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Research Article

Oxygenase Enzyme Activity and Compound Profile in Hydrocarbon Bioremediation by *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* Consortium

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Abstract

Hydrocarbon contamination is one of the most significant environmental problems, including in the marine environment. Several methods, such as chemical and physical remediation, have been constructed to remediate pollutants but remain side effects and have a relatively high cost. Bioremediation is an alternative method for degrading contaminants due to its eco-friendliness and low cost. During bioremediation, the enzyme oxygenase is produced by bacteria to degrade pollutants. However, the oxygenation enzyme's activity and compound profile during this bioremediation process are still sparsely explored. This study aimed to analyze oxygenation enzyme activity and compound profiles during the hydrocarbon bioremediation process by bacterial consortia of *Pseudomonas aeruginosa* and *Rhodococcus erythopolis*. The materials of this research were the oxygenase enzyme activity, compound profile, and amount of BOD and COD during the bioremediation process. The results showed that the monooxygenase enzyme has activity between 0.257 and 3.859 U/mL. Meanwhile, the dioxygenase enzyme has activity between 0.579 and 5.402 U/mL. The final incubation compound profile found that Hexadecanoic acid methyl ester ($C_{17}H_{34}O_2$) decreased by up to 47.66%. Moreover, BOD and COD reductions were 68.59% and 67.51%, respectively. This study concluded that oxygenation enzymes were produced to degrade pollutant compounds during the hydrocarbon bioremediation process. Further research is needed to improve the effectiveness of bioremediation by enhancing enzyme activity.

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1. Introduction

Nowadays, environmental contamination has been increasing alarmingly. Massive amounts of organic and inorganic compounds are introduced into the environment endlessly because of human needs, technological advances, and irresponsible application of agrochemicals in agricultural activity (Adipah *et al*., 2018; Ogbuka *et al*., 2022). One of the contaminants that have been paid attention to by scientists worldwide is petroleum hydrocarbon. Environmental pollution caused by petroleum hydrocarbon has become a severe problem worldwide. While petroleum hydrocarbon is helpful for human life, it causes problems due to its toxicity if it leaks into natural environments such as marine and soil. One of the oil spill accidents occurred in Gulf of Mexico. The oil spill incident from BP (British Petroleum) on the Gulf Coast of Mexico from April to July 2010 caused almost 600,000 tons of crude oil to spill along the Gulf Coast (Gao *et al*., 2023). All these spills have caused tremendous damage to ecological and environmental systems, especially to many plant species, a wide array of animals, and human health, altering the coast's aesthetics for tourism and recreation. In addition, some compounds that are classified as PHCs (petroleum hydrocarbons) are better known as BTEX (benzene, toluene, ethylbenzene, and xylene) (Asemoloye *et al*., 2020; Sirotkin and Harrath, 2017). In terms of harmful effects on health, benzene is one of the most concerning compounds due to its carcinogenic effect. Long-term exposure to benzene may cause bone marrow abnormalities. Ethylbenzene is considered carcinogenic since its inhalation increases the incidence of renal, testicular, and liver tumors. Toluene and xylene exhibit acute and chronic toxicity in the central nervous systems of humans and animals (Adipah *et al*., 2018; Varjani and Upasani, 2021; Yaashikaa *et al*., 2019).

Bioremediation is a pollution remediation process using microorganisms (Chen *et al*., 2019; Masithah *et al*., 2011). Bioremediation aims to break down or degrade pollutants into less toxic or non-toxic materials (Dzionek *et al*., 2016). Bioremediation is a method that utilizes living creatures' capability to diminish, deteriorate, and eliminate environmental pollutants, including marine and terrestrial ecosystems, thereby decreasing the harm to human health by rehabilitating the ecosystem to its normal condition. The foundational concepts of bioremediation require reducing the solubility, redox reactions, and adsorption of contaminants from the polluted environment (Kurniawan and Fukuda, 2023). The achievement of this method also relies on the origin of the pollutant, which may be sewage, nuclear waste, greenhouse gases, agrochemicals, heavy metals, and hydrocarbons (polycyclic aromatic hydrocarbon). Bioremediation technologies are based on redox reactions (chemical oxidation and reduction reactions). These reactions alter the chemical compound with the addition of reagents, producing an increase in the degradation and removal of contaminants, transforming them into less toxic, immobile, or inanimate compounds (Emanuele *et al*., 2017; Kriwy and Uthicke, 2011; Roche *et al*., 2017; Tam *et al*., 2009).

During the bioremediation process, enzymes produced by microorganisms modify toxic pollutants by changing the chemical structure of the pollutant (Karigar and Rao, 2011). Bioremediation is highly dependent on microbes (Azubuike *et al*., 2016). The bioremediation mechanism has the aim of increasing the rate of decomposition, inactivating microbes that can survive the presence of toxic pollutants, and utilizing these microbes to decompose pollutants so that they become elements that are beneficial to the surrounding environment (Paglarini *et al*., 2019). According to the implementation site, bioremediation technologies can be divided into two groups. They are *ex-situ* or *in-situ* bioremediation (Dzionek *et al*., 2016; Emanuele *et al*., 2017). The *in-situ* bioremediation process suggests a purification treatment of pollutants at their origin (without transferring the pollutant to another place). The excavation process is not required, generating little or no destruction in soil formation. This bioremediation has been profitably applied in the treatment process of hydrocarbons, heavy metals, and dyes (Kapahi and Sachdeva, 2019).

On the other hand, *ex-situ* bioremediation requires the excavation of the contaminants and, afterward, transferring them to another treatment place. Hence, to determine the appropriate application method that should be used, specific considerations such as the pollutant's classification, the pollution rate, and the contaminated site's typology must be examined. The common *in-situ* and *ex-situ* physicochemical treatments for remediation of petroleum hydrocarbons (PHCs, known as fossil fuel) need costly strategies and frequently result in the incomplete degradation of the pollutants. Consequently, in the last two decades, alternative purification technology-dependent biological treatments have been increasingly accepted as standard practice since they are more efficient in optimizing side products and conserving natural resources, as well as being more cost-effective) (Jabbar *et al*., 2022; Olukanni *et al*., 2014).

One of the roles of bioremediation is to remediate hydrocarbon pollution. Hydrocarbon compounds are

dangerous environmental pollutants (Abdel-Shafy and Mansour, 2016). One source of pollutant hydrocarbon compounds in the sea is the activity of dismantling ships in coastal areas (Tornero and Hanke, 2016). Total petroleum hydrocarbons (TPH) can be classified into three categories: aliphatic, alicyclic, and aromatic. In addition, aromatic compounds can be polyaromatic or monoaromatic (Sirotkin and Harrath, 2017). The components of monoaromatics are benzene, toluene, ethylbenzene, and xylem isomers (Prenafeta-Boldú *et al*., 2002). Petroleum hydrocarbons' natural degradation is a complicated process that relies on the natural environment and the availability of petroleum hydrocarbons (the number of hydrocarbons in oil-contaminated hydrocarbons). Several hydrocarbon degradation variables have been investigated (Kebede *et al*., 2021). The limitation of the availability of microorganisms is one of the essential factors that influence the natural degradation of oil contamination in the natural environment. Petroleum hydrocarbon composition attaches to soil constituents, which are hard to diminish or purify. Therefore, hydrocarbons differ in their vulnerability to microbial treatment. The sensitivity of petroleum hydrocarbons to microbial removal can be generally arranged as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Varjani and Upasani, 2021). Several constituents may not be removed at all, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs).

Degradation by using microorganisms is the prime and most sophisticated natural treatment by which one can purify petroleum hydrocarbon contaminants from the natural environment. The identification of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reported by Das and Chandran *et al*. (2011). They observed the immense bioremediation of alkyl aromatics in marine sediments, which happened especially to distinguishable biodegradation of the n-alkane group of the petroleum hydrocarbon fractions, and the biological agents, namely, *Rhodococcus, Sphingomonas, Pseudomonas, Mycobacterium, Burkholderia, and Arthrobacter*, were discovered to be in charge of alkyl aromatic degradation. The biodegradation of petroleum hydrocarbons in a contaminated tropical stream in Lagos, Nigeria, was reported (Mustapha and Halimoon, 2015). Various bacterial strains, namely *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Alcaligenes* sp., *Acinetobacter lwoffi*, *Flavobacterium* sp., *Micrococcus roseus*, and *Corynebacterium* sp., were used from the polluted streamer ecosystem, which could decrease petroleum hydrocarbons (Geetha *et al*., 2018; Luo *et al*., 2022; Rahman *et al*., 2022).

In bioremediation, the main principle is to use the metabolism of microorganisms (dos Santos *et al*., 2011). Microorganisms produce enzymes to degrade and utilize hydrocarbons as a source of carbon and energy (Das and Chandran, 2011). Microorganisms utilize hydrocarbons as carbon substrates, increase their population, and degrade them into harmless compounds such as CO_2 and H_2O (dos Santos *et al*., 2011). Microorganisms capable of degrading compounds in hydrocarbons are known as hydrocarbonoclastic bacteria that can express oxygenase enzymes. Oxygenase is a hydrocarbon-oxidizing enzyme (Włóka *et al*., 2017). In recent years, the term has been used to refer to certain bacteria that can utilize almost exclusively hydrocarbons as a source of energy and carbon, generally called hydrocarbonoclastic bacteria. A unique group of oil-degrading marine gammaproteobacterial species, the marine hydrocarbonoclastic bacteria belonging to the Oceanospirillales, has received attention for several years, and it is common sense that these bacteria play an essential role in the natural degradation of major marine pollutants, including marine crude oil. These bacteria immobilize a couple of organic acids (acetate and pyruvate) and instead feed on various aliphatic hydrocarbons. Several of these remarkable marine bacteria, including *Alcanivorax, Oleispira, Oleiphilus, Neptunomonas, Thalassolituus, Marinobacter, and Cycloclasticus* species, have been tracked down in the sea environment all over the world, always appearing in very small abundances (Amin *et al*., 2022; Chettri *et al*., 2022; Radwan *et al*., 2019; Syakti *et al*., 2019).

Pseudomonas aeruginosa and *Rhodococcus erythopolis* can degrade hydrocarbon waste (Das and Chandran, 2011). Both bacteria are hydrocarbonoclastic bacteria. Microorganisms that can produce biosurfactants are hydrocarbonoclastic marine bacteria (Syakti *et al*., 2019). These bacteria are found in marine waters contaminated with oil and could degrade hydrocarbon compounds (Dwinovantyo *et al*., 2016). Several pieces of information also mention that *Pseudomonas* is the most popular biological agent capable of using hydrocarbons as carbon and energy sources and producing biosurfactants. *P. aeruginosa* is largely examined for generating glycolipid-type biosurfactants among the Pseudomonas family. However, glycolipid-type biosurfactants are also reported from other species like *P. putida* and *P. chlororaphis*. Biosurfactants are involved in increasing oil surface area, and that amount of oil is available for bacteria to use for energy (Chachina *et al*., 2016; Chettri *et al*., 2022; Silva *et al*., 2020). In addition, several researchers have also observed that biological agents from the genus *Rhodococcus* could degrade petroleum hydrocarbon fractions, including

long-chain hydrocarbons. *Rhodococcus* also assists in the ability to overhaul harmful competencies such as phenanthrene. Furthermore, *Rhodococcus* has been utilized and proposed for the construction of a purification method in petroleum hydrocarbon-contaminated environments due to its ability to degrade oil in the oil-polluted environment (Pi *et al*., 2017; Shintani *et al*., 2019).

Oxygenase enzymes are divided into several groups, such as monooxygenase enzymes and dioxygenase enzymes (Silva *et al*., 2020). Bacteria use oxygenase enzymes in direct contact with substrates containing hydrocarbons and thus can be used as carbon sources for their growth (Juhasz *et al*., 2014; Sujadi *et al*., 2020). Oxygenase is an enzyme produced by bacteria in the biodegradation of hydrocarbons. Moreover, the ability of bacteria to degrade hydrocarbons is seen in the pattern of decreasing hydrocarbon concentration and in the degradation process of the different types of compounds. Microbial creatures reveal various functional processes correlated to crude oil removal, such as having numerous catabolic genes, which improve the way to target petroleum hydrocarbons and break down hydrocarbon substrates. Several genes are involved in petroleum hydrocarbons' alteration and metabolism processes, such as alkB and alkJ, which encoding alkane monooxygenase and aliphatic alcohol dehydrogenase, respectively.

However, during the petroleum hydrocarbon bioremediation process, the activity of the oxygenase enzymes and the compound profile are rarely explored. Therefore, exploration related to this activity must be carried out to develop the most efficient biodegradation process in the future. This study aims to analyze the activity of oxygenation enzymes and compound profiles during the hydrocarbon bioremediation process by a consortium of bacteria, *Pseudomonas aeruginosa* and *Rhodococcus erythopoli*s.

2. Materials and Methods

2.1 Research Description

The bacteria were isolated from hydrocarbon-contaminated sea water in Prigi Harbor, Trenggalek City, East Java Province, Indonesia. Several examinations have been accomplished in analyzing oxygenation enzyme activity and compound profiles during the hydrocarbon bioremediation process. Oxygenase enzyme activity test, gas chromatography-mass spectrometry analysis, and Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) were conducted at the Central Laboratory of Life Sciences, University of Brawijaya, Indonesia.

2.2 Oxygenase Enzyme Activity Test

The initial stage of the test was that the sample was centrifuged at a speed of 5,000 rpm, then separated into the supernatant. Then, the pellet was suspended in 1 mL of 20 mM Tris-HCl buffer, pH 7.4. The pellets were then sonicated using an ultrasonic disintegrator and centrifuged for 10 minutes at 8,000 rpm at 4ºC. The supernatant obtained was then used to test the enzyme activity by the Bradford method (Kielkopf *et al*., 2020). The reaction mixture contained 20 mM Tris-HCl buffer, 0.1 mM NADH, hexadecane, or naphthalene solution (1% hexadecane or naphthalene in 80% DMSO), and a 20 µL crude extract of the enzyme. The reaction was started by adding 2 µL of hexadecane solution to the reaction mixture. The mixture was then homogenized using a vortex for three seconds and incubated for five minutes.

The absorbance value was obtained by measuring the sample at a wavelength of 340 nm (Huang *et al*., 2022). The number of enzymes is calculated using the following equation:

Absorbance of absolute test =
Absorbance of sample – Absorbance of blank ..Eq 1

$$\Delta A_{340} = \text{Absorbance of initial NADH} - \text{Absorbance of absolute test} \quad \text{...Eq 2}$$

$$\text{Enzym Activity}\left(\frac{U}{ml}\right) = \frac{\Delta A_{340} x V_e (mL)}{\alpha_{340} (mL\ \mu mol^{-1} cm^{-1}) x\ V_s (mL) x\ t\ (minutes) x\ l\ (cm)} \quad \text{...Eq 3}$$

α340 : Molar absorptivity of NADH (6,22 mL µmol^{-1}cm^{-1})
Ve : Total volume of reaction
Vs : Volume of sample
t : Incubation time
l : Pathlength (0,05 cm)

2.3 Analysis of Gas Chromatography-Mass Spectrometry

Analysis of the hydrocarbon compound profile was carried out using the GC-MS test. The GC-MS test was carried out before and after treatment. Hydrocarbon extraction is carried out to separate hydrocarbon compounds from the medium. Extraction of hydrocarbon compounds using the liquid-liquid chromatography method with hexane as the mobile phase.

One hundred ml of the sample was put into a separatory funnel. Then, 100 ml of hexane was put into a separatory funnel filled with liquid BSM medium. The separating funnel was shaken several times vigorously, then allowed to stand until two layers formed. The liquid medium (bottom layer) is removed from the separatory funnel and collected in an Erlenmeyer flask. The liquid medium was again extracted with the same procedure. Hexane was removed from the separatory funnel and filtered using filter paper sprinkled with 1 g of Na_2SO_4. The filtrate obtained is heated in an oven to evaporate the remaining hexane. After all the hexane has evaporated, the filtrate that has been obtained is weighed. The filtrate was dissolved with 1 ml of hexane, transferred to a vial, and then stored for analysis.

The extraction of hydrocarbon compounds from the liquid medium with bacterial isolates was conducted. However, it was centrifuged at 5,000 rpm for five minutes to separate the bacterial cells and reduce the emulsion during extraction. Gas chromatography analysis was performed with a Shimadzu GC-MS QP 2010 equipped with a flame ionization detector (FID) and a capillary column Rtx-1MS 100% dimethyl polysiloxane (30 m x 0.25 mm), J & W Scientific, SA, USA. Helium was used as the carrier gas, and the flow rate was maintained at 1.54 ml/min. One µl was injected at an injection port temperature of 280°C, oven column temperature 50°C, pressure 90.7 bar, column temperature maintained at 50°C for three minutes, then increased to 260°C for 10 minutes. Hydrocarbon compounds measured using GC-MS analysis can be grouped at a specific temperature range. The GC/MS instrument analysis resulted in Total Ionic Chromatogram (TIC) spectra showing each compound's percent area and MS spectra in lines with specific m/z values. The percentage area of each compound indicates the concentration of the compound. In contrast, the m/z value indicates the character of the detected compound, so the detected compound can be characterized by the approach method of fragmentation (Brattoli *et al*., 2013).

2.4 Analysis of Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD)

The BOD quantification was done using the iodometric method (SNI) (Indonesian Standard for Quantification of BOD). 6869.72-2.000). First, the water sample (500 mL) was diluted inside a container (beaker glass) using distilled water and then aerated for two hours until the volume reached 2.000 ml. After that, samples were removed into six bottles (Winkler bottles). Then 1 ml of manganese sulfate and 1 ml of alkaline iodide were put in the Winkler BOD bottle from day 0 to day 5. The solution was allowed to stand for 5-10 minutes until lumps formed; 5 ml of concentrated sulfuric acid was added and then homogenized. Finally, 50 mL of the sample was dripped with a starch indicator and titrated with sodium thiosulfate until the blue color disappeared and the titrant volume was obtained. The following formula was used to calculate dissolved oxygen (DO) and BOD:

$$BOD = DO_0 - DO_5 \quad \text{...Eq 4}$$

$$DO\ (mg/L) = \frac{V\ Thiosulfat \times N\ Thiosulfat \times 1000 \times BeO_2 \times P}{V\ Sampel} \quad \text{..Eq 5}$$

Where V is volume, DO_0 is dissolved oxygen at day 0, DO_5 is dissolved oxygen at day 5, BeO_2 is the oxidation number of BeO_2 (8), and P is dilution.

COD determination was carried out using spectrophotometry according to SNI (Indonesian Standard for Measurement of COD). 6869.72-2009. The 100 ml sample was added with 4 N sulfuric acid and 5 ml and 10 ml of calcium permanganate, respectively. The resulting solution was heated, and 10 ml of oxalic acid was added. The obtained sample was titrated using calcium permanganate in a hot state until a rose-colored sample was obtained, and the titrant volume was acquired. The following formula is used to calculate

$$COD{:}COD = \frac{(A-B) \times N\ FAS \times 1000 \times BeO_2 \times P}{V\ Sampel} \quad \text{Eq 6}$$

Where A is the volume (ml) of the blank titrant, B is the sample titration's volume (ml), N is the normality of FAS (Ferro Ammonium Sulfat), BeO_2 is the oxidation number of BeO2 (8), and P is the dilution.

3. Results and Discussion

3.1 Oxygenase Enzyme Activity

The production of oxygenase enzymes consisting of monooxygenase enzymes and dioxygenase enzymes was carried out by growing bacteria on media containing hydrocarbon substrates without adding hydrocarbons, with a cultivation time of 14 days. The test activity of oxygenase enzymes consists of monooxygenase enzymes and dioxygenase enzymes from the bioremediation process of *Pseudomonas aeruginosa* and *Rhodococcus erythopolis* bacteria at a hydrocarbon concentration of 30 ppm. This test was carried out by the spectrophotometric method using ELISA. The test results for the activity of the

monooxygenase and dioxygenase enzymes are changed over incubation time with different number of enzyme activity (Figure 1 and Figure 2). One unit of activity for the monoxide and dioxide enzymes is the number of enzymes that require 1 mol of NADH to oxidize the substrate.

Monooxygenase enzymes have activities between 0.257 and 3.859 U/mL. Meanwhile, the dioxygenase enzyme has an activity between 0.579 and 5.402 U/mL. The highest activity was shown in incubation on the seventh day. At this time of incubation, bacterial growth experienced a stationary phase. The stationary phase of bacteria occurs when cell growth is inhibited due to cell density, the accumulation of secondary metabolites that are toxic to cells, and oxygen limitation, which causes bacteria to secrete enzymes with the highest activity under these conditions. The lowest activity occurred during the incubation time of day 0 because of the absence of substrate used by bacteria as a carbon source and the absence of bacterial adaptation to the substrate. There was no increase in the number of bacteria in the stationary phase. The number of cells that grew was equal to those that died because food reserves started running low. In this phase, the bacteria will produce secondary metabolites as a defense mechanism against their environment and other microorganisms (Djenar and Mulyono, 2017).

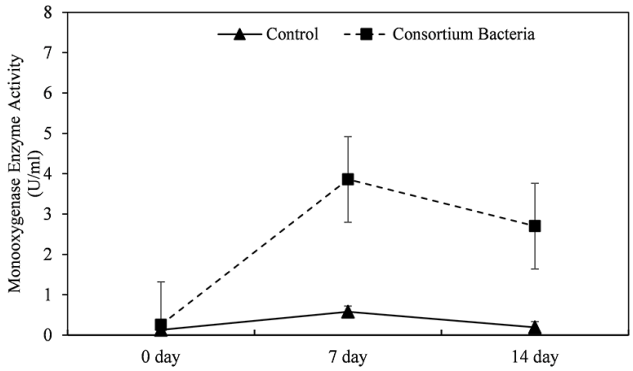

Figure 1. Monooxygenase enzyme activity

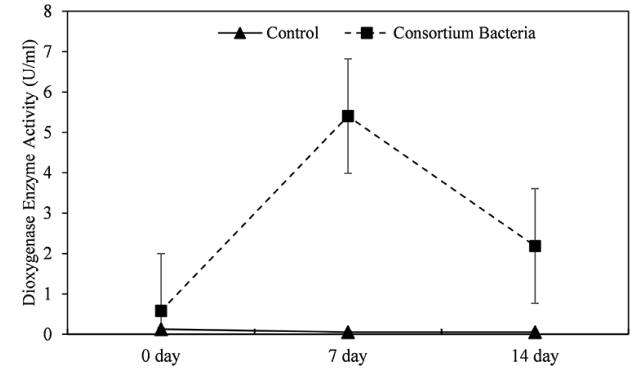

Figure 2. Dioxygenase enzyme activity

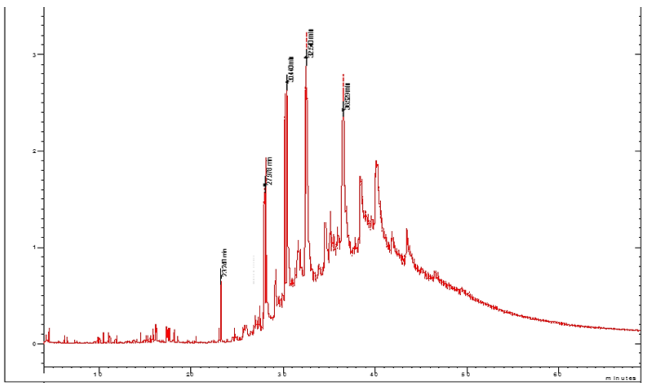

Figure 3. Result of GC-MS analysis before bioremediation treatment

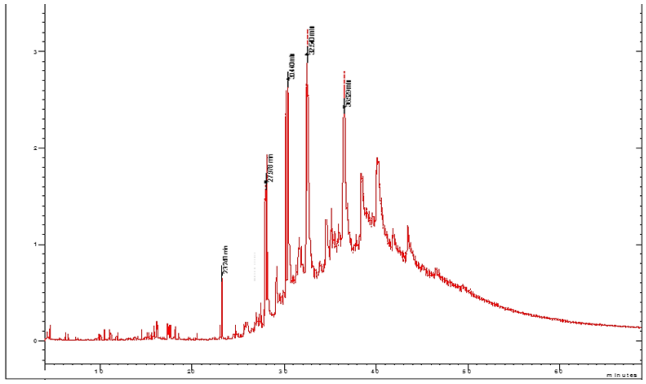

Figure 4. Result of GC-MS analysis after bioremediation treatment

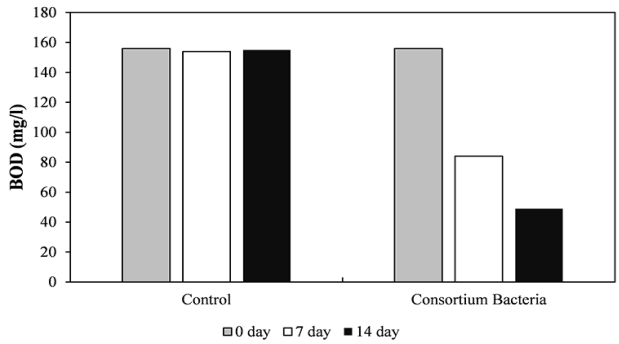

Figure 5. BOD Value during bioremediation process

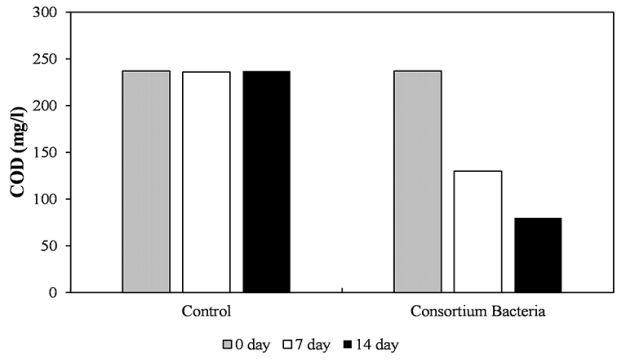

Figure 6. COD Value during bioremediation process

Table 1. Hydrocarbon degradation profile

Compound	Control		Consortium Bacteria		Decrease in Area (%)
	Retention Time	Average Area	*Retention Time*	Average Area	
n-Undekana ($C_{11}H_{24}$)	10.009	836411	-	-	100
1,2,3,5-Tetrametilbenzena ($C_{10}H_{14}$)	10.976	162319	-	-	100
2-Metilantrasena ($C_{15}H_{12}$)	16.164	282389	-	-	100
1-Metilpenantrena ($C_{15}H_{12}$)	17.340	1775093	-	-	100
Asam Oleat ($C_{30}H_{48}O_3$)	-	-	23.240	327245	-
n-Tetrakosana ($C_{24}H_{50}$)	25.984	329241	-	-	100
n-Oktadekana ($C_{18}H_{38}$)	27.978	1791797	27.732	1401364	21.79
2,6-Heptanedione ($C_7H_{12}O_2$)	28.148	3872986	-	-	100
2,5-Hexanedione ($C_6H_{10}O_2$)	30.440	2817843	30.805	2303023	18.27
Hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$)	32.540	4616597	32.087	2416327	47.66
3,6-Octanedione ($C_8H_{14}O_2$)	34.240	3164372	-	-	100
n-Heneicosane ($C_{21}H_{44}$)	36.529	323577	36.131	204533	36.79
8-Heksilpentadekana ($C_{21}H_{44}$)	38.152	2657531	-	-	100
Tetrakontana ($C_{40}H_{82}$)	39.752	395286	-	-	100
n-Tetratriakontana ($C_{34}H_{70}$)	42.762	140696	-	-	100

The bacterial consortium (in this research, the proportion of consortia is 50 %) secretes its extracellular enzymes to break down complex organic compounds into simpler ones (Allison *et al*., 2014). The simple compounds formed will be able to enter the cell through active transport, diffusion, or osmosis to be used as a source of nutrients for the ongoing metabolism of bacteria (Wignyanto *et al*., 2009). As bacterial cells increase, more enzymes are released (Allison *et al*., 2014). If the number of enzymes released is balanced with the volume of pollutants, a total reduction can occur, and the waste degradation process takes place ideally (Tamoor *et al*., 2021).

3.2 GC-MS Compound Profile

Hydrocarbon degradation analysis was conducted to determine the ability of bacterial isolates to use hydrocarbon compounds as a carbon source. This ability can be seen in changes in the concentration of the initial and final hydrocarbon compounds. Use gas chromatography with a mass spectrometer to identify unknown compounds (Kaur, 2019).

In this study, the consortium of bacteria (*Pseudomonas aeruginosa, Rhodococcus erythopolis*) showed the highest percentage of decreased concentration (Figure 3 and Figure 4). The reduction in hydrocarbon compounds was calculated by comparing

the sample area before bioremediation with the treatment area after bioremediation at almost the same retention time (Table 1).

The GC-MS analysis of hydrocarbons before bioremediation showed that the hydrocarbon compounds consisted of 14 compounds. These compounds were indicated by peaks number 1 to number 14 which appeared at the retention time of the 16th minute to the 34th minute. These compounds consist of short-chain hydrocarbons ($\leq C_9$), medium-chain hydrocarbons (C_{10}-C_{24}), and long-chain hydrocarbons ($\geq C_{25}$) in both linear and cyclic forms (Table 1). The results of GC-MS hydrocarbons after bioremediation *with Pseudomonas aeruginosa* and *Rhodococcus erythopolis* bacteria lasted for 14 days.

On the 14th day, there was a significant decrease in hydrocarbon peaks compared to day 0. Long-chain hydrocarbons ($\geq C_{25}$), medium-chain hydrocarbons (C_{10}-C_{24}), and short-chain hydrocarbons ($\leq C_9$), which were detected on day 0, decreased in area, and some compounds were even lost significantly on day 14. The hydrocarbon compounds indicated by peaks number 1 to number 6, peak number 10, and peak number 12 to number 14 were not found again on day 14 (Figure 2). These results indicate that *Pseudomonas aeruginosa* and *Rhodococcus erythopolis* consortium bacteria can noticeably degrade long-chain, medium-chain, and short-chain hydrocarbons.

Following the results of the gas chromatogram of hydrocarbons after bioremediation on day 14, it showed low peaks, compared to previously high peaks in used oil before bioremediation on day 0. The presence of compounds detected as having low peaks seems to result from the degradation of high-molecular-weight compounds detected by gas chromatography or a collection of fractions resulting from the degradation of compounds that have a decreased peak area (Wu *et al*., 2017). Hexadecenoic acid methyl ester has the molecular formula $C_{17}H_{34}O_2$. The decrease in these compounds to 47.66% is thought to be because bacteria can directly use and degrade these compounds for metabolism. Bacteria will degrade hydrocarbon compounds into alcohol by monooxygenase enzymes, converting hydrocarbons into alcohol (Katam *et al*., 2018). Then the alcohol will be converted into an aldehyde, which will be converted into an acid. Finally, the acid will enter the oxidation pathway (Silva *et al*., 2020). The higher the enzymatic activity in biodegradation treatment, the better the transformation of alkane hydrocarbons into alcoholic compounds by a group of bacterial consortia. An alkane monooxygenase enzyme could oxidize a sizeable aromatic ring molecule, chelate metals occur in asphaltene composition, and break down the internal binding between asphaltene molecules and large aliphatic chains. Therefore, there is an opportunity that the bacterial strains in every of the consortium have several metabolic genes that improve the production of catalysators, alkane monooxygenase enzymes for the breaking down of specific alkane chain lengths into alkanols (Berhanu *et al*., 2023; Gao *et al*., 2023; Rabus *et al*., 2016). Bioremediation is a promising approach for contaminant purification. Several recent studies, especially for lab-scale bioremediation, have been carried out. However, the application of the bioremediation method on a larger scale has not yet been performed. Furthermore, scaling up bioremediation is hardly needed. Scale-up bioremediation should consider several factors to maintain the efficiency of its process, including the availability of nutrients, the type of contaminant, and the appropriate biological agent. By considering those factors, hopefully scale-up bioremediation can be applied in the future.

3.2.1 BOD and COD

In the analysis of treating the bacterial consortium (*Pseudomonas aeruginosa* and *Rhodococcus erythopolis*) with a hydrocarbon concentration of 30 ppm on day 0, the BOD concentration of 156 mg/L decreased significantly on the 14th day to 49 mg/L, with the most significant decrease in BOD concentration of 68.59% (Figure 5). The BOD value in the hydrocarbon treatment decreased due to the hydrocarbon-degrading bacteria decomposing hydrocarbons. A low BOD value indicates a small amount of hydrocarbon residue. This low value is because BOD is an indicator that measures the amount of oxygen needed to decompose pollutants in waste (Kurniawan *et al*., 2020). The greater the number of pollutants, the greater the oxygen needed to decompose, so the BOD value is enormous (Rabus *et al*., 2016). If the pollutant in the waste has been decomposed by degrading heterotrophic bacteria, the amount will be less, and the oxygen needed is also less, so the BOD value is low (Sujadi *et al*., 2019; Kurniawan *et al*., 2020).

In the treatment of the bacterial consortium (*Pseudomonas aeruginosa* and *Rhodococcus erythopolis*) with a hydrocarbon concentration of 30 ppm on day 0, the COD concentration of 237 mg/L decreased significantly on the 14th day to 77 mg/L, with the most significant decrease in BOD concentration of 67.51% (Figure 6). The COD value decreased due to hydrocarbon-degrading bacteria capable of decomposing hydrocarbons in wastewater. Chemical Oxygen Demand (COD) is the amount of chemical oxygen required to oxidize organic matter in wastewater

(Dewinta *et al*., 2020). COD measures waste pollution by using organic substances that can naturally be oxidized through biological processes, resulting in reduced oxygen dissolved in wastewater (Febriansyah *et al*., 2022). This test does not distinguish between biodegradable materials and non-biodegrabdable materials. A low COD value in effluent indicates better wastewater quality (Kurniawan and Yamamoto, 2013). Several natural degradation treatments are conducted in aerobic conditions due to oxygen availability. Oxygen is essential for living microorganisms, whereas another degradation treatment requires anaerobic conditions. In general, the availability of oxygen can improve hydrocarbon and petroleum secretion. Some research shows that 90% of petroleum hydrocarbons were degraded under aerobic conditions, while 25% were degraded under anaerobic conditions. The attainability of oxygen is an essential indicator of the biodegradation of petroleum hydrocarbons. A sufficient oxygen number is needed to improve the speed of microbial activity. In addition, the aerobic natural degradation of aliphatic hydrocarbons requires three steps. The first step is the oxidation reaction, which needs an oxygen molecule and is catalyzed by an enzyme; the second is the primary alcohol, which is generated by the oxidation of methyl set; and the last is alcohol, which is then subjected to perpetual processes of oxidation to generate aldehyde, which is frequently converted into fatty acid. Commonly, the oxygen molecule is decreased to water, whereas petroleum hydrocarbons are oxidized to create energy, CO_2, and cell mass (Bacosa *et al*., 2022; Brimberry *et al*., 2023; Kebede *et al*., 2021; Wang *et al*., 2011).

4. Conclusion

This study showed that the consortium (*Pseudomonas aeruginosa* and *Rhodococcus erythopolis*) at a hydrocarbon concentration of 30 ppm had monooxygenase and dioxygenase enzyme activities. Microorganisms need these enzymes to biodegrade hydrocarbons and catalyze hydrocarbon oxidation reactions. The monooxygenase enzyme has an activity between 0.257 and 3.859 U/mL. Meanwhile, the dioxygenase enzyme has an activity between 0.579 and 5.402 U/mL. The compound decrease reached 47.66%, presumably because bacteria can directly use and degrade these compounds for metabolism. In addition, there was a decrease in BOD of 68.59% and a decrease in COD of 67.51%. This study concludes that oxygenation enzymes are produced during the hydrocarbon bioremediation process to degrade pollutant compounds. Furthermore, several considerations are needed to improve the bioremediation process by adding nutrients and increasing the number of bacteria. The application of large-scale bioremediation should be carried out soon to reach a good and healthy environment.

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Authors' Contributions

Each author's contribution is as follows, Adi Tiya Yanuar; conceptualized, investigated, curated the data, and worked on a formal analysis and methodology. Abd. Aziz Amin, Lutfi Nimatus Salamah, and Frentina Murti Sujadi; investigated, curated the data, and worked on a formal analysis and methodology. Ekwan Nofa Wiratno; worked on a formal analysis and reviewed the manuscript. Citra Satrya Utama Dewi; investigated, curated the data, and worked on a formal analysis and methodology. Andi Kurniawan; conceptualized, worked on a methodology, and supervised the research. All authors discussed and contributed to the final manuscript.

Conflict of Interest

The author declares that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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