Isolation and Screening of Diesel Degrading Bacteria from Ship Dismantling Facility at Tanjungjati, Madura, Indonesia

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Abstract. The ship dismantling industry is a cause of contamination of the environment by diesel. The objectives of this study were to isolate and screen diesel degrading bacteria from diesel contaminated areas. Diesel contaminated seawater and soil samples were collected from a ship dismantling facility at Tanjungjati, Madura, Indonesia. Isolation was conducted with an aseptic technique and growing the mixture culture was carried out based on the pour plate method. After 24 h of incubation, thirteen bacteria strains were isolated from diesel contaminated seawater and soil samples from the area of study. The isolated bacteria were identified based on morphological characterization. Mostly gram positive bacteria were found. The isolated bacteria were screened by using nutrient agar medium containing various diesel concentrations (0%, 5%, 10%, and 15% (v/v)). The result of the screening test showed that the bacteria coded EL and CT displayed the best resistance and highest growth in diesel polluted medium. It was shown that both of them potentially have a higher capability of utilizing diesel as carbon and energy source than the others.

Keywords: *contamination; marine environment; resistance bacteria; seawater; soil.*

1 Introduction

Ship dismantling is a source of diesel pollution in coastal areas. The main activity is the dismantling and cutting apart of ships. Indirectly, remaining diesel oil that was used as engine fuel for the ships will be released into the seawater and soil [1]. One ship dismantling facility in Indonesia is located in Tanjungjati, Madura, Indonesia [2]. Its activity has caused seawater and soil pollution as indicated by the seawater and soil having become muddy and black. Every year about 1500 tons of diesel are released into the sea from this activity.

Received January 30th, 2018, Revised February 2nd, 2018, Accepted for publication March 2nd, 2018.
 ISSN: 2337-5779, DOI: 10.5614/j.eng.technol.sci.2018.50.1.7

Diesel is an engine fuel that contains rich hydrocarbons, ranging from C_8-C_{26}, and polyaromatic hydrocarbons (PAHs) [3]. Diesel is a seawater and soil contaminant [4]. Worldwide, about 1.7-8.8 million metric tons of diesel are released into the aquatic environment and soil each year [5]. Reference [6] states that the maximum concentration of PAH in seawater is 0.003 mg/L. Diesel spillage causes serious damage to the marine ecosystem. The components of diesel are toxic for the environment and potentially carcinogenic [3].

One of the methods that can be used to reduce diesel pollution is bioremediation [4]. Bioremediation is a biotechnology that utilizes the metabolism of microorganisms to reduce diesel contamination of seawater and soil. In a previous study, several bacterias were isolated from diesel contaminated areas, i.e. *Psuedomonas, Micrococcus, Staphylococcus, Bacillus, Flavobacterium, Acromobacter, Klebsiella, Actinomycetes, Acetobacter, Rhodococcus* [7]. Olawale, *et al.* [8] state that bioremediation has many advantages for detoxification of toxic substances because it is an environmentally friendly and simple method. Bioremediation depends on the ability of microorganisms to degrade diesel pollutants [9]. Some bacteria isolated from diesel contaminated areas have the ability to produce enzymes for degrading and utilizing diesel as carbon and energy source [10]. Furthermore, some microorganisms have the ability to produce biosurfactant to increase the solubility of diesel.

Indigenous microorganisms isolated from diesel contaminated areas have more efficient capability in degrading diesel [11]. The objectives of this study were to isolate diesel degrading bacteria from an area close to the ship dismantling facility in Tanjungjati and determining resistant bacteria based on bacteria growth capability screened for various diesel concentrations.

2 Materials and Methods

2.1 Samples Collection

Diesel contaminated seawater and soil were collected at the coast near the ship dismantling facility in Tanjungjati, Madura, Indonesia. Seawater and soil samples were aseptically collected below surface at a depth of 20 cm [12] and three different randomly selected points. The distance between each sampling point was about 15 m (Figure 1). The seawater samples were collected into sterilized bottles and the soil samples were collected into sterilized plastic bags. At the location, pH and temperature of the seawater and soil samples were measured by pH meter (Cyberscan pH 510, Singapore) and thermometer (OneMed, Indonesia), seawater salinity was measured in a laboratory by salinity

meter (pH Onlab, USA). All collected samples were stored in an icebox at 4 °C [13] and then transferred to the laboratory for further analysis.

To determine the components of diesel in the seawater and soil samples, all samples were extracted with organic solvent. From the seawater samples, 10 mL was extracted with 20 mL of n-hexane solvent (Fulltime, USA) in two stages using separation funnels (Pyrex, Germany) to remove cellular materials [4]. For the soil samples, 2 grams were extracted by using an ultrasonic method along with 2 grams of anhydrous sodium sulfate (Merck, Germany) and 25 mL of n-hexane solvent for 30 minutes and then filtered through glass wool [14]. The sample extracts were transferred to vials and then kept at 4 °C until being analyzed with gas chromatography (HP 6890, USA).

Figure 1 Sampling locations (T = soil sampling location, L = seawater sampling location).

2.2 Isolation of Diesel Degrading Bacteria

Isolation of diesel degrading bacteria was performed using a serial dilution method [15]. A hundred mL of each seawater sample was mixed into a 500-mL sterilized Schott bottle (Pyrex, Germany). Ten grams of each soil sample were suspended in 100 mL of 0.85% NaCl in a sterilized 250 mL Erlenmeyer (Pyrex, Germany) [11]. After that, all samples were shaken by a rotating shaker (Memert, Germany) at 150 rpm for 1 hour [16]. One mL of each sample was transferred into a tube containing 9 mL of 0.85% NaCl, followed by serial dilutions up to 10^{-8}. 0.1 mL of 10^{-6}, 10^{-7}, and 10^{-8} dilutions were spread on the surface of a nutrient agar plate and then incubated in an incubator (Ogawa Seiki,

Japan) at 37 °C for 24 hours [4]. After the incubation period, different colonies appeared on the agar surface. These were counted using a bacteria colony counter (Topac, USA) and streaked onto nutrient agar plates for purification and identification. After that the isolated colonies were transferred into nutrient agar slants and stored in a refrigerator at 4 °C for further analysis [7].

2.3 Morphological Characterization of Diesel Degrading Bacteria

Morphological characterization of the isolated bacteria was conducted based on physical characterization [7]. Physical characteristics such as size, shape, form, elevation, margin, appearance, pigmentation and gram staining were determined based on Harley-Prescott [15].

2.4 Screening of Diesel Degrading Bacteria

Screening of diesel degrading bacteria was carried out using a streak plate method [17]. Nutrient agar medium containing various concentrations of diesel was streaked with isolated bacteria onto the surface of nutrient agar medium. The diesel concentrations used for screening the diesel degrading bacteria were 0%, 5%, 10%, and 15% (v/v) [4,13]. The streaked diesel contaminated nutrient agar was then incubated in an incubator at 37 °C for 24-48 hours [17].

The result of screening was determined by qualitative and quantitative analysis [16]. The qualitative analysis was done based on the physical appearance of the isolated bacteria. The quantitative analysis was done based on the surface area percentage of bacterial growth in the various diesel concentrations of contaminated nutrient agar medium and comparing it to the nutrient agar plate surface area by using a bacteria colony counter (BBC). The surface area of bacteria growth was measured by using a bacteria colony counter. The plates containing diesel and appearing bacteria were placed on the BBC. The number of squares (1 x 1 cm) on the BBC were counted to obtain the amount of surface area with bacteria growth.

3 Results and Discussions

3.1 Physicochemical Properties of Diesel Contaminated Seawater and Soil

Physicochemical properties of the diesel contaminated seawater and soil, i.e. pH, temperature, salinity, and soil colour, were analyzed; the results are shown in Table 1. The components of diesel in seawater and soil were analyzed using gas chromatography; the results are shown in Table 2. Table 1 shows that the

seawater in coastal Tanjungjati, Madura, Indonesia is brackish water. The salinity range of brackish water is 5-30‰.

Table 1 Seawater and soil sample analysis.

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)	< 3	3.2	3	33.7	38.6	36.8
Salinity (‰)	15.8	16	17.8	-	-	-
Soil Colour	-	-	-	Thick brown	Thick brown	Thick brown

Table 2 Components of diesel in seawater and soil.

No	Components of diesel
1	Naphthalenane (C_{10})
2	Cyclohexane (C_6)
3	Capnellane (C_{15})
4	Tricosane (C_{23})
5	Octadecane (C_{18})
6	Tetracosamethylcyclododecasiloxane (C_{24})
7	Hexadecane (C_{16})
8	Heptadecene (C_{17})
9	Octadecamethylcyclononasiloxane (C_{18})
10	Isochiapin B
11	Bergamotane (C_{15})

Table 2 shows that the components of diesel in the seawater and soil samples were hydrocarbons ranging from C_6-C_{24}, such as cyclohexane, naphthalenane, hexadecane, octadecane, etc. Reference [3] states that diesel contains rich hydrocarbons ranging from C_8-C_{26} and polyaromatic hydrocarbons (PAHs). Also, reference [7] states that diesel contains a large amount of alkane hydrocarbon chains ranging from C_{10}-C_{20}.

3.2 Isolation of Diesel Degrading Bacteria

Thirteen bacterial strains were isolated from diesel contaminated seawater and soil by serial dilution. The isolated bacteria were identified based on morphological characterization (Table 3). The percentage of isolated gram positive bacteria (54%) was higher than that of gram negative bacteria (46%). Gram positive bacteria dominate in diesel contaminated areas [12]. This is because gram positive bacteria have stronger cells than gram negative bacteria, which allows them to grow in various conditions. This is different from the observation by [17], where it is stated that the amount of gram negative bacteria

isolated from diesel contaminated soil was higher than the amount of gram positive bacteria. Furthermore, [16] also states that the amount of gram negative bacteria isolated from diesel contaminated seawater was higher than the amount of gram positive bacteria.

Table 3 Morphological characterization of isolated bacteria.

Bacteria code	Morphological Characterization							
	Gram staining	Shape	Form	Elevation	Margin	Appearance	Pigmentation	Diameter (mm)
Soil Samples								
AT	-	Coccus	Circular	Convex	Entire	Shiny	White	2
BT	-	Rod	Circular	Raised	Entire	Shiny	White	1
CT	+	Coccus	Circular	Convex	Entire	Shiny	White	3
ET	-	Coccus	Punctiform	Flat	Entire	Shiny	White	1
FT	+	Rod	Punctiform	Flat	Entire	Dull	White	0.5
GT	-	Coccus	Circular	Convex	Entire	Dull	White	0.5
HT	-	Rod	Circular	Convex	Undulate	Dull	White	1
Seawater Samples								
AL	-	Rod	Circular	Convex	Entire	Dull	White	0.5
BL	+	Rod	Circular	Convex	Undulate	Dull	White	0.1
CL	+	Rod	Punctiform	Convex	Undulate	Shiny	White	0.1
DL	+	Rod	Circular	Convex	Entire	Shiny	White	1
EL	+	Coccus	Circular	Convex	Entire	Shiny	Yellow	0.1
FL	+	Rod	Circular	Convex	Entire	Dull	White	1

The distribution of bacteria isolates obtained from different points indicated the presence of active metabolism of microorganisms in diesel contaminated areas [12]. This strongly suggests that isolated bacteria from diesel contaminated areas should be used to degrade diesel in seawater and soil because such bacteria isolates can utilize the diesel as carbon and energy source.

3.3 Screening of Diesel Degrading Bacteria

The capability of isolated bacteria utilizing diesel as carbon and energy source was identified by using a screening method. Thirteen bacteria isolates were inoculated onto nutrient agar plates containing various diesel concentrations (0%, 5%, 10%, and 15% (v/v)). Every microorganism has a different capability to degrade diesel depending on its condition and metabolism and the diesel concentration [7]. The purpose of the screening test was to select two bacteria that showed strong growth in the diesel contaminated nutrient agar medium. The two selected bacteria were used in a diesel degradation test, in single and

consortium culture. The result of the screening test was determined by qualitative and quantitative analysis (Table 4 and Figure 2).

Table 4 Bacteria Growth in various diesel concentrations 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	+++++	+	+	-

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

According to Table 4, the results of the qualitative and quantitative analyses of the screening test showed that the bacteria coded EL and CT were the most resistant strains and had the highest growth levels in diesel contaminated medium. The growth percentages of the bacteria coded EL and CT were about 61-80% and 41-60% in diesel contaminated medium without color change. This indicates that the capabilities of the bacterial isolates to utilize diesel were different from one another [18]. Table 4 also shows that the higher the diesel concentration in the medium, the lower the bacterial growth. This is caused by the toxicity of high diesel concentrations, which causes stress and shock, affecting bacterial growth [4,12]. Using this method, it could be seen that microorganisms had more growth when the diesel concentration decreased. Each microorganism has its own level of tolerance to diesel because diesel can act as their carbon and energy source. On the other hand, diesel can also be toxic to microorganisms. According to [17], microorganisms prefer to grow when the concentration of hydrocarbons is low, but every organism has its own level of tolerance [22].

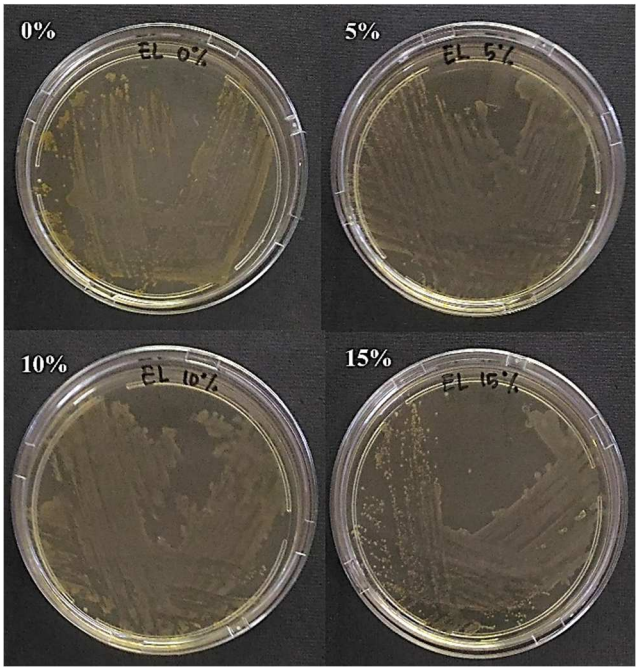

Figure 2 Results of screening test for bacteria coded EL with various diesel concentrations.

The results of this study were compared with those from previous studies. Prakash, *et al.* [19] isolated 59 bacteria strains from petroleum contaminated soil, of which only *Bacillus sp., Pseudomonas sp.,* and *Micrococcus sp.* could grow in 1% of diesel and were capable to utilize and degrade diesel. Mujahid, *et al.* [17] report that they isolated twelve bacterial strains from petroleum contaminated soil, of which only *Pseudomonas sp.* and *Micrococcus sp.* could grow in nutrient agar medium containing 0.25% of hydrocarbon, i.e. naphthalene, phenanthrene, biphenyl, anthracene, and xylene. Furthermore, Bhasheer, *et al.* [12] isolated five bacterial strains from diesel contaminated soil, of which only *Staphylococcus* and *Pseudomonas* could grow efficiently in 10% (v/v) of diesel.

As for the morphological characteristics, the bacteria coded EL and CT were gram positive bacteria with colonies appearing circular, entire, shiny and convex. The difference between the bacteria coded EL and CL was in their color and shape. The color and shape of the bacteria coded EL were yellow and coccus-shaped with irregular clusters. Meanwhile, the color and shape of the bacteria coded CT were white and coccus-shaped with regular 'grape-like' clusters. According to Holt, *et al.* [20], the Micrococcus genus is a gram positive bacteria with colonies appearing circular, entire, and convex; coccus-

shaped with irregular clusters; and the color is yellow. Furthermore, Holt, *et al.* [20] state that *Staphylococcus* genus is a gram positive bacteria with colonies appearing circular, entire, and convex; coccus-shaped forming regular and 'grape-like' clusters; and the color is white.

Based on the result of morphological characterization, the bacterial strains coded EL and CT were predicted as belonging to the *Micrococcus* and *Staphylococcus* genii, because both bacteria were coccus-shaped and gram positive. Identification of the selected bacteria will be conducted in the follow-up of this research using a biochemical test based on Bergey's *Manual of Determination Bacteriology* and biomolecules. The two isolated bacteria coded EL and CT will be used for further research using biostimulation and bioaugmentation techniques. These bacteria will be inoculated into diesel contaminated artificial seawater and soil based on the characteristics of seawater and soil samples from the preliminary study. According to [4,17,21], isolated bacteria from diesel contaminated seawater and soil are able to use diesel as carbon and energy source for their metabolism.

4 Conclusion

Diesel-degrading bacteria were successfully isolated from diesel contaminated seawater and soil. In this study, thirteen bacteria strains with potential to degrade diesel were isolated. According to the result of the screening test, the bacterial strains coded EL and CT were the most resistant and showed the highest growth percentages in diesel contaminated medium. These bacteria are predicted as belonging to the *Micrococcus* and *Staphylococcus* genii. Both of them have potential to be used as diesel degrading bacteria in soil or seawater bioremediation.

Acknowledgements

The authors would like to thank the Ministry of Research, Technology, and Higher Education of Republic of Indonesia through PUPT 2017 No. 629/PKS/ITS/2017 for funding this research.

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