

# Activities and molecular characterization of petroleum hydrocarbons degrading rhizobacteria from mangrove plants (*Rhizophora* sp.) in Kulon Progo, Yogyakarta, Indonesia

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**Abstract.** Benget VV, Retnaningrum E. 2020. Activities and molecular characterization of petroleum hydrocarbons degrading rhizobacteria from mangrove plants (*Rhizophora* sp.) in Kulon Progo, Yogyakarta, Indonesia. *Biodiversitas* 21: 21-27. The increasing number of human population density around mangrove ecosystems has resulted in its pollution, particularly oil spills. Rhizobacteria have metabolic abilities that make them possible to live in polluted environmental conditions and the potential to be biological agents for oil remediation. However, investigations of rhizobacteria especially from rhizospheres of mangrove plants (*Rhizophora* sp.) and their potential use in remediation remains limited. Therefore, the purposes of this research were to investigate the ability of selected isolate in the degradation of petroleum hydrocarbons and identify its isolate using 16S rRNA gene. Observation of that degradation ability was conducted by inoculating the isolate in Bushnell-Haas Minimal Salt (BHMS) medium containing crude petroleum 0.01 %. After 10 days of incubation, the degradation hydrocarbons were analyzed by measuring Total Petroleum Hydrocarbons (TPH) using Gas Chromatography-Flame Ionization Detector (GC-FID) method. Four rhizobacteria isolate from rhizospheres of mangrove plants (*Rhizophora* sp), NMKP1, NMKP2, NMKP3, and NMKP4 were analyzed based on their growth in Bushnell-Haas Minimal Salt (BHMS) medium containing crude petroleum 0.01 %. The results revealed that NMKP4 isolate had the highest specific growth rate. In addition, it could degrade those hydrocarbons pollutants as much of 98.72 % within 10 days. Based on molecular characterization showed that NMKP4 isolate was in the same cluster as *Pseudomonas* sp. CO03.

**Keywords:** 16S rRNA, GC-FID, mangroves, oil spills, *Pseudomonas*, *Rhizophora*

## INTRODUCTION

Petroleum is an important energy source because petroleum has a significant percentage in meeting world energy consumption. Indonesia is one of oil-producing countries in the world. BP Statistical Review of World Energy (2018) data shows Indonesia produces 949,000 barrels of oil per day. Most petroleum hydrocarbon compounds are known to be carcinogenic, mutagenic, and classified as major environmental pollutants by US Environmental Protection Agency. Content of these contaminants has a long-term effect on ecosystem and increases presence of toxicity in organisms to the top of food chain (Khan et al. 2018). Biodegradation of hydrocarbon-contaminated soils, using microorganisms that have potential to degrade or detoxify these contaminants, has been proven as an environmentally friendly and low-cost method. Oil-degrading microorganisms have an enzyme system and metabolic abilities, allowing them to resist polluted environmental conditions (Meng et al. 2018; Ji et al. 2019).

Mangroves are intertidal wetland swamps that lie along the coastline of tropical and subtropical regions that have large ecological and economic interests. However, throughout world mangroves are the most threatened environment of damage. In addition, mangrove forest has

decreased its quality due to oil pollution, especially during their petroleum exploration (Chai et al. 2019; Garcia et al. 2019). Extent to impact of environmental damage is still unknown but may be a major problem in the future. A method to restore these environmental conditions is very important to be developed to prevent damage mangrove ecosystems.

Mangrove forest area in Kulon Progo, Yogyakarta, Indonesia is also inseparable from threat of damage. In general, most of the mangrove area's environment is located in areas with high anthropogenic influence and therefore mangrove environment is also inseparable from exposure to pollutants, such as exposure to oil spills. The oil pollutants found in mangrove area come from the activities of shrimp farms that are also located in the area. The oil pollutant comes from the use of a diesel engine that functions as an aerator for ponds. In addition, oil pollutants also come from motorized boats owned by residents along river estuary in the mangrove area. Oil pollutants are sourced from refueling activities of motorboat.

Rhizosphere is an ideal area for growth of soil microorganisms that are generally dominated by bacteria, actinomycetes and fungi (Renu et al. 2019). This area is also rich exudates that are produced by plants through the process of root secretion. These exudates are then decomposed by microorganisms that produce energy and

precursor compounds. Then, these precursor compounds can be utilized by themselves and plants (Haichar et al. 2014; Sasse et al. 2018). Hence, the rhizosphere microorganisms play a role in the nutrient cycle and also in the process of soil formation. Therefore, plant growth greatly affects the activity of microorganisms, as well as controlling biodiversity of root pathogens (Clairmont et al. 2019; Wei et al. 2019). In addition, the rhizobacteria were also reported to be able to degrade of that hydrocarbon pollutants in the soil environment (Retnaningrum and Wilopo 2018). Based on those reasons, this study aims to obtain the best rhizobacteria species from mangrove plants (*Rhizophora* sp) at Kulon Progo, Yogyakarta which has ability to degrade petroleum hydrocarbons and to identify it by 16S rRNA gene.

## MATERIALS AND METHODS

### Location and sample collection

Soil samples were collected from the rhizosphere of mangrove plants (*Rhizophora* sp) located at Wanatirta Mangrove Forest, Kulon Progo, Yogyakarta. Rhizospheric soil samples were taken at one point randomly where there was exposed to oil pollutants which characterized by the presence of oil shine above the water surface where the mangrove plants grew. A 5 g of rhizospheric soil samples were taken at the root part, then filtered using a sieve with 2 mm diameter sieve hole (Rajaei et al. 2013). The soil samples obtained were then stored at -20 ° C.

### Analysis of rhizospheric soil samples

Soil samples were analyzed based on pH values and Total Petroleum Hydrocarbon (TPH). Measurements of pH values were conducted using a pH meter. Previously pH meter was calibrated using a buffer solution pH 4.0 and 7.0, then washed with distilled water and dried with tissue. The pH values of the samples were then measured 3 times to obtain the representative data. For measuring TPH, soil sample previously was homogenized and was then taken as much as 5 g and put into a large test tube. The soil sample was then extracted with 10 ml of n-hexane and allowed to stand until the solution separates completely. The top layer was then taken and injected as much as 1 µL in Shimadzu GC-2010 which was equipped with a Flame Ionization Detector (FID), using a RTX-5 column, 30 m long (Srivastva et al. 2017). The standard and extractant were also injected under the same conditions as much as 1 µL in Shimadzu GC-FID. The TPH levels were then calculated using the following formula (Mishra et al. 2001):

$$TPH (\%) = \frac{\text{Sample area} - \text{extractant area}}{\text{Std. area} - \text{extractant area}} \times DF \times \text{Std. conc. (1000 mg/L)}$$

Where:

DF was dilution factor

### Isolation and phenotypic characterization of rhizobacteria from *Rhizophora* sp.

The petroleum hydrocarbons degrading rhizobacteria from *Rhizophora* sp were isolated from the rhizospheric soil samples by inoculating them into Bushnell-Haas Minimal Salt Broth Medium (BHMS) with addition of 0.01% crude oil. The culture was then incubated at room temperature, on a rotary shaker (180 rpm) for 7 days. The culture was then subcultured two times into fresh medium of BHMS with addition 0.01 % crude oil. The adaptive rhizobacteria cultures were then purified into BHMS agar using pour plate method. Purified rhizobacteria isolates were characterized phenotypically by analyzing their cell shape, gram staining and colony properties (color, shape, elevation, margin and inner structure).

Gram staining method was carried out to identify the rhizobacteria cells based on their different cell wall constituents using 2 kinds of stain. Firstly, a small amount of isolate culture was spread on the surface of the slide. The smear was then fixed physically by heat to the surface of the slide. As much as 2-3 drops of crystal violet were dropped on a surface of slide sample for 60 seconds. The slide sample was then washed using water and dried. A total of 2-3 drops of Lugol solution was dropped over the slide sample for 60 seconds. Slide sample was washed with water and dried. Slide sample was further dropped 2-3 drops of an alcohol-acetone solution for 30 seconds, then washed again and dried. Furthermore, the slide sample was dripped with a safranin solution of 2-3 drops for 60 seconds, then washed and dried. Finally, the slide sample was then observed under a light microscope.

### The growth of rhizobacteria isolates from *Rhizophora* sp.

The growth of rhizobacteria isolated from *Rhizophora* sp. was measured by their specific growth rate by culturing them into BHMS medium with addition of crude oil 0.01 %. Each rhizobacteria isolate was inoculated into 250 ml liquid BHMS medium with addition of crude oil 0.01 %. The specific growth rates of isolates can be measured by analyzing their growth at an interval time of 12 hours for 72 hours using total plate count (TPC). Finally, the specific growth rate can be calculated using the equation reported by Maier and Pepper (2009).

$$\mu = 0.693 \times \frac{\log N_t - \log N_0}{0.301 \times t}$$

Where:

k : The constant of growth rate (generation/time)

N<sub>0</sub>: The initial number of bacteria during the exponential phase (CFU/mL)

N : The number of bacteria after growing during t-time (CFU/mL)

t : The growth time at exponential phase (seconds)

### Activity of rhizobacteria isolates on petroleum hydrocarbons degrading

Selected rhizobacteria isolate was cultured in BHMS medium with addition of 0.01 % crude oil and incubated on a rotary shaker at a speed of 180 rpm at room temperature. After 10 days of incubation, the pH value and TPH of culture were analyzed. For analysing the TPH of culture, firstly it was extracted using 40 ml dichloromethane in separating funnel. Solution mixture was then stirred for 3 minutes with a rest period of 10 minutes. Extracted sample was then reacted with 10 mg Na<sub>2</sub>SO<sub>4</sub> in a glass funnel coated with glass wool. Finally, the sample was collected into a flask and washed using dichloromethane. The top layer then was taken and injected as much as 1 µL into GC-FID. Preparation and measurement of standard, extractant and extracted samples were carried out in the same procedure as in the previous TPH analysis.

### Molecular identification of rhizobacteria isolated from *Rhizophora* sp

The overnight of the rhizobacteria culture in the Nutrient Broth (NB) medium was prepared for extracting DNA using the Quick-DNA™ Fungal/Bacterial Miniprep (Zymo Research). The extract of DNA was then suspended in 500 µL of buffer TE. The purity of DNA extracted was measured at A<sub>260/280</sub> wavelengths using UV-Vis Nanodrop-Spectrophotometer (Thermo Scientific) with three replication.

The DNA further used as DNA template in PCR reactions. The 16S rRNA gene was amplified by using the primer pairs 27F / 1429R (27F: 5'-AGA GTT TGA TCC TGG CTC AG-3', 1429R: 5'-TAC GGY TAC CTT GTT ACG AGA CTT -3'). Amplification was carried in several cycles of reaction including predenaturation at 95° C for 3 minutes, amplification of 30 cycles included denaturation at 95°C for 30 seconds, annealing at 57°C for 1 minute, extension at 72°C for 1 minute 40 seconds and cooling at 4°C.

The PCR product samples were detected qualitatively using electrophoresis with 1 % of agarose (w/v) and visualized using UV Transluminator. This PCR product was then sequenced by Sanger method. Sequencing results were compared with database on NCBI website (National Center for Biotechnology) through the BLAST tools ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Furthermore, phylogenetic tree was constructed using Neighbor-Joining (NJ) method with MEGA 7 program.

### Data analysis

The experimental data were statistically analyzed with SPSS version 12. The means of tested parameters were analyzed by Duncan's Multiple Range Test (DMRT) and ANOVA ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

### Rhizosphere soil samples from *Rhizophora* sp.

Rhizosphere soil samples were taken at one point randomly from mangroves area in Kulon Progo, Yogyakarta, Indonesia where there was exposure to oil pollutants which characterized by the presence of oil glints above the surface of water where the plants grew. From the measurements, it showed that the pH and TPH values of samples were 7.26 and 109.87 mg/mL (0.01%), respectively. These results could describe that the mangrove area was contaminated hydrocarbon. However, *Rhizophora* sp. could grow well which might be correlated with the presence and activities of rhizobacteria population in the mangrove ecosystem. The pH values of sample tend alkaline, this indicated the presence of mineral salt content in the area (Ceccon et al. 2019). These measurement results were also important for preparing medium for isolating and culturing rhizobacteria isolates.

### Phenotypic characters of rhizobacteria isolated from *Rhizophora* sp.

Four isolates of petroleum hydrocarbons degrading rhizobacteria were obtained, each coded NMKP1, NMKP2, NMKP3, and NMKP4. These isolates were able to use crude oil for their C source. The observation results of phenotypic characters isolates were displayed in Table 1. The cell morphology of NMKP1 and NMKP3 isolates were rod-shaped, whereas NMKP2 and NMKP4 isolates had short rod-shaped. Based on the Gram staining results, all rhizobacteria isolates showed Gram-negative. In addition, all isolates showed the same white color, irregular shape, effuse elevation and smooth inner structure of their colonies. These results matching with the profile of *Pseudomonas* sp which explained in Bergey's Manual Systematic of Bacteriology (Brenner et al. 2005).

**Table 1.** Morphological characters of rhizobacteria isolated from *Rhizophora* sp.

Characters		Isolate			
		NMKP1	NMKP2	NMKP3	NMKP4
Cell morphology	Shape	Rod-shaped	Short rod-shaped	Rod-shaped	Short rod-shaped
	Gram stain	Negative (-)	Negative (-)	Negative (-)	Negative (-)
Colony morphology	Color	White	White	White	White
	Shape	Irregular	irregular	Irregular	Irregular
	Elevation	Effuse	effuse	Effuse	Effuse
	Inner structure	Smooth	Smooth	Smooth	Smooth

### The growth of rhizobacteria isolates from *Rhizophora* sp.

The number of rhizobacteria cells varied with time incubation and the kind of isolate (Figure 1). The number of NMKP1, NMKP2, NMKP3 and NMKP4 isolates at 12 hours growth were  $2.18 \times 10^7$ ,  $1.525 \times 10^7$ ,  $1.86.5 \times 10^7$  and  $0.505 \times 10^7$  CFU/mL. During 12-24 hours incubation, all isolates showed flat growth curves. These were probably because each isolate synthesized an enzyme to degrade hydrocarbons which occurred in a lag phase. In addition, in these phase cells were also synthesized enzymes needed by their cells for metabolic processes. All isolates entered the exponential phase and reach its peak in different time incubation and cells number. The highest number cells of NMKP1, NMK3, and NMK4 isolates were  $2.345 \times 10^7$ ,  $3.065 \times 10^7$  and  $3.15 \times 10^7$  CFU/mL, respectively which were reach at 48 hours incubation. Whereas, NMKP2 isolate was reached the highest cells number of  $2.105 \times 10^7$  CFU/mL at 36 hours incubation. During these peak curves, the rhizobacteria divided rapidly and increased logarithmic according to time. (Madigan et al. 2012).

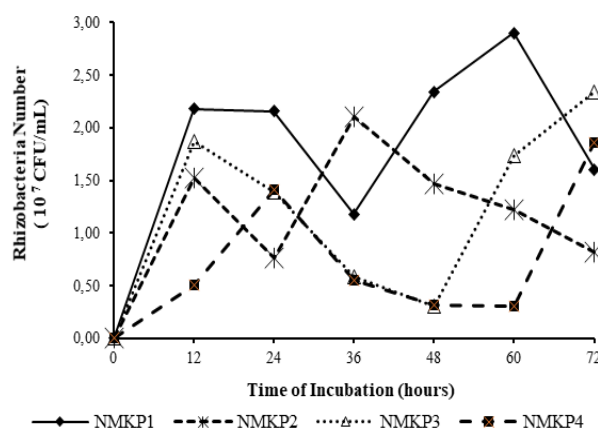
Those growth observations could be further calculated to obtain their growth kinetics, as shown in Table 2. The highest specific growth rate ( $\mu$ ) of NMPK4 isolate revealed that isolate could grow and adapt quickly in the crude oil exposure. Furthermore, this isolate was selected and measured its activity in BHMS medium with 0.01 % crude oil addition.

### The activity of rhizobacteria isolates on petroleum hydrocarbons degrading

The activity of selected rhizobacteria isolates (NMPK4) on petroleum hydrocarbons degrading was determined by culturing its isolate in medium BHMS containing 0.01 % crude oil at 10 days incubation. During time of incubation, isolate had an ability to use hydrocarbon compounds as carbon sources. This ability could be seen from the changes in hydrocarbon compounds concentration. Total Petroleum Hydrocarbon (TPH) is a method to determine the value of petroleum hydrocarbons content in liquid media which can be measured using Gas Chromatography. The results showed that the TPH of culture after 10 hours incubation was 0.00014% ( $p < 0.05$ ) which shown in Table 3. There was a decrease which equals 98.72% in TPH levels after 10 days of incubation. These results indicate that NMKP4 isolate could degrade petroleum hydrocarbons well. These results were in accordance with a previous researcher who reported that rhizobacteria isolate from mangrove plants at

Rio de Janeiro, Brazil was able to reduce TPH values by up to 97% (Carmo et al. 2011) Therefore, it could be said that rhizosphere of mangrove plants have a great potential for obtaining novelty bacterial isolates which have an ability to degrade petroleum hydrocarbons.

The measurements of pH cultures showed that it was decreased significantly ( $p < 0.05$ ). This decreasing in pH culture was caused by rhizobacteria activity which uses and degrades hydrocarbons for its metabolism. During hydrocarbons degradation process, their compounds will be degraded by bacteria into alcohol using monooxygenase which converts hydrocarbons to alcohol. Then the alcohol will be converted into aldehyde which will be converted to acid. The acid will enter  $\beta$ -oxidation pathway. Therefore, the acid produced from this metabolic process affects pH in culture media (Al-Hawash et al. 2018).



**Figure 1.** The growth of rhizobacteria isolates from *Rhizophora* sp in BHMS medium with 0.01 % crude oil addition during 72 hours incubation

**Table 3.** The change of TPH and pH of NMPK4 culture after 10 days incubation

Measurements	Time Incubation (days)	
	0	10
Total Petroleum Hydrocarbon (TPH) (%)	0.01	0.00014
pH	7.53	7.51

**Table 2.** Growth kinetics of rhizobacteria isolates from mangrove plants (*Rhizophora* sp.)

Rhizobacteria isolate	N0 (CFU/mL)	N (CFU/mL)	K (Generation/second)	$\mu$
NMKP1	$2.180 \times 10^6$	$2.900 \times 10^7$	0.0086	0.005
NMKP2	$1.525 \times 10^5$	$2.105 \times 10^5$	0.0194	0.010
NMKP3	$1.865 \times 10^5$	$3.065 \times 10^5$	0.0199	0.010
NMKP4	$5.050 \times 10^4$	$3.150 \times 10^6$	0.0734	0.050

Note: K was the constant of growth rate, N0: the initial number of rhizobacteria during the exponential phase, N: the number of rhizobacteria after growing during t-time, t: the growth time at exponential phase and  $\mu$ : the specific growth rate.

**Table 4.** Results of measurement purity sample DNA isolates NMKP4

Parameters	Result
Concentration (ng/ $\mu$ L)	307.50 $\pm$ 1.500
DNA purity value (A260/280)	1.91 $\pm$ 0.002
Volume ( $\mu$ L)	30.00 $\pm$ 0.060

### Molecular identification of NMKP4 isolate with 16S rRNA gene

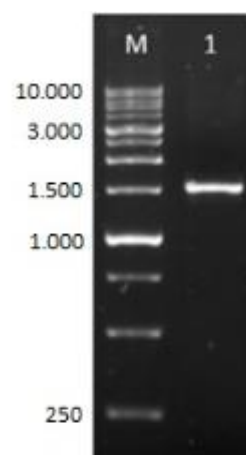
Molecular identification was carried out using ribosomal gene as a molecular marker to confirm the identity of the selected strain. Previously, rhizobacterial DNA was extracted and checked its purity and concentration using UV-Vis Nanodrop-Spectrophotometer spectrophotometer (Sambrook et al. 1989). The results showed that DNA extract of NMKP4 isolate had a ratio 1.91 (Table). This ratio value was still in the range of 1.8 to 2.0 included in the pure category. If the ratio < 1.8 indicated it DNA extract was contaminated by phenol and solvent. Whereas, if ratio > 2.0 indicated it DNA extract was contaminated by proteins and other compounds, such as RNA.

The DNA extract of NMKP4 isolate was then used as templates for amplification PCR using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1429R (5'-TAC GGY TAC CTT GTT ACG AGA CTT -3'). These pair of primer 27F/1429R could amplify almost all of 16S rRNA genes (Osborne et al. 2006). The PCR products then visualized using electrophoresis which displayed in Figure 2. The amplified product showed a single band approximately 1500 bp, corresponding to the expected size of the 16S rRNA genes (Janda and Abbott 2007). This PCR product was finally sequenced with both of 27F and 1429R primers. Some systems could determine nucleotide sequences through reading one primer, but reading with two primers gives more accurate results.

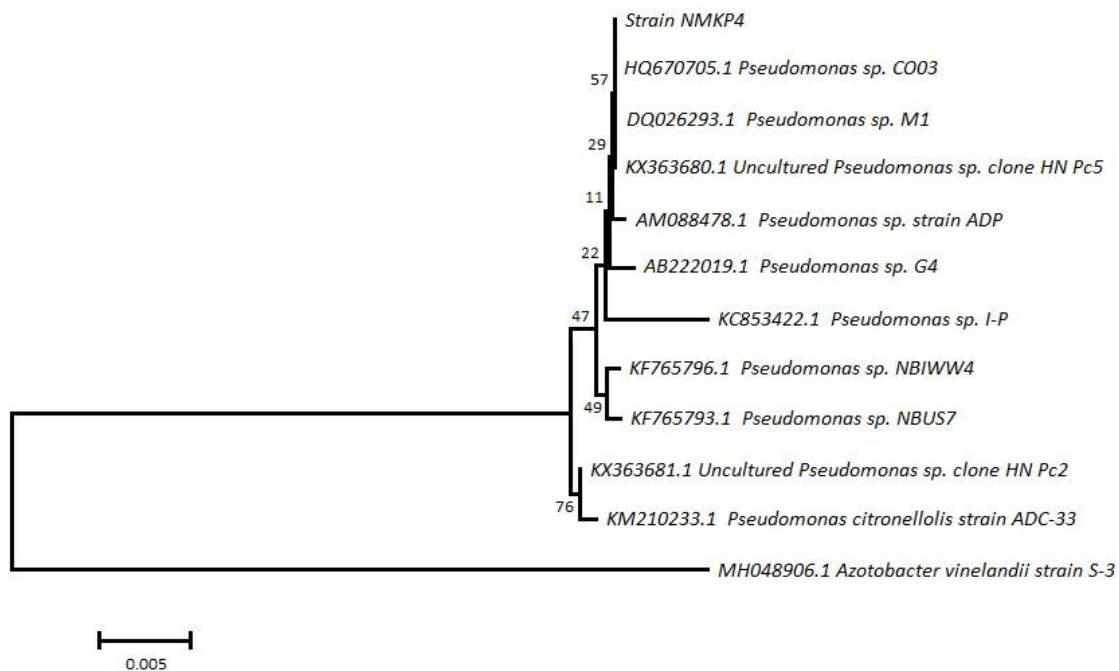
The sequencing results were then compared to reference sequences which could compare their identity values, between the sequence of sample isolates (Query) and reference sequence (Sbjct) throughout sequence examined

(Table 5). The results revealed that NMKP4 isolate had a similarity of 99.14% with *Pseudomonas* sp. CO03. As explained by Miller et al. (1990) identities were presentations of compatibility between sequences queries (sequences that are owned) with database nucleotide sequences. According to Dighe et al. (2004) and Greenwood et al. (2012) if the similarity of 16S rRNA coding genes base sequence more than 97 % could be considered the same species. Therefore, this NMKP4 isolate was identified as *Pseudomonas* sp. This molecular identification result of NMKP4 isolate was congruence with previous phenotypic characterization.

The sequence data of 16S rRNA gene could describe the degree of similarity between two taxa. Therefore, it could be used to construct phylogenetic trees that showed the relationship among ancestors and kinship of organisms (Godini and Fallahi 2019). This 16S rRNA gene was already used widely for constructing phylogenetic trees. In addition, those 16S rRNA genes were found in all prokaryotes. This 16S rRNA gene also had variable and conserve areas that reflected phylogenetic relationships among species (Van de Peer 2009; Mitreva 2017).

**Figure 2.** Results of NMKP4 isolate DNA amplification with 27F and 1429R primers. (M: Marker, 1: NMKP4 isolate)**Table 5.** BLAST results of 16S rRNA gene sequence NMKP4 isolate

Homologous species	Identities	Accession Number	Source of isolates	References
<i>Pseudomonas</i> sp. CO03	99.14%	HQ670705.1	Tobacco rhizosphere	Demaneche et al. (2011)
<i>Pseudomonas</i> sp. M1	99.07%	DQ026293.1	River sediments	Santos et al. (2007)
<i>Pseudomonas</i> sp. strain ADP	99.07%	AM088478.1	Garden soil	Lang et al. (2007)
Uncultured <i>Pseudomonas</i> sp. clone HN Pc5	99.00%	KX363680.1	Sandflies	Li et al. (2016)
<i>Pseudomonas</i> sp. NBIWW4	98.93%	KF765796.1	Activated sludge	Tan et al. (2015)
<i>Pseudomonas</i> sp. NBUS7	98.93%	KF765793.1	Activated sludge	Tan et al. (2015)
<i>Pseudomonas</i> sp. G4	98.86%	AB222019.1	Crude petroleum-contaminated soil	Ozaki et al. (2006)
Uncultured <i>Pseudomonas</i> sp. clone HN Pc2	98.79%	KX363681.1	Sandflies	Li et al. (2016)
<i>Pseudomonas citronellolis</i> strain ADC-33	98.64%	KM210233.1	Activated sludge	Ntougias et al. (2014)
<i>Pseudomonas</i> sp. I-P	98.50%	KC853422.1	Wastewater treatment plant	Xu et al. (2013)



**Figure 3.** The results of phylogeny tree construction NMKP4 isolate

For constructing phylogeny tree, this analysis was carried out using Maximum Likelihood method due to several reasons explained by previously researchers. This method reconstructed of phylogenetic tree based on DNA sequences which include automorphic, plesiomorphic, and synapomorphic characters (Van de Peer 2009). In addition, Heng (2006) and Heath et al. (2006) stated that Maximum Likelihood could apply sequence evolution models which were ideal for building phylogeny tree using sequences data.

In most phylogeny tree structuring incorporated outgroups into analysis. This outgroup was outside of group tested but still had a relationship with the test group. In this analysis, *Azotobacter vinelandii* strain S-3 was chosen as outgroup. This selection was choose based on the fact that the test group (*Pseudomonas* sp) and outgroup (*Azotobacter vinelandii* strain S-3) was classified in the same Family of Pseudomonadaceae.

The result of phylogeny tree construction as displayed in Figure 3, it revealed that NMKP4 isolate was in the same cluster as *Pseudomonas* sp. CO03. This was consistent with the results of analysis using BLAST algorithm which showed NMKP4 isolate had a similarity of 99.14% to partial sequence of *Pseudomonas* sp. CO03. These result of the similarities was greatest compared to other comparative isolates. Thus it could be concluded that petroleum hydrocarbons degrading rhizobacteria from mangrove plants (*Rhizophora* sp.) in Kulon Progo, were identified as *Pseudomonas* sp. Previous study conducted by Carmo et al. (2011) also reported that one of hydrocarbons degrading rhizobacteria isolates from mangrove in Rio de Janeiro was identity with *Pseudomonas* sp. Whereas, other isolates were identities with *Azospirillum* sp., *Acinetobacter* sp.,

*Paenibacillus* sp., and *Ralstonia* sp. Therefore, based on these investigations, it could be concluded that NMKP4 isolates from rhizospheres of mangrove plants in Kulon Progo had the best ability to grow in BHMS medium containing petroleum hydrocarbons 0.01% and ability to degrade those pollutants as much as 98.72% for 10 days. This isolate clearly characterized molecularly based on 16S rRNA gene was same cluster as *Pseudomonas* sp CO03.

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