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RESEARCH ARTICLE

Biodegradation of crude oil by *Ralstonia pickettii* under high salinity medium

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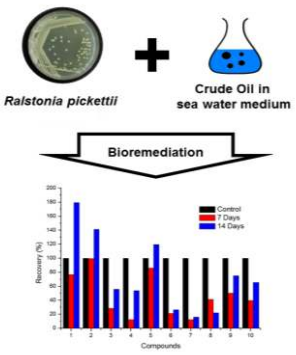
Graphical abstract



Abstract

Bacterium *Ralstonia pickettii* has ability to survive and thrive in low nutrient condition as well as a capability to remediate some pollutants and using them as carbon and energy source. In this study, the ability of *R. pickettii* on biodegradation of crude oil under high salinity medium was investigated. *R. pickettii* was pre-incubated in nutrient broth (NB) medium and then, washed and transferred to artificial seawater medium. Crude oil was added to each culture and incubated for 7 and 14 days. The biodegradation of crude oil was analysed using Gas chromatography mass spectrometry (GC-MS). The result showed that *R. pickettii* had successfully degraded the crude oil in the high salinity artificial seawater. The incubation on 7 and 14 days did not show a significant effect on the number of the degraded compounds. The optimum recovery percent was obtained from the derivation of 2,6,10,14-tetramethyl hexadecane with the recovery percentage of 12.7% and 16.0% for 7 and 14 days respectively. This study indicates that *R. picketti* can be potentially used for bioremediation of crude oil under high salinity environments.

Keywords: Biodegradation, *Ralstonia pickettii*, crude oil, high salinity medium

INTRODUCTION

The exploration activity of petroleum hydrocarbon has been accompanied by accidental spills and chronic pollution in marine ecosystems (Mapelli et al., 2017). More than 90% of oil pollution comes from non-accidental natural and anthropogenic sources, including routine ship operations and also run off from land-based sources (Li et al., 2016; Daffonchio et al., 2013). Oil spills pollutions may contain various hydrocarbons and several polycyclic aromatic compounds (Onwurahet et al., 2007). Major hydrocarbon classes found in oil spills are normal alkanes, branched alkanes, cycloalkanes, isoprenes and aromatics. Some priority contaminants of oil spills contain some compounds such as benzene, hexane, heptane, isobutane, isopentane, polycyclic aromatic hydrocarbon (PAHs), BTEX (benzene, toluene, ethyl benzene and xylene), resin and asphaltenes (Plaza et al., 2008).

Oil spills can have extensive environmental impact in the marine environment that may affect organisms found therein by direct toxicity or by physical smothering (Perry and Guthrie, 1980). Oil spills generally, can cause various damages to the marsh vegetation. The component of hydrocarbon derived from petroleum is toxic and interfere the reproduction, development, growth, and behaviour of marine biota, even causes death from a number of marine biota (Onwurahet et al., 2007). Oil spilled at sea forms a surface slick that usually becomes more viscous and creates water-in-oil emulsion. Oil in water causes depletion of dissolved oxygen due to transformation of the organic component into inorganic compounds, loss of biodiversity through a decrease in amphipod population that is important in food chain, and eutrophication. Short-term toxicity in fishes includes lymphocytosis, epidermal hyperplasia, haemorrhagic septicaemia (Beeby, 1993), whereas in mammals, it possesses an anticoagulant potency (Onwurahet et al., 2002). It was estimated that some tens of thousands of seabirds were killed as a result of spilled oil in sea (Dunnet, 1982).

Several techniques are developed for the oil spill response including mechanical recovery, burning, solidifiers, dispersants and bioremediation (Dave and Ghaly, 2011). One of the preferred methods is bioremediation since it does not contain side effects (using natural biological agents) and is able to be performed *in situ* (Marquez-Rocha, 2000). On the other side, bioremediation is a cost-effective and sustainable biotechnology for the treatment of contaminated coastal and marine sites (Kumar et al., 2011). Generally, bioremediation involves the use of microorganisms and their enzymes for the degradation and transformation of pollutants into another form which less toxic to the environment (Dua et al., 2002). Some studies about bioremediation of oil by bacteria have been reported. Shahaby et al. (2015) reported that *Actinomyces odontolyctus* AF-104-MH, *Bacillus thuringiensis* AT5, *Pseudomonas aeruginosa* AF11-GT, and *Pseudomonas stutzeri* AT 11 have the capability to degrade petroleum oil. Besides, Marzan et al. (2017) reported that *P. aeruginosa*, *Bacillus* sp., and *Serratia* sp. have significant bioremediation potential for oil spillage accident. The ability of bacteria to degrade petroleum oil pollutants is based on their capability to consume hydrocarbon compound in petroleum oil as carbon sources for their metabolism to produce energy (Paniagua-Michel and Rosales, 2015).

One of the bacteria that is able to be used as bioremediation agent is *Ralstonia pickettii*. Some studies about pollutant bioremediation by *R. pickettii* have been reported. Bucheli-Witschel et al. (2009) reported that *R. pickettii* PKO1 has capability to degrade benzene in the presence of the alternative substrate succinate. Zhang et al. (2011) reported that *R. pickettii* L2 able to degrade chlorobenzene (CB) as the sole source of carbon and energy. Plaza et al. (2005) reported that *R. pickettii* SRS combined with *Alcaligenes piechaudii* SRS was able to degrade hexane (one of aliphatic hydrocarbons) by 80%. In addition, these bacteria can also produce biosurfactants. The ability of *R. pickettii* to degrade pollutants is also supported by their ability to produce biosurfactants to enhance their bioremediation ability (Plaza et al., 2005). Biosurfactant plays an important role in bioremediation application in oil-contaminated environments (Whanget et al., 2008). Moreover, *R. pickettii* is capable to degrade many aromatic hydrocarbons and using them as both carbon and energy source. *R. pickettii* has ability to survive and thrive in low nutrient condition and has never been detected as a phytopathogen or as an animal pathogen (Ryan et al., 2007). Furthermore, Hundt et al. (1998) reported that *R. pickettii* SBUG 290 has the ability to degrade biaryl compounds in mineral salt medium. It was indicated that *R. pickettii* has bioremediation ability under low nutrient conditions that could have potential to be a bioremediation agent in low nutrient condition (Whanget et al., 2008).

Based on studies that have been done recently, *R. pickettii* has never been grown in the high salinity water. Thus, it is very important to investigate its ability in the high salinity medium (artificial ocean water). In this study, the capability of *R. pickettii* on bioremediation of oil spills in high salinity medium (artificial ocean water) was investigated. The bioremediation of oil spills by *R. pickettii* was monitored using gas chromatography mass spectrometer (GC-MS).

EXPERIMENTAL

Materials

The crude oil in this study was collected from Oil Site in Cepu-Indonesia. Bacteria culture *R. pickettii* NBRC 102503 was purchased from NITE Biological Resource Centre (NBRC), Chiba, Japan. Nutrient Agar (NA) and Nutrient Broth (NB) were purchased from Merck, Darmstadt, German. Sodium Chloride (NaCl), Magnesium Chloride ($MgCl_2$), Sodium Sulphate (Na_2SO_4), Calcium Chloride ($CaCl_2$), Potassium Chloride (KCl), Sodium Bicarbonate (NaHCO), Potassium Bromide (KBr), Boric Acid (H_3BO_3), Strontium Chloride ($SrCl_2$), Sodium Fluoride (NaF) were purchased from SAP chemicals, Indonesia. Dichloro diphenyl trichloroethane (DDT) as internal standard was purchased from Tokyo Chemical Industry Co. Methanol, *n*-hexane and acetone were purchased from Anhui Fulltime Specialized Solvent & Reagent Co., Ltd (Anhui, China).

Preparation of artificial sea water

Artificial Seawater was prepared based on ASTM D1141-98, "Standard Practice for the Preparation of Substitute Ocean Water". Artificial seawater (1 L) was prepared by following this method: 24.5 g NaCl and 4 g Na_2SO_4 anhydrous were dissolved in 800 mL water. Stock solution A (20 mL) ($MgCl_2.6H_2O$ 55.6 g L^{-1}; $CaCl_2$ 57.9 g L^{-1}; $SrCl_2.6H_2O$ 2 g L^{-1}) was added slowly with vigorous stirring. Then, 10 mL of stock solution B (KCl 69.5 g L^{-1}; $NaHCO_3$ 20 g L^{-1}; KBr 10 g L^{-1}; H_3BO_3 2.7 g L^{-1}; NaF 0.3 g L^{-1}) was added. Artificial seawater was diluted to 1 L and the pH was adjusted to 8.3 by adding NaOH 0.1 N in which the pH range of seawater is 7.5-8.4 (Hundt et al., 1998).

Preparation of liquid culture and *R. picketti* regeneration

Bacterium culture *R. pickettii* NBRC 102503 was maintained on NA, which was incubated at 37°C. The colony was inoculated into 10 mL NB in Falcone tube. The cultures were pre-incubated at 37°C for 30 hours with incubator shaker at 180 rpm.

Bioremediation of petroleum waste

Pre-incubated culture bacterium was centrifuged for 10 minutes, decanted and replaced with 10 mL sterile water medium. The sterile water medium was decanted and replaced by 10 mL sterile artificial seawater. Furthermore, 50 µL crude oil was added to each inoculated flask. Each flask was flushed with oxygen and sealed with a glass stopper and sealing tape to prevent sample volatilization. The cultures were incubated statically for 7 and 14 days at 30°C. As a control, the cultures were terminated by autoclaved (121°C, 15 min) after pre-incubation. The experiments were performed in triplicate.

Bioremediation of petroleum waste

After incubation process, the culture was homogenized with 10 mL of methanol and 50 µL of DDT 5 mM in dimethyl sulfoxide (DMSO) (final concentration 0.25 µmol) as internal standard. The mixture was washed with 5 mL of acetone and centrifuged at 3000 rpm for 10 min. The biomass and supernatant were separated. The supernatant was filtered with Whatman filter paper No. 41. The filtrates were evaporated at 64°C and extracted with 100 mL *n*-hexane and the organic fractions were collected and dried over anhydrous sodium sulphate. The extracts were evaporated at 68°C and concentrated to dryness under reduced pressure. The extracts were analysed using GCMS. GCMS was performed using an Agilent Technologies 7890 GC System linked to an Agilent Technologies 5975C VL MSD Detector with a 30 m × 50 µm × 0.25 µm Agilent 19091S-433 column. The oven temperature was programmed to start from 80°C and held for 2 min and then, the temperature was increased to 280°C at 5°C min^{-1} and held for 15 min.

RESULTS AND DISCUSSION

Preparation of artificial sea water

In this study, the bioremediation of crude oil by *R. pickettii* under high salinity medium was investigated. The medium used in the process of crude oil remediation was artificial seawater. Meanwhile, the original seawater from the ocean contains impurities and other microbes that can disrupt the study. Artificial seawater was used as a medium in crude oil bioremediation to simulate the capability of *R. pickettii* on oil spills remediation in marine environment, although artificial seawater has low nutrients.

Bioremediation of crude oil by *R. pickettii*

The ability of *R. pickettii* to degrade crude oil under high salinity medium was investigated using GCMS. The result of GCMS analysis is shown in Figure 1. Figure 1 shows that chromatogram of control has higher intensity than that of 7 and 14 days' treatments. The decreased of chromatogram intensity in 7 and 14 days' treatments indicated that *R. pickettii* was able to degrade crude oil compounds under high salinity medium.

This result is similar with the previous research by Plaza et al. (2008). Plaza et al. (2008) reported that R. *pickettii* SRS is able to degrade crude oil in sterile mineral medium. Besides, *R. pickettii* is also reported has the ability to degrade some pollutant compounds. Al-Zuhair (2012) reported that *R. pickettii* has been successfully used to degrade phenolic compounds. Bucheli-Witschel (2009) reported that *R. pickettii* is able to degrade benzene compounds. Besides, *R. pickettii* also able to degrade lantadene (pentacyclic triterpenoid hepatotoxin, Sharma et al., 1997) as well as DDT (Setyo et al., 2018).

The ability of *R. pickettii* to degrade crude oil might be associated with their ability to produce some enzymes for their metabolism. Some bacteria have the ability to produce degradative enzymes such as oxidoreductases, peroxidases, hydrolases and laccases, which are actively involved in bioremediation process (Sharma et al., 2018). Some studies reported the involvement of enzymes on pollutants bioremediation by *R. pickettii*. Zhang et al. (2011) reported that cathechol-1,2-dioxygenase enzyme is involved in chlorobenzene degradation by *R. pickettii* L2. Hatta et al. (1999) reported that hydroxyquinol 1,2-dioxygenase enzyme is involved in 2,4,6-trichlorophenol degradation by *R. pickettii* DTP0602. Riyan et al. (2007) also reported that *R. pickettii* also able to produce hydroxylase enzyme of 2,4,6-trichlorophrnol-4-dechlorinase, which plays the role in the degradation of thrichlorophenol. It indicated that the pollutants degradation by *R. pickettii* is involving enzymatic process.

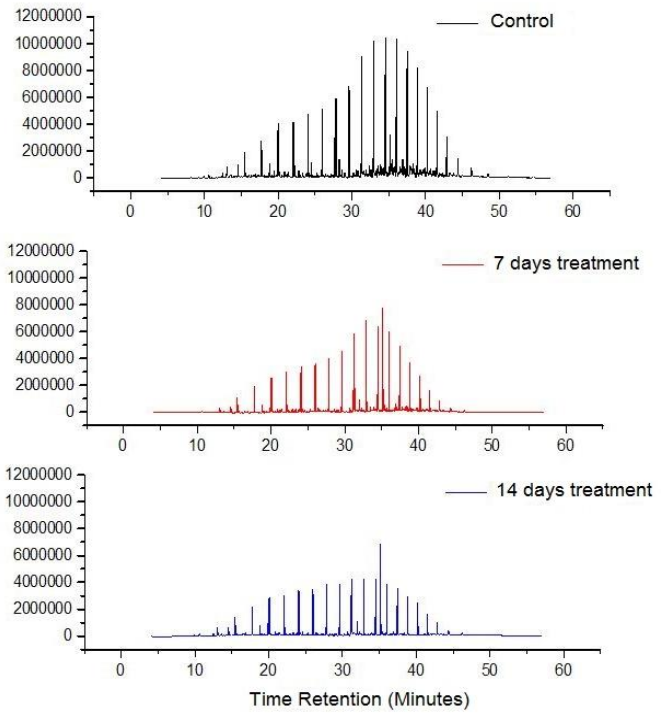


Fig. 1 Chromatograms of crude oil degradation, (a) control, (b) 7 days' treatment (c) 14 days' treatment.

In addition to the enzymatic degradation mechanism, *R. pickettii* can also degrade petroleum compounds by producing biosurfactants. Plaza et al. (2005) reported that *R. pickettii* also able to produce biosurfactants, which involved on bioremediation of soil contaminated with petroleum hydrocarbon. Biosurfactants also play the role in pollutants bioremediation by microorganisms (Banat et al., 2010). Biosurfactants can reduce the surface tension and oil interface using high salinity water. The decrease of petroleum surface tension can increase petroleum solubility so that it is easier to be degraded (Plaza et al., 2008).

Crude oil recovery

The crude oil recovery is shown in Figure 2**.** The result of chromatogram data was plotted in the diagram of petroleum ratio recovery towards the degradation time (7 and 14 days) in order to find out the amount of petroleum components recovered after the process of bioremediation. The amount of the recovered compounds indicated the number of the compound that can be degraded. The lower the recovery of the compound, the higher the compound can be degraded. In this study, some crude oil compounds were detected such as tetradecane; dibenzothiophene; hexadecanoic acid; 9,12-octadecadienoic acid; heneicosane; tricosane; 2,6,10,14-tetrametyl hexadecane; N-(2-trifluoromethylphenyl)-pyridine-3-carboxamide, oxime; tetratetracontane; and tetracosane. As shown by Harayama et al. (1999) and Mc Genity et al. (2012), crude oil is a natural heterogeneous mixture of hydrocarbons such as long-chain aliphatic hydrocarbons, carboxylic acid compounds, branched hydrocarbon compounds, aromatic hydrocarbons and pyridine compounds.

Based on Figure 2, most of the total crude oil components recovery on day 14 was higher than day 7, especially for alkane compounds such as tetradecane. In 7 days' incubation, simple structure compounds such as alkane compounds of C14-C16 was initially degraded, afterwards, during the 14th day of degradation, long chain alkane compounds of C24-C44 begun to degrade into alkane with shorter hydrocarbon chain such as C14-C16, which resulted in an accumulation of the compound with simpler structure. As reported by Leahy and Colwell (1990), microbial degradation of oil involves attacking on aliphatic or aromatic fractions of the oil; with high molecular weight compounds are converted to simpler or low molecular compounds. Thus, the accumulation of lower molecular compounds of crude oil was higher in 14 days' incubation.

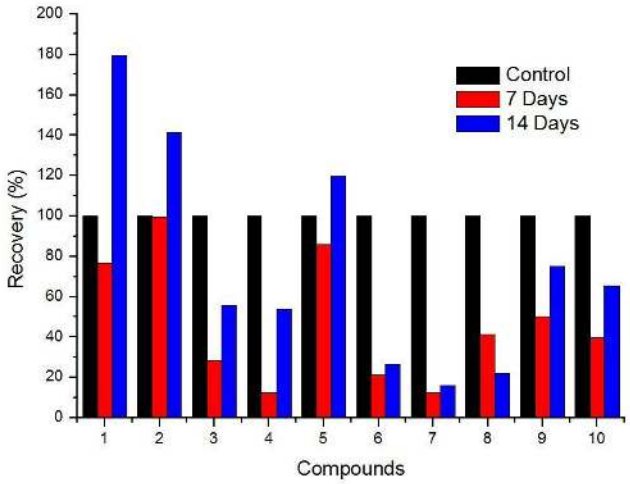


Fig. 2 Recovery of crude oil (Name of each of compounds is described in Table 1).

Table 1 shows some compounds that can be degraded by *R. pickettii* during incubation period of 7 and 14 days. Majority of the petroleum components are long-chain aliphatic hydrocarbon compound of C14-C44, carboxylic acid compound (hexadecanoic acid), branched hydrocarbon compound (2,6,10,14-tetramethyl- hexadecane), aromatic hydrocarbon (dibenzothiphene), and pyridine (N-(2-trifluoromethylphenyl)-pyridine-3-carboxamide, oxime).

Table 1 Recovery percentage of the compounds from the degraded crude oil.

Compounds	Recovery (%)	
	7 days	14 days
Tetradecane	76.5	179.2
Dibenzothiophene	99.8	141.4
Hexadecanoic acid	28.4	55.7
9,12-octadecadienoic acid	12.4	53.6
Heneicosane	85.9	119.8
Tricosane	21.3	26.6
2,6,10,14-tetramethyl- hexadecane	12.7	16.0
N-(2-trifluoromethyl phenyl)-pyridine-3-carboxamide, oxime	41.2	21.9
Tetratetracontane	49.9	74.8
Tetracosane	39.5	65.2

The compound with optimum recovery at degradation time of 7 and 14 days was 2,6,10,14-tetramethyl hexadecane with the recovery of 12.7% and 16.0%, respectively. This petroleum component is a branched hydrocarbon compound that is classified as a compound with quite resistant or difficult to be degraded by some microorganisms (Rontani, 1997). It can be assumed that *R. pickettii* is one of the microorganisms that can use branched hydrocarbon compound as the source of carbon and energy.

According to Silva et al. (2007), the degradation mechanism of 2,6,10,14-tetramethyl hexadecane also known as phytane, can be performed by oxidation mechanism of the mono alkyl group or di-terminal with the aid of oxygenase enzyme. The oxidized alkyl group turns into a primary alcohol group then the group is oxidized back into the form of carboxylic acid and may be further oxidized into a short-branched amino acid. The short-branched amino acid can be further metabolized through oxidation involving enzymatic formation by carboxylation and decarboxylation as well as production of carboxylic acid by β-oxidation processes.

The most recovered compound (very little amount of degradation) is dibenzothiophene with the recovery of 99.6% for 7 days. The compound is a cyclic aromatic one that owns a very stable bond, thus, it is difficult to change into a simpler form.

CONCLUSION

R. pickettii is capable of degrading some of the petroleum components, such as long-chain aliphatic hydrocarbon C14-C44,

carboxylic acid compound, branched hydrocarbon compound, aromatic hydrocarbon, and pyridine. The optimum recovery was obtained from 2,6,10,14-tetramethyl hexadecane during 7 days' incubation. This study indicates that *R. picketti* can be potentially used for bioremediation of crude oil under high salinity environments.

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