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Isolation and Screening of Diesel-Degrading Bacteria from the Diesel Contaminated Seawater at Kenjeran Beach, Surabaya

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Abstract

Samples of contaminated seawater by diesel were taken at Kenjeran Beach Surabaya using aseptic technique. Isolation was conducted using serial dilution and spread method on nutrient agar (NA) media. The all bacteria colony were devided in to group based on with morphological characterization and gram staining. After that, those bacterial colonies were tested individually in NA media containing different concentration of diesel (2, 4, 6, 8, and 10%) for up to 7 days at 30°C. The results showed that eight bacterial strains were isolated from diesel contaminated seawater in Kenjeran Beach Surabaya. Screening on diesel showed that all the isolation bacteria were capable of degrading diesel and bacteria with code of B and E haves highly percentage growth in compared to other bacterial isolation. In conclusion, bacteria with code of B and E have potential to be used in diesel bioremediation in contaminated seawater.

Keywords: bacteria; colony; diesel; isolate; seawater

1. Introduction

Kenjeran Beach Surabaya, as the east coastal areas of East Java, is a waterfront area that is prone to spills/contamination of crude oil because of there were many fishermen using traditional diesel boat to catch fish. Diesel is one of the sources of pollution in the environment. Spillage in the marine condition affects the aquatic living environment (Karthika *et al*., 2014).

Hydrocarbon contamination in seawater has been regarded as an increasingly serious international concern for environmental reasons because it can damage the capacity of the coastal environment. Physical, chemical and thermal methods have commonly been employed to clean up theoil-contaminated sites. However, these techniques are relatively expensive and also require site restoration (Roy *et al*., 2014). There is an increased interest in promoting ecofriendly methods in the process of cleaning oil-polluted sites. Many species of microorganisms including bacteria, yeasts and fungi obtain both energy and tissue-building material from hydrocarbon (Bhasheer *et al*., 2014). This is possible because microorganisms have enzyme system to degrade and utilize diesel oil as a source of carbon and energy (Panda *et al*., 2013). Microorganisms play important roles in the degradation of hydrocarbon in terrestrial and aquatic ecosystems (Sun *et al*., 2010).

Hence in this investigation indigenous bacteria which degrade diesel was isolated from diesel contaminated seawater at Kenjeran Beach and screened for endurance bacteria towards various concentration of diesel.

2. Materials and Methods

2.1 Sample collection

The diesel polluted sea water samples were collected from Kenjeran Beach, Surabaya (latitude: 7° 13’25.35” S; longtitude: 112° 47’ 26.16” E) in February, 2016. Kenjeran Beach is one of the traditional fishing boat dock in east Surabaya. Although the amount of oil spills are a few, if it happen continuously it will cause the occurrence of contamination. Fig. 1 showed sampling sites near the fishing boat dock. The samples were collected using sterile bottles, which has sterilized use autoclave. The sample bottles were inserted into the seawater below the surface of the water contaminated diesel at a depth of 0-20 cm. The bottle was opened in water and allowed to fill with seawater until it is full and then closed at the positions are still under water. After that, the sample bottles were transferred to the laboratories to measure pH by using pH meter (Naila, 2015), the salinity of the seawater was determined by salinity meter (Diaz-Herrera *et al*., 2006) and

Figure 1. Sampling sites in Kenjeran Beach, Surabaya

the temperature of seawater was determined by thermometer (Garcia-Benadi *et al*., 2015).

2.2 Isolation of bacteria from seawater sample

The samples were measured to determine pH, temperature, and salinity. The samples were be shake by using shaker Innova 2000 for 1 h at a speed of 150 rpm. The aim of shaking samples were be homogenized seawater contained in that seterile bottles. After the sample was shaken for 1 h, 1 mL sample was taken with a pipette measure that has been sterilized and diluted from 10^{-1} to 10^{-7} by using a tube containing 9 mL of 0.85% sterile saline. Pipette 0.1 mL of the dilution 10^{-6} and 10^{-7} and pipetted onto the center of an agar media (Nutrient Agar) (Merck, USA). After that, 0.1 mL was spread over the agar surface by using Spread Plate Method. Briefly pass the ethanol-soaked spreader through the flame to burn off the alcohol and allow it to cool inside the lid of a sterile petri plate. After the entire surface of the plate has been covered, invert the plates and incubate for 24 to 48 h at 30°C.

2.3 Morphological characterization of bacteria

Identification of selected isolate for pure culture was studied based on different bacterial colony characteristics on agar media, gram staining, and microscope with 40x magnification. The morphological of colony bacteria including colony form, elevation, margin, texture, pigmentation were carried out based on Harley-Prescott (2002).

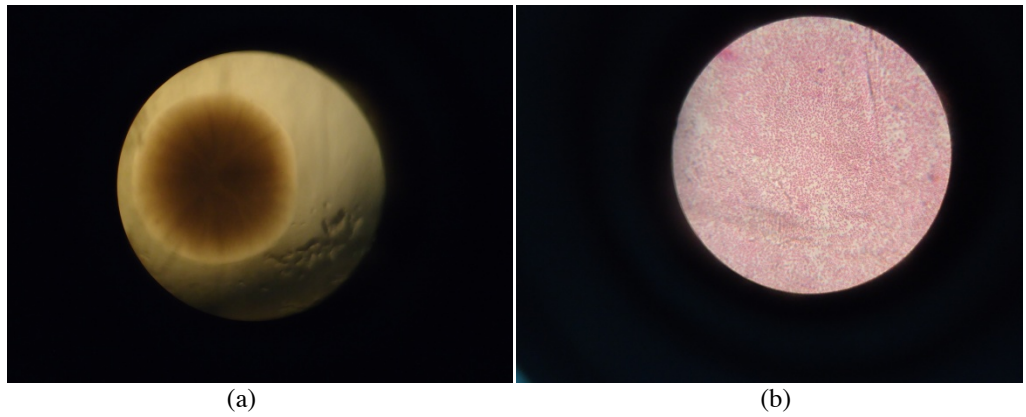
2.4 Screening of bacteria for diesel-degrading

Screening of bacteria for degradation of diesel was conducted using spread plate method based on Gayathri *et al*. (2014). Media for screening of pure culture used NA media (Nutrient Agar) (Merck, USA) and variation of diesel concentration. The concentrations of diesel were 2, 4, 6, 8, and 10% (v/v) plus the control plate without diesel. Prepare Nutrient Agar plates and spread with various concentration of diesel. After that, a sterile NA plate was be spreaded with one code of bacterial culture. All spreaded NA media were kept in incubator (Ogawa Seiki, Japan) at 30°C for overnight and observe result. If cultures show growth in the presence of diesel, it meant that culture have properties to degrade diesel. Overall, there were 8 pure cultures obtained from isolation bred to NA media and diesel. Qualitative and quantitative observations were conducted to determine the resistance of bacteria in various concentration of diesel. The qualitative was based on their ability to grow in medium containing crude oil (diesel) as carbon source (Roy *et al*., 2014). The quantitative observation was the percentage calculation that based on extensive growth of colony growth on agar media by using bacterial colony counter (Harley-Prescott, 2002).

3. Results and Discussion

3.1 Seawater sample properties

Collected seawater samples were analyzed for physical properties of seawater. The pH of diesel



(a) (b)

Figure 2. Morphological properties of bacterial code F: (a) colonies of bacterial code F in microscope and (b) gram-negative bacterium of bacterial code F

polluted seawater was 6.87 ± 0.015. The temperature was noted 31.67 ± 0.289°C and the salinity of seawater was 23,98 ± 0.043 ‰. The values of pH, temperature and salinity at the sampling sites are typical of tropical ocean in Indonesia. Comparing with earlier study showed that one of coastal waters in Indonesia (Jepara) has salinity range 28.44‰, pH value of water sample was 7.28 and temperature was 28.44°C (Sabdono, 2009). The difference occurs because the sampling location near to the fishing boat dock that can effect to the low salinity.

3.2 Isolation of bacteria

Based on the results, a total of fourteen numbers of colonies grew in one petridish and it called as mix culture. The pure culture was obtained by microcope to see the differents of colonies bacteria. All bacteria in mix culture were devided into 8 group of bacteria based on the observation using microscope in 40x magnification. Eight bacterial strains were isolated to identification morphological characterization and gram staining.

3.3 Morphological characterization of bacteria

The isolated of pure culture from seawater sample was named as A to H. Colony morphology microscopic observation reveled that the bacterial colony collected from seawater has different morphology (Table 1). Fig. 2 showed the morphological properties of bacterial code F in microscope and gram staining. Results showed the percentage presence of higher gram negative bacteria (62,5%) than gram positive bacteria (37,5%). The previous studies have also shown that the gram negative bacterial strains isolated from hydrocarbon contaminated seawater was higher than gram positive bacteria (Deng *et al*., 2014). Almost the color of all the bacterial colonies were white. The previous studies have also shown the white color of the bacterial colonies (Deng *et al*., 2014).

3.4 Screening of bacteria on diesel

Diesel oil is a complex mixture of hydrocarbons of different lengths ranging from C8 to C22 and

Table 1. Bacterial morphology

Character	Bacterial Code							
	A	B	C	D	E	F	G	H
Gram staining	-	+	-	-	+	-	+	-
Form	Rhizoid	Circular	Rhizoid	Circular	Circular	Circular	Circular	Circular
Elevation	Raised	Convex	Raised	Convex	Convex	Raised	Raised	Raised
Margin	Erose	Entire	Undulate	Entire	Undulate	Entire	Curled	Entire
Pigmentation	White	White	White	White	White	Yellow	White	White
Texture	Rough	Smooth	Rough	Smooth	Smooth	Smooth	Smooth	Smooth
Optical property	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Appearance	Dull	Shiny	Shiny	Shiny	Shiny	Dull	Dull	Dull
Diameter (cm)	0,4	0,3	0,2	0,6	0,3	0,3	0,4	0,3

Table 2. Growth of Bacterial Strains on Nutrient Agar Plates in the Presence of Diesel at Day 7

Bacterial code	Concentration of diesel (%)				
	2	4	6	8	10
A	++	++	++	+	+
B	+++	+++	+++	+++	++
C	+++	++	+++	++	++
D	++	++	++	++	++
E	+++	+++	+++	+++	+++
F	++	++	++	++	++
G	++	++	++	++	++
H	++	++	++	++	++

Key: + = less growth, ++ = moderate growth, +++ = heavy growth, - = no growth

includes paraffins, olefins, and aromatic compounds. With the variation of hydrocarbon compounds in its composistion, diesel stands as a great substrate for screening hydrocarbon degrading capababilities in microorganism (Naila, 2015).

The ability to utilize diesel degradation capability was determined by observing growth in NA media and diesel as carbon source. The most successful isolate was selected for studying effects of different concentration of diesel. The eight isolates were individually cultured in NA media containing different concentration of diesel (2, 4, 6, 8, and 10%) for up to 7 days at 30^{o}C. The effect of varying concentrations of diesel was tested to find the optimum concentration for the isolate. The perentage of growth bacteria from each isolate was calculated from plates with bacterial colony counter.

The results of qualitative observation showed in Table 2. From the eight isolates, bacterial code B showed the highest growth in diesel of varying concentrations and bacterial code E showed the next highest growth in diesel. Thus, the distribution of bacterial isolates obtained from the sampling source indicates the common occurrence of metabolically active strains in areas that are contaminated with hydrocarbons strongly suggested the ability of these bacteria to utilize the hydrocarbons as their carbon and energy source (Bhasheer *et al*., 2014).

The results of quantitative observation showed in Table 3. Based on percentage of growth bacteria, the percentage showed almost all bacterial isolates has percent growth of more than 50% for all concentrations. This confirms that all the isolates were capable of degrading diesel. It has been shown that there is a high correlation between cellular growth and diesel assimilation in microbes (Bhasheer *et al*., 2014).

Generally, the entire carbon source is assimilated by the bacterium for growth and reproduction. For bacterial growth diesel acts as a carbon source but at certain concentration, diesel can be toxic to microorganisms due to the solvent effect of diesel which destroys cell membrane.

This result was comparable to the result observed by Naila (2015) and Shukor *et al*. (2009). Generally, the entire carbon source is assimilated by the bacterium for growth and energy and an increase of percentage is regarded as an indicator of degradation with higher percentage correlating with higher amount of diesel being degrading. The high concentration of diesel could be harmful to the bacterial population in enclosed system due to its solvent effect that may damage the bacterial cell membrane. In the Naila (2015) observation, the highest concentration tested was 6% (v/v) diesel. Therefore, the tolerance of diesel for the bacterial may exceed 6% (v/v). A study showed some growth up to a concentration of 10% (v/v) diesel. This may be considered as a high hydrocarbon concentration, with a potential toxicity towards most of the microorganisms. The isolates, purified through this study should tolerate high hydrocarbon concentrations and their potential toxicity. Indeed, the high selection pressure should lead to the isolation of suitable isolates for the bioremediation of oil hydrocarbons (Aldisi *et al*., 2016).

Table 3. Percentage of Growth Bacteria at Day 7 using Bacterial Colony Counter

Diesel concentration	Bacterial code							
	A	B	C	D	E	F	G	H
2%	75,64%	89,21%	82,55%	63,83%	90,21%	79,73%	70,75%	75,96%
4%	76,65%	85,11%	74,76%	69,86%	86,85%	76,68%	63,57%	77,05%
6%	65,09%	82,34%	81,24%	62,41%	84,66%	77,65%	72,87%	74,87%
8%	44,06%	86,44%	75,01%	58,23%	81,37%	72,36%	58,62%	60,67%
10%	47,70%	76,98%	68,14%	54,31%	81,86%	75,81%	52,77%	58,26%

Based on the results of gram staining, bacterial code B was predicted as the genus of *Micrococcus* because the shape of bacteria was coccus. Bacterial code E was predicted as the genus of *Bacillus* because this bacteria has rod-shaped. Identification of bacteria will be identified for further study by using biochemical/biomolecular.

4. Conclusion

The present study was designed to identify endurance the diesel degrading microorganism from the diesel polluted seawater. The seawater samples were serially diluted to find the bacterial colony. The isolated colonies were devided into group based on the morphological characterization and gram staining. Eight group of colony were tested in NA media containing various concentration of diesel. According to the screening studies it was concluded that bacterial code B and E have shown higest percentage of bacterial growth in 7 days. It was indicating that two bacterial code were more resistance with diesel compared with six other isolate bacteria. Isolation of bacterial code B and E have potential to be used for diesel bioremediation and bacterial identification for the next study to determine the spesies of those bacteria.

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