-alkanes and could reduce aromatic hydrocarbon like naphthalene andbenzen. Hamzah et al. [8] reported that *A. lwoffii* could degrade and utilize short chain ($C_{11} – C_{17}$) n-alkanes up to 28% up to 24 hours. Based on Figure 8 showed that *V. alginolyticus* could degrade short chain ($C_5 – C_{21}$) n-alkanes. It was indicated that both of bacteria could utilize diesel as carbon source and energy [11].

To improve diesel degradation, the optimum condition of pH, temperature, and salinity should be achived. In previous study, the optimum pH and temperature of *A. lwoffii* to degrade diesel were 6.5 and 30 – 37°C[8, 17].Yuan et al. [26] reported that *A. lwoffii*could growth well on 5‰ of salinity. Abdel-El-Haleem [1]also reported that*A. lwoffii*tend to have bad growth in high salinity medium. Different with *V. alginolyticus*, it has optimum pH, temperature, and salinity to degrade diesel and produce biosurfactant on 7.5; 28°C; and 20‰[9, 11]. Surendran et al. [23]reported that *V. alginolyticus* was halophilic bacteria that growth on high salinity between 15 - 35‰.

Biodegradation of the diesel was not only related to their chain lengths and physicochemical properties, but also related to characteristics of bacteria[7]. Emulsification was found in the MSM medium containing diesel, suggesting that *A. lwoffii* and *V. alginolyticus* could be capable of producing emulsifying agents or biosurfactants. Howover, *V. alginolyticus* could be more produce biosurfactanon high salinity than *A.lwoffi*.It was reported by Yuan et al. [26]and Hu et al. [9] that the optimum salinity of *A. lwoffii* and *V. alginolyticus* producing biosurfactant were 5‰ and 20‰.It has been reported that different chain-length hydrocarbons are selectively dissolved by emulsifying agents[15].

CONCLUSION

It was concluded that diesel degrading bacteria can be isolated from diesel contaminated seawater and soil. Thirteen bacteria strains were isolated which had the potential to degrade diesel.The optimum concentration was found to be 10% (v/v) of diesel and bacteria coded AT and EL have highest growth with 8.01 and 8.13 log CFU/mL for 7 days incubation and were identified as *Acinetobacter lwoffii* and *Vibrio alginolyticus*. The result showed that *V. alginolyticus* and *A. lwoffii* were able to degrade diesel about 26.78% and 1.85% for 14 days of incubation at initial diesel of 10% (v/v).Therefore, *V. alginolyticus* may employed as an excellent degrader to develop one cost-effective and eco-friendly method for the bioremediation of marine environments polluted by diesel. The results will be used for further investigation in biostimulation and bioaugmentation technique.

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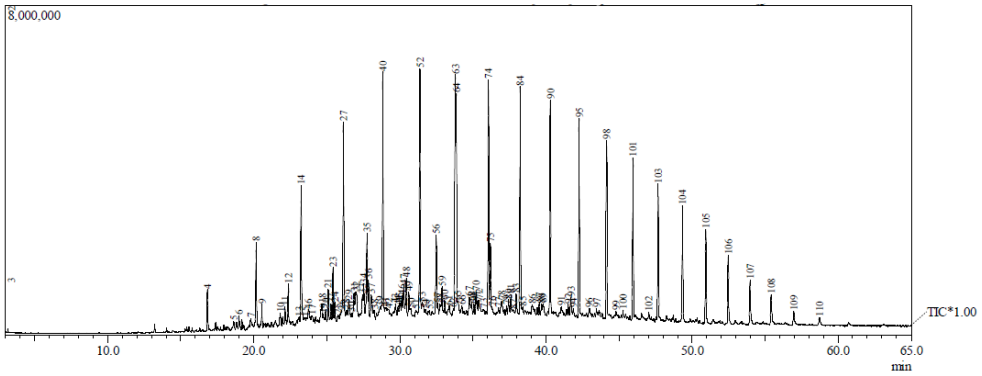

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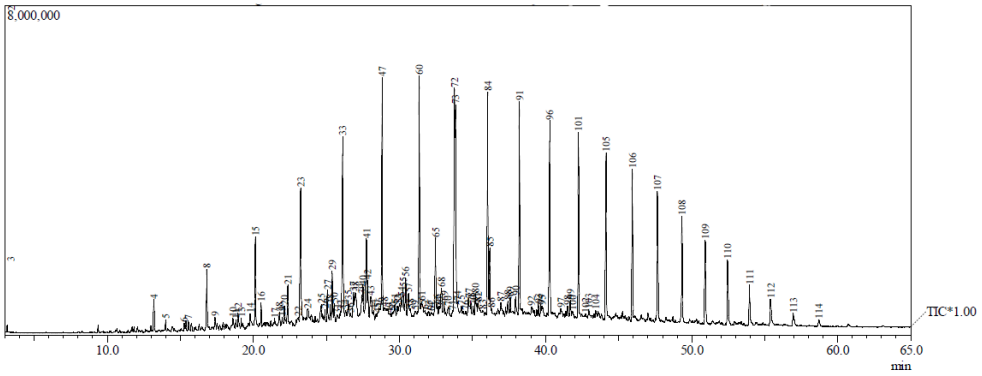
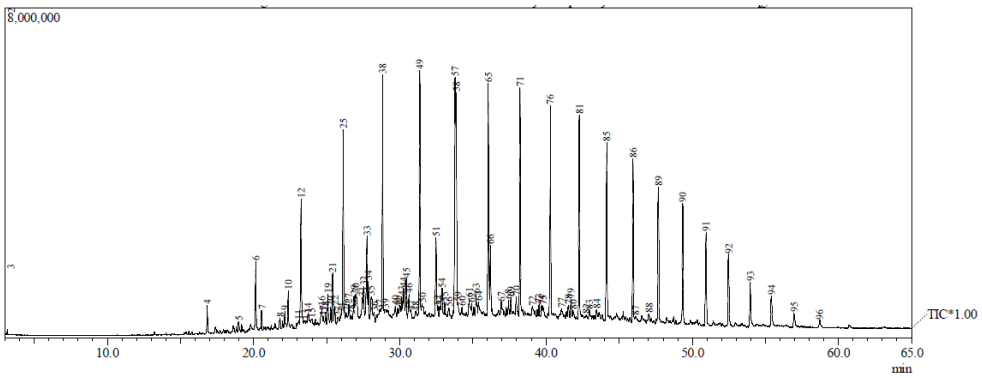


(b)

Figure 8. a) GC-MS for control diesel and **b)** GC-MS for diesel after biodegradation by A. lwoffii



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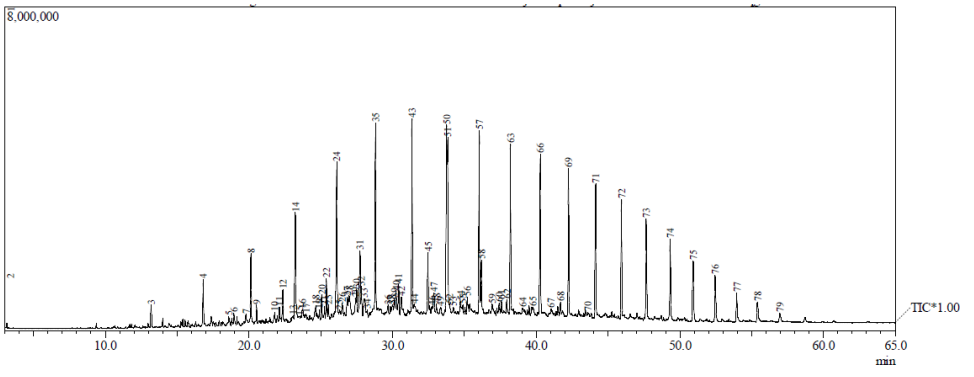

(b)

Figure 9. a) GC-MS for control diesel and **b)** GC-MS for diesel after biodegradation by *V. alginolyticus*

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