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Identification of Diesel Resistant Bacteria that Isolated from Ship Dismantling Area in Madura Coastal

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Abstract- Ship dismantling activity is one industry that caused diesel contamination in environment. One of technology that can be used to remediate diesel contaminated area is bioremediation. Bioremediation can be conducted using bioaugmentation technique. The objective of this study was to identifity of diesel resistant bacteria using biochemicals test methods. The seawater and coastal soil samples were collected from area study using a sterilized bottles. All samples were shaker at 150 rpm for 1 h, after that samples were taken and serially diluted from 10^{-1} to 10^{-7}. The diluted sample was inoculated on nutrient agar plates by pour plate method. The plate was incubated for about 24-48 hours and the growth of microorganisms was noted. Bacteria with difference of colony morphologies were selected. The cell morphology was determined microscopically after Gram-stain preparation. The isolates were identified using Microbact GNB 12A and 12B (Oxoid, UK) identification kit. This is a miniaturized computer aided identification system for the identification of organisms belonging to the genus Acinetobacter and Vibrio.

Keywords: *bacteria, bioremediation, coastal, seawater, ship dismantling*

1. Introduction

The increasing of development in many fields can cause many pollutions on soil, air, water and seawater. Oceans are the sinks for the pollutants traveled through several sources such as rivers, streams, coastal restaurants, industries and inland transportation (Kiranmai and Anima, 2017). Many ships are needed for transportation in river and ocean. Actually, large-capacity ships have a life time of approximately 20 years. After that time, the ships should be destroyed. Ship dismantling is a activity to cut a big ship become a small part depending on a functions of that part such as steel material, plate material and ship engine. Although this activities of ship dismantling and cutting has some advantages for human beings at that area but it can caused pollution such as many spills of diesel and oil on soil and seawater near that location.

Ship dismantling activity is one industry that cause diesel contamination in environment. One of technology that can be used to remediate diesel contaminated area is bioremediation. Bioremediation can be conducted using natural attenuation, bioaugmentation technique and biostimulation technique. There are many reasons to use microorganisms for biodegradation. Bacteriological degradation and remediation is economical and exhibits high competence without secondary pollution (Mulani et al., 2017). The potential and selected microbes can alter raw and crude oils in beneficial ways and the resulting end products are comparatively safer to the environment and all living beings. These microbes utilize waste material as carbon substrate, increase their population, and ultimately biodegrade hydrocarbon products to nontoxic products, such

as H_2O and CO_2 (Prakash et al., 2014). The presence of hydrocarbon degrading microorganisms i.e bacteria play an effective role in bioremediation of any spilled hydrocarbons in the contaminated environment. Hydrocarbon degraders are ubiquitous in the marine environment (Paniagua-Michel and Rosales, 2015). Marine bacteria are of great interest as novel and rich sources of biologically active products. Marine bacteria are found in seawater, sediments and marine macro organisms (Arunkumar and Karthik, 2013).

The aim of the research was to identify the potentially bacteria for biodegradation of diesel that be isolated from ship dismantling area in Madura Coastal. This research is a part of main research in bioremediation in contaminated coastal area due to ship dismantling activity. The results will be used in further investigation.

2. Materials and Methods

2.1. Isolation of Bacteria at Diesel Contaminated Coastal Area

Isolation of bacteria was conducted at Tanjung Jati coastal in Madura Island. Fig. 1 showed the location of Tanjung Jati coastal. This isolation method is according to reference of Thi et al. (2012), Pranowo and Titah (2016), Kiranmai and Anima (2017) with modification. Approximately 10 g of soil coastal at three sampling locations were suspended using 100 mL sterile saline water in sterile botols and 100 ml of seawater from same locations were put in sterile botols. All samples were taken in a cooling box and were send to the laboratory as soon as possible. After all samples arrived at laboratory, all samples were shaken vigorously in an shaker (Innova 2000, Eppendorf, Jerman) at 150 rpm for 1 h. After all particles had been settled for 1 min, 1 mL of the homogeneous suspension was added to dilution tubes or a bottle containing 9 mL of sterile physiologis solution (8.5 g NaCl/1000 mL) to make a serial dilution (10^{-1} until 10^{-7}). The suspensions (0.1 mL) were plated onto a Nutrient Agar or NA (Merck, USA) medium by a serial dilution using the pour plate technique. All plates were incubated at 37 ^{o}C in an incubator (Ogawa Seiki, Japan), and were observed for 1 week.

After it was incubated, a single colony was picked up and streaked onto a fresh NA medium to obtain a pure culture. Bacteria isolates were selected to represent distinct types based on differences in colony morphology such as colony form (Herley and Prescott, 2002). The general form of the colony can be determined through eye vision on the top of the colony and observed those colonies using microscope with 40X of magnification. Gram-staining of the isolates were examined after 24 or 48 h (depending on the tested isolates) of incubation on NA agar plates (Titah et al., 2011).

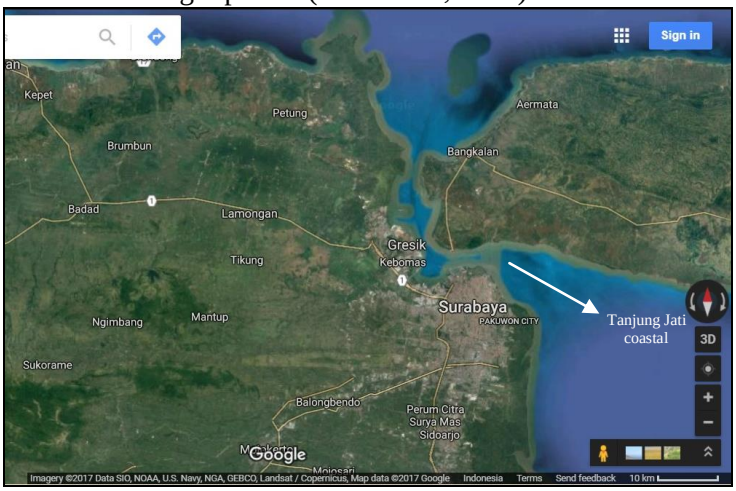


Fig. 1 Map of Tanjung Jati coastal

2.2. Identification of Bacteria

Bacterial identification was conducted using a biochemical methods. Microbact Identification Kits (Microbact^{TM} GNB 12A and 12B) (Oxoid, UK) were used in this research and based on book of Bergey's Manual of Determinative Bacteriology (Holt, 1992). Each kit contains 12 (12A, 12B & 12E) or 24 (24E) miniature biochemical tests. Organism identification was based on pH change and substrate utilisation. Clinical used only: Microbact^{TM} Gram-negative 12A (strip format) and 12E (microplate format) may be used alone for the identification of oxidase-negative, nitratepositive glucose fermenters (comprising 15 genera) and was useful for screening pathogenic Microbact^{TM} Gram-negative 12B can be used in conjunction with 12A for the identification of oxidase-positive, nitrate-negative, glucose non-fermenters (miscellaneous Gram-negative bacteria - MGNB) and Enterobacteriaceae. Meanwhile, Microbact^{TM} Gram-negative 24E is a combination of the tests in 12A(or12E) and 12B in microplate format.

First, selected bacteria were re-grown on NA slant media. After the bacteria was18-24 h, some biochemicals test were conducted. Gram staining was carried out to determine the gram of bacteria and the oxidase test were conducted to determine the type of identification kit. 10 mL bacteria suspension was made from 1 to 3 colonies of bacteria with physiologis solution of 0.85% NaCl. About 250 µL of bacteria suspension was filled to all hole on microbact kit. 4 drops of bacterial suspension were be added to each well and 2 drops of Mineral Oil (MB1093A) were added to black wells. After that, microbact kits were sealed and stored in incubator at 37^{O}C for 24 h. Microbact kits were taken from incubator, and those were added with appropriate reagents (Table 1). The results were analyzed using programe of MicrobacTM Gram Negative Identification System.

Table 1. Addition of reagents to MicrobactTM Gram-negative Identification Sytems

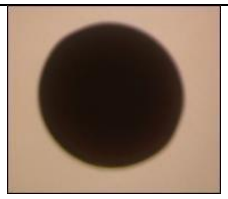
System	Well	Reagent	Quantity	Time to read
12A (12E) or 24E	8	Indole	2 drops	2 mins
12A (12E) or 24E	10	VPI & VPII	1 drop each	15-30 mins
12A (12E) or 24E	12	TDA	1 drop	Immediately

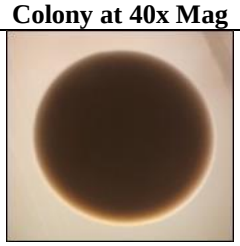
3. Results and Discussion

3.1. Isolation of Bacteria at Diesel Contaminated Coastal Area

Based on the results, total colony number at soil coastal and sea water samples were 6.3x1^{8} CFU/mL and 1.47x10^{9} CFU/mL, indicating that many colonies of bacteria could grow from sample at contaminated locations. After preliminary observation on colonies using microscope with magnification of 40X (Table 2), eight colonies of bacteria from soil coastal samples were be selected to inoculate on fresh NA medium. Meanwhile, seven colonies of bacteria from sea water samples were be selected to inoculate on fresh NA medium. The selections were conducted based on difference of shape colony and difference of color colony. The difference of shape colony and difference of color colony were indicated that strain of bacteria was different. It was predicted belonging to difference of bacterial species too.

Table 2. Summary of bacterial colony isolation that have difference of shape and color colony

No	Type of Sampel	Code of Bacteria	Colony at 40x Mag
1	Soil coastal	AT	
2	Soil coastal	BT	
3	Soil coastal	CT	
4	Soil coastal	DT	
No	**Type of Sampel**	**Code of Bacteria**	**Colony at 40x Mag**
5	Soil coastal	ET	
6	Soil coastal	FT	

No	Type of Sampel	Code of Bacteria	Colony at 40x Mag
7	Soil coastal	GT	
8	Soil coastal	HT	
9	Brackish water	AL	
10	Brackish water	BL	
No	**Type of Sampel**	**Code of Bacteria**	**Colony At 40x Mag**
11	Brackish water	CL	
12	Brackish water	DL1	

No	Type of Sampel	Code of Bacteria	Colony at 40x Mag
13	Brackish water	DL2	
14	Brackish water	EL	
15	Brackish water	FL	

3.2. Identification of Bacteria

Based on our previous study, screening of diesel on all bacteria code (data is not showed), it were selected two bacteria with code of AT and EL for identification. The selection reasons of AT and EL due to those bacteria have high resistance to diesel. Table 3 showed the biochemicals test results for identification using Microbact Identification Kits (Microbact^{TM} GNB 12A and 12B). Based on the results, the bacterial code of AT was *Acinetobacter lwoffii* and the bacterial code of EL was *Vibrio alginolyticus*. The accuracy for each identified bacteria were 92.85% and 99.91%.

Table 3. Biochemicals test results for identification

No	Characteristic	Code bacteria	
		AT	EL
1	Oxidase	-	+
2	Motility	-	+
3	Nitrate	-	+
4	Lysine	-	-
5	Ornithine	-	-
6	H_2S	-	-
7	Glucose	-	-
8	Mannitol	-	-
9	Xylose	-	-
10	ONPG	+	+
11	Indole	+	-
12	Urease	-	-
13	VP	-	+
14	Citrate	-	-
15	TDA	-	-

No	Characteristic	Code bacteria	
		AT	EL
16	Gelatin	-	-
17	Malonate	-	-
18	Inositol	-	-
19	Sorbitol	-	-
20	Rhamnose	-	-
21	Sucrose	-	-
22	Lactose	-	-
23	Arabinose	-	-
24	Adonitol	-	-
25	Raffinose	-	-
26	Salicin	-	-
27	Arginine	-	-
28	Gram Staining	Negative	Negative
29	Shape of Colony	Coccus	Bacillus
30	Species	*Acinetobacter lwoffii*	*Vibrio alginoyiticus*
31	Accuracy	92.85%	99.91%

Acinetobacter spp. are ubiquitous gram negative and non-fermenting coccobacilli that have for long been described from various environmental sources such as fresh water, seawater, wastewater, and soil (Al Atrouni et al,. 2016). *V. alginolyticus* is a ubiquitous bacteria isolated in seawater and appears to be present in seawater in larger numbers than other Vibrio species (Chen et al., 2011).

Other research reported that bacteria of *Burkholderia sp., Moraxella sp., Vibrio sp., Yersinia sp., dan Acinetobacter sp.* can be isolated from contaminated seawater and soil with diesel and heavy metal (Bhasheer *et al*., 2014; Mujahid *et al*., 2015; Isiodu *et al*., 2016). Some bacterial genera, such as *Acinetobacter, Burkholderia, Gordonia, Dietzia, Brevibacterium, Aeromicrobium, Celeribacter, Mycobacterium* and *Sphingomonas*, isolated from petroleum-contaminated soil have proved to show potential for hydrocarbon degradation. These bacteria can produce biosurfactants, active agents which emulsify these hydrocarbons (El Hanafya et al., 2016). According to Hamzah et al. (2010), *A. lwoffii* that be isolated from soil contaminated with hydrocarbon can degrade cruide oil.

4. Conclusion

Result showed that total colony of bacteria at soil coastal sample and sea water sample were $6.3x10^8$ CFU/mL and 1.47×10^9 CFU/mL, respectively. Total of eight colonies of bacteria from soil coastal sample and seven colonies of bacteria from sea water sample can be isolated. Identification results of two bacterial code that have potential for diesel biodegradation were *Acinetobacter lwoffii* and *Vibrio alginolyticus*. The accuracy for identified bacteria were 92.85% and 99.91%, respectively. Two species of this bacteria will be used to further research on diesel biodegradation.

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