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Naphthalene degradation by *Pseudomonas* sp. strain LBKURCC149 with the addition of glucose as co-substrate

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Abstract. *Novianty R, Antika B, Saryono, Awaluddin A, Pratiwi NW, Juliantari E. 2022. Naphthalene degradation by* Pseudomonas *sp. strain LBKURCC149 with the addition of glucose as co-substrate. Biodiversitas 23: 5654-5661.* Naphthalene is one of the pollutants that cause carcinogens. The biodegradation of carcinogenic naphthalene toxic compounds needs to be done to control pollution. *Pseudomonas* is one of several bacterial species that can degrading naphthalene. This study aimed to test the effectiveness of *Pseudomonas* sp. isolated from the land area of the Joint Operating Body (JOB) of PT. Siak Bumi Pusako-Pertamina Hulu in degrading naphthalene with glucose as a co-substrate. The potency of three isolates of *Pseudomonas* sp. LBKURCC148, LBKURCC149, and LBKURCC150 was tested in Minimal Media (MM) containing 0.2 mM naphthalene and incubated for seven days. The Optical Density (OD), pH, decreased concentration of naphthalene and degradation ability parameters were analyzed using UV-Vis spectrophotometer. The results showed that *Pseudomonas* sp. LBKURCC149 was able to degrade naphthalene by 13.95%. Naphthalene analysis using HPLC resulted in a decrease in the peak area of the sample as compared to the control. Based on these findings *Pseudomonas* sp. LBKURCC149 could be recommended to use to degrade naphthalene pollution.

Keywords: Biodegradation, glucose, naphthalene, *Pseudomonas*

INTRODUCTION

Naphthalene is one of 16 Polycyclic aromatic hydrocarbons (PAHs) classified as a priority pollutant frequently found in nature by the United States Environmental Protection Agency (EPA). Naphthalene is used as the main model in PAH bioremediation studies due to its simple structure and soluble properties compared to other PAHs. This compound has a low molecular weight consisting of two benzene rings. Naphthalene is also one of the dominant PAH groups contained in petroleum and coal (Farini et al. 2017). These bicyclic aromatic hydrocarbons and their derivatives are considered the most dangerous compounds in the oil fraction (Raissa et al. 2012). Naphthalene causes pollution in nature which often comes from the disposal of petroleum waste and oil spills. At certain levels, it inhibits respiration in mitochondria, resulting in the inhibition of oxygen consumption in some organisms. Naphthalene poisoning causes hemolytic anemia and nephrotoxicity (Wijayaratih 2001).

One of the ways to overcome pollution is by involving biological agents, namely biodegradation. Biodegradation is the decomposition or overhaul of organic compounds by utilizing microorganisms (Yudono et al. 2013; Fareed et al. 2017). Indigenous microorganisms that are petrophilic can degrade components from simple hydrocarbons by bacteria (Novianty et al. 2020a) and fungi (Sari et al. 2019) to more complex PAHs. One of the toxic PAH compounds, naphthalene, can be degraded by microorganisms such as bacteria without glucose (Novianty et al. 2020a) and fungi (Novianty et al. 2020b). The addition of glucose co-substrate as a carbon source also increases the biodegradability of naphthalene by fungi (Fitrida et al. 2020b) and without glucose (Fitrida et al. 2020a). Indigenous *Pseudomonas* sp. LBKURCC148, LBKURCC149, and LBKURCC150 are local biota originating from an environment polluted by petroleum in the area of the Joint Operating Body (JOB) of PT. Siak Bumi Pusako-Pertamina Hulu needs to be tested for its effectiveness in degrading the toxic compound naphthalene (Novianty et al. 2021).

There are several bacterial species reported for naphthalene degradation (Rabani et al. 2020). The common genus of naphthalene is *Pseudomonas* (Abarian et al. 2018; Tirkey et al. 2021). The effectiveness of biodegradation can be increased by manipulating environmental parameters that create optimum conditions to support microbial growth in degrading pollutant compounds more quickly. One of them is the addition of glucose as a co-substrate which can increase the growth of each isolate (Ambarsari et al. 2015; Li et al. 2021). Therefore, it was important to investigate the ability of these indigenous bacterial isolates to degrade complex hydrocarbon compounds, particularly naphthalene, when glucose was added.

MATERIALS AND METHODS

Study area

The cultures of *Pseudomonas* sp. were obtained from the land area of the Joint Operating Body (JOB) of PT. Siak Bumi Pusako-Pertamina Hulu, Siak District, Riau Province, Indonesia (Figure 1).

Isolation and characterization of bacteria

The bacteria used in this study were isolated at Enzyme, Fermentation and Biomolecular Research Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Science, Riau University. The screening of bacteria as biodegradation agents for crude oil were determined by using Bushnell Haas medium containing 5% (v/v) crude palm oil. The materials used were Nutrient Agar (NA) media (Merck Cat. No. 1.05450), Nutrient Broth (NB) media (Merck Cat. No. 1.06649), Minimal Media (MM) consisting of: $(NH_4)_2SO_4$, K_2HPO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, aqua DM, 0,02% tween 80, glucose 10 g/L and pH 7, naphthalene (Merck), hexane, acetone, NaCl, NaOH, aluminum foil and alcohol antiseptic 70%.

Rejuvenation of each bacterial isolate on nutrient agar (NA) media

Bacterial isolates were taken from pure cultures using an ose needle. This was done so that the ose's tip touched the medium surface. Bacterial isolates were then inoculated on an inclined NA medium and incubated for one day at room temperature of 22-25°C.

Preparation of starter for each bacterial isolate on nutrient broth (NB) media

A total of 1-2 oses of each bacterial isolate were inoculated into 100 mL of NB media in a 250 mL Erlenmeyer. The inoculum was incubated for 24 hours in the incubator of 37°C.

Naphthalene biodegradation

Preparation of naphthalene stock solution and Minimal medium (MM)

A 10 mM naphthalene stock solution was created by weighing and dissolving 0.128 g of naphthalene in 50 mL of acetone in a 100 mL volumetric flask, adding acetone to the mark homogenized. MM was used to test the degradation of naphthalene by bacterial isolates. MM was made by dissolving 2 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 1 g KH_2PO_4, 0,2 g $MgSO_4.7H_2O$, 0,01 g $FeSO_4.7H_2O$, 0.02% tween 80, glucose 10 g/L and pH 7 in 1000 mL aquadest. The media was sterilized in an autoclave at 121°C pressure 15 psi for 15 min after cold MM solution of 0.2 mM naphthalene was added. After one day of incubation, the media was ready to use if there was no contamination.

Naphthalene biodegradation test in MM

The biodegradation test was carried out using 30 mL sterile MM, to which 0.2 mM naphthalene was added as a carbon and energy source (Pawar et al. 2013). Furthermore, each bacterial isolate as much as 5% v/v of the cell suspension (OD610nm=0.5) was inoculated into the media. This experiment was carried out in three replicates. Naphthalene biodegradation test was incubated for 7 days on a rotary shaker at 150 rpm at room temperature. Each inoculum was monitored at days 0, 1, 3, 5, and 7 by measuring cell growth through Optical Density (OD) at a wavelength of 600 nm and pH using pH meter.

The optimum wavelength of naphthalene was determined beforehand using a UV-Vis spectrophotometer. The concentration was calculated using a standard curve of naphthalene with hexane as a blank solution. Before the residual concentration of naphthalene was measured, naphthalene was first extracted by adding 30 mL of hexane to the sample, then centrifuged at 1200 rpm for 5 min to separate the organic phase and the aqueous phase.

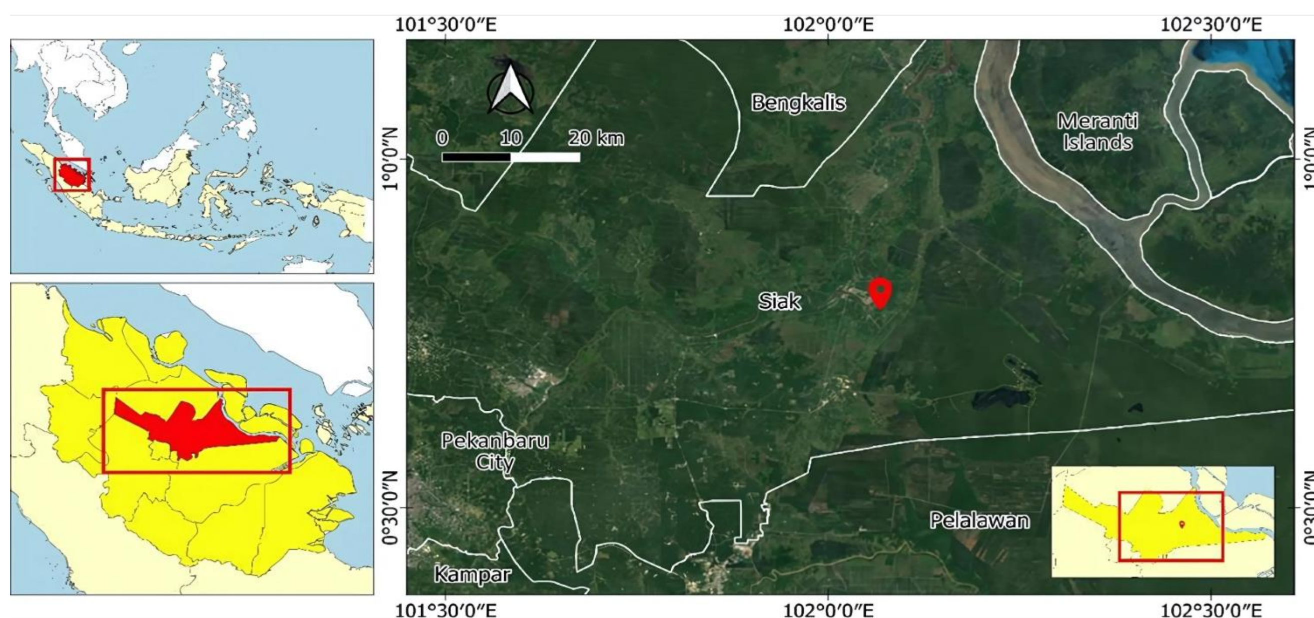

Figure 1. Study area in Riau Province, Indonesia

Then 1 mL of the solution in the organic phase was taken, and the absorbance was measured on days 0, 1, 3, 5, and 7 of incubation. As a control, MM with naphthalene (without bacterial isolate) was used (Pawar et al. 2013). The percentage of naphthalene biodegradation was calculated using the following equation (Deng et al. 2015):

$$\text{Degradation}\ (\%) = \frac{(\text{Residual amount in blank control} - \text{residual amount in sample})}{(\text{Residual amount in blank control})} \times 100$$

Naphthalene analysis using HPLC

The isolates with the highest percentage of biodegradation were analyzed for naphthalene using HPLC instruments. Naphthalene was extracted using hexane and solvent was evaporated at room temperature. After the solvent evaporated, naphthalene was dissolved in acetonitrile for analysis using reverse phase HPLC. A total of 20 L of naphthalene solution was injected into the HPLC Shim-pack VP-ODS column (250 × 4.6 mm). Samples were analyzed for 20 minutes using acetonitrile and water using a gradient elution system with 70% acetonitrile for first 5 min, then increased to 90% at 5 to 15 minutes and reduced again to 70% for 20 minutes. UV light detector used at a wavelength of 290 nm. The selection of this wavelength was based on preliminary measurements using a UV spectrophotometer (Hendayana, 2006).

Data analysis

The data obtained from this study were analyzed descriptively in graphs and statistically analyzed using Duncan's advanced multiple-distance test at 5% level using the SPSS program version 23.

RESULTS AND DISCUSSION

Isolation and characterization of bacteria

The sample was obtained from the land of the Joint Operating Body (JOB) of PT. Siak Bumi Pusako-Pertamina Hulu, Siak Regency, Riau Province with purposive sampling method. NA (Nutrient Agar) media was used for bacterial cultivation.

Three isolates were obtained from the isolation-coded *Pseudomonas* sp. strain LBKURCC148, *Pseudomonas* sp. strain LBKURCC149 and *Pseudomonas* sp. strain LBKURCC150. The characterization results showed that, generally, the isolates were Gram-negative bacteria. The colony morphology of three isolates is presented in Table 1. Screening results on Bushnell Haas medium showed that crude oil containing *Pseudomonas* sp. strain LBKURCC149 has decreased, indicating that the crude oil has been degraded.

Parameters optical density (OD) during the degradation process

The growth of each isolate in liquid MM in the presence of glucose and the addition of 0.2 mM naphthalene was observed through the OD parameter during the incubation time (Figure 2). The growth of isolates was observed based on the turbidity of the solution (Pawar et al. 2013). Each isolate showed a positive growth response in liquid MM in the presence of naphthalene.

Each bacterial isolate undergoes a different exponential phase in *Pseudomonas* sp. LBKURCC149 experienced the fastest exponential phase on day one of the incubation periods, while in *Pseudomonas* sp. LBKURCC148 and LBKURCC150 until the fifth day of the incubation period, then underwent a stationary phase (Figure 2). These finding show the adaptability and ability of the isolates to use naphthalene as a substrate. Based on the results of Duncan's test (Table 2), it can be seen that in the media with the addition of glucose, generally isolates had significantly different OD values with increasing incubation time.

pH parameter during the degradation process

The pH parameter is an important factor that affects the activity of microorganisms and affects the rate of degradation. The decrease in pH in liquid MM in the presence of glucose and the addition of naphthalene resulted in a change in pH from 7 to 4.12 in each isolate. The changes in pH of medium indicated the presence of growth and metabolites produced by microbes (Figure 3). Statistical analysis showed that generally in each isolate, there was a significantly different change in pH (Table 3).

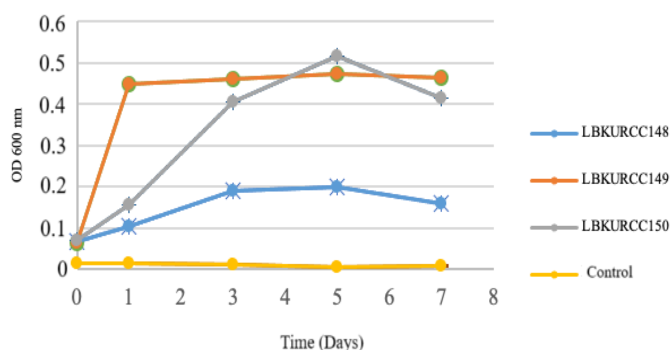

Figure 2. OD value of the isolate in MM solution over the course of the incubation

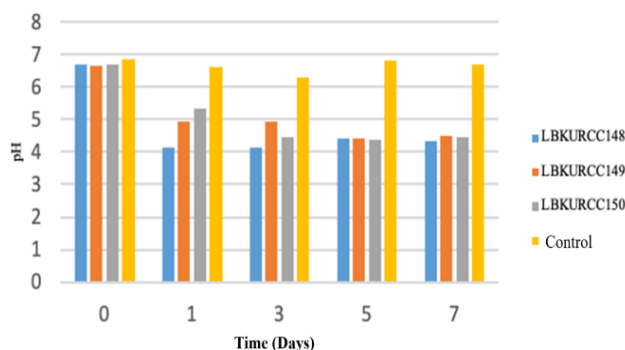

Figure 3. pH of isolates in liquid MM during the incubation time

Table 1. Colony morphology

Isolates	Morphology				
	Colony margin	**Colony elevation**	**Color**	**Shape**	**Gram stain**
LBKURCC148	Smooth	Flat	Cream	Bacilli	Negative
LBKURCC149	Smooth	Convex	Orange	Coccus	Negative
LBKURCC150	Smooth	Convex	White	Bacilli	Negative

Table 2. OD value during incubation time based on Duncan test

Incubation time	Isolates			
	***Pseudomonas* sp. LBKURCC148**	***Pseudomonas* sp. LBKURCC149**	***Pseudomonas* sp. LBKURCC150**	**Control**
0	0.067±0.002[a]	0.064±0.018[a]	0.069±0.017[a]	0.014±0.011[a]
1	0.104±0.003[b]	0.448±0.053[b]	0.156±0.017[b]	0.014±0.002[a]
3	0.189±0.029[c,d]	0.459±0.028[b]	0.404±0.013[c]	0.014±0.006[a]
5	0.198±0.010[d]	0.473±0.036[b]	0.515±0.070[d]	0.007±0.002[a]
7	0.159±0.024[c]	0.462±0.047[b]	0.414±0.033[c]	0.009±0.002[a]

Note: a,b,c,d different letters within a column indicate significant differences at $P \leq 0.05$.

Table 3. The pH value of the isolate media during incubation time

Incubation time	Isolates			
	***Pseudomonas* sp. LBKURCC148**	***Pseudomonas* sp. LBKURCC149**	***Pseudomonas* sp. LBKURCC150**	**Control**
0	6.69±0.10[c]	6.64±0.08[b]	6.68±0.22[c]	6.83±0.23[b]
1	4.15±0.07[a]	4.91±0.35[a]	5.34±0.83[b]	6.59±0.28[a,b]
3	4.12±0.01[a]	4.94±1.05[a]	4.46±0.09[a]	6.30±0.17[a]
5	4.40±0.20[b]	4.42±0.31[a]	4.37±0.26[a]	6.82±0.03[b]
7	4.33±0.11[a,b]	4.48±0.15[a]	4.45±0.31[a]	6.70±0.10[b]

Note: a,b,c,d different letters within a column indicate significant differences at $P \leq 0.05$.

Decrease in naphthalene concentration during the degradation process

The concentration of naphthalene degradation during the incubation time can be determined by using a standard curve of naphthalene with a blank solution of hexane and decreasing the concentration of naphthalene. Figure 4 indicates that the concentration of naphthalene gradually reduced up to the seventh day of incubation. *Pseudomonas* sp. LBKURCC149 showed the highest decreased concentration of 0.148 mM. Based on the results of Duncan's test, there was no significant difference in each isolate (Table 4).

The percentage degradation results were observed at the end of seven-day incubation (Table 5). In the addition of glucose, *Pseudomonas* sp. LBKURCC149 was the best isolate in degrading naphthalene by 13.95% while the other isolates did not reduce naphthalene concentration.

Naphthalene analysis using HPLC

This test was carried out on *Pseudomonas* sp. LBKURCC149 isolates which showed the highest ability to degrade naphthalene with the addition of glucose and compared with controls. Result showed that the peak height, and area of *Pseudomonas* sp. LBKURCC149 with control decreased from 45,256 to 36,219 at a wavelength of 291 nm. The peak that appeared at the retention time of 3.4 was suspected as acetone which was the initial solvent of naphthalene. However, there were 2 additional dominant peaks in the control in this medium, which were probably impurities (Figure 5).

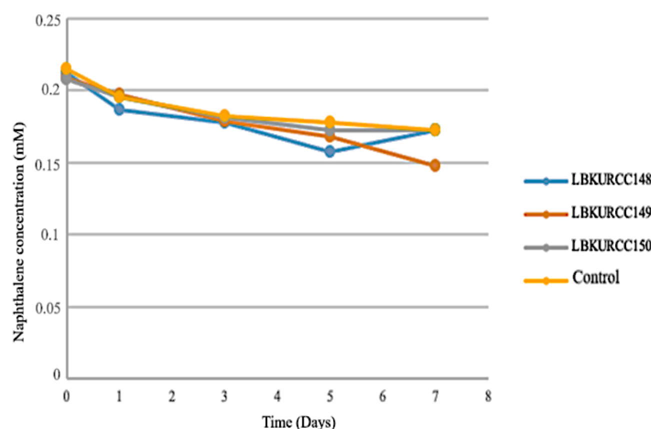

Figure 4. Decrease in naphthalene concentration during the incubation time

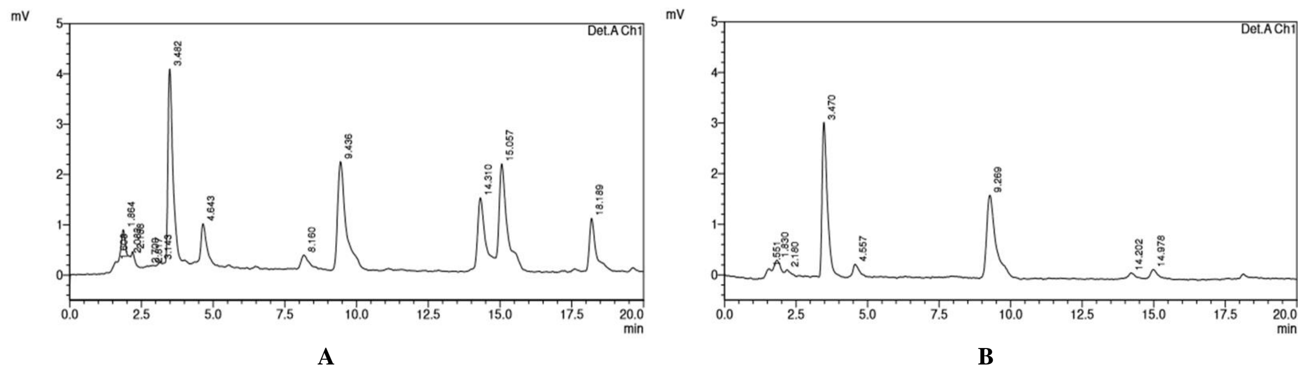

Figure 5. HPLC Chromatogram in media with glucose, (A) control dan (B) *Pseudomonas* sp. LB KURCC149

Table 4. Duncan test results from naphthalene concentration

Incubation time	Isolates			
	***Pseudomonas* sp. LBKURCC148**	***Pseudomonas* sp. LBKURCC149**	***Pseudomonas* sp. LBKURCC150**	**Control**
0	0.212±0.033[a]	0.193±0.058[a]	0.199±0.073[a]	0.215±0.002[c]
1	0.187±0.041[a]	0.189±0.054[a]	0.185±0.069[a]	0.195±0.001[b]
3	0.177±0.030[a]	0.168±0.073[a]	0.173±0.049[a]	0.182±0.002[a,b]
5	0.157±0.052[a]	0.151±0.065[a]	0.167±0.036[a]	0.178±0.017[a]
7	0.173±0.028[a]	0.149±0.055[a]	0.146±0.059[a]	0.172±0.008[a]

Note: a,b,c,d different letters within a column indicate significant differences at $P \leq 0.05$.

Table 5. Degradation percentage of naphthalene at seven days incubation

Isolates	Percentage of degradation (%)
Pseudomonas sp. LBKURCC148	0
Pseudomonas sp. LBKURCC149	13.95
Pseudomonas sp. LBKURCC150	0

Naphthalene is one of the dominant pollutants found in petroleum and is classified as one of 16 carcinogenic pollutants (Bonfa et al. 2011; Ghosal et al. 2016). Naphthalene is used as a biodegradation model for hydrocarbon compounds to test the ability of microorganisms to degrade PAHs, because it is the simplest and most water-soluble PAHs when compared to other PAHs (Chang et al. 2014; Hussein and Mansour 2016). The media used in this study was Minimal Media (MM) with the addition of tween 80. The function of tween 80 is as a surfactant that can increase the solubility of naphthalene so that it is easier to use by microorganisms (Nielsen et al. 2016). The addition of surfactants can increase the process of degradation of naphthalene and anthracene (Mesbaiah et al. 2014). Furthermore, in the optimization media for bacterial growth, the media is added with glucose as an additional carbon source to induce the growth of microorganisms and is expected to increase the ability of microorganisms to degrade hydrocarbons (Juliani et al. 2016; Xu et al. 2018).

PAH biodegradation process is influenced by several factors, namely microbial properties, number of microbial cells, nutrients, oxygen, pH, PAH substrate concentration and PAH properties (Bisht et al. 2015; Hatef and Khudeir 2016). Therefore, in this study, to determine the ability of microbes to degrade substrates, it is known through increasing the number of cells, decreasing the pH of the media and decreasing the concentration of PAH substrates, especially naphthalene (Ghosal et al. 2016).

The growth of microorganisms in liquid MM was indicated by an increase in the turbidity of solution (Sudrajat 2015) when calculated by spectrophotometer. Each isolate was able to adapt and use a hydrocarbon substrate for its growth. According to Sharah et al. (2015) exponential phase cell division occurs at a constant rate, mass doubles at the same rate, metabolic activity is constant, allowing microorganisms to grow very quickly. Then, each isolate experienced a stationary phase. In the stationary phase cell biomass reaches its maximum number, but the growth rate is constant between living and dead bacteria. This is due to reduced nutrients resulting in competition for nutrients, causing some cells to die and others to continue to grow. This is what causes the number of cells remain constant. In this phase, metabolic products are also formed and tend to be toxic to microbes.

The addition of glucose to the media had a positive effect on the growth rate of each isolate when compared to the media without glucose. It can be seen that the OD of microbial growth without glucose under optimal conditions was low, while in the presence of glucose the OD of microbes under optimal conditions was high, especially

Pseudomonas sp. LBKURCC149. This proved that in the presence of glucose, each isolate was able to use glucose to optimize its growth in the media. The addition of glucose as a source of co-substrate increases the growth of microorganisms significantly (Bren et al. 2016). Glucose can increase microbes at it is a carbon source with a simpler structure and lighter than naphthalene so that glucose is more easily used by microbes for growth and metabolism (Juliani et al. 2016).

pH is one of the parameters whose information can be used to support the degradation of contaminant compounds or not (Azubuike et al. 2016). pH is an essential environmental parameter that affects activities like cell membrane transport and catalytic reaction balance. It should be taken into consideration for improving process control of the bioremediation system (Al-Hawash et al. 2018). The decrease in pH in the medium during the incubation period indicated a chemical change in the hydrocarbon substrate caused by enzymes produced by microorganisms (Atlas 1981; Novianty et al. 2021). The initial pH in this study was made in such a way with the addition of NaOH so that the pH of the media became 7. This was done to support the optimum microbial growth environment, pH 7 is also the optimum pH of *Pseudomonas* sp. in degrading naphthalene (Hatef and Khudeir 2015; Aina et al. 2018).

According to the results of the analysis of pH data on media without glucose, it was observed that naphthalene was the only carbon source and there was a decrease in pH from an average of 7 to 6. Janbandhu and Fulekar (2011) also reported that the pH of media decreases and become more acidic from 7 to 5. This indicates that organic acids are produced by microbes from the use of the existing naphthalene substrate. Meanwhile, in the media with glucose as a co-substrate, the pH of the media decreased from an average of 7 to 4. This may be because glucose was used as a co-substrate by microbes to grow more and resulted in more organic acids being formed as a result of metabolism microbes so that the pH in the media decreased significantly.

Naphthalene degradation was high at the beginning of incubation until the last day of incubation (7th day). From the data on the percentage of degradation in the media without glucose, each isolate was known to be able to degrade naphthalene for growth (Feijoo-Siota et al. 2008). The difference in the degradation ability of each isolate was caused by differences in the viability and adhesion of cells. *Pseudomonas* sp. LBKURCC148 had a high degradation of naphthalene indicated by a decrease in the high concentration of naphthalene, it may be suspected that its high viability and high cell adhesion can easily accept hydrocarbon molecules through its cell membrane so that (mass transfer) will increase the rate of naphthalene degradation.

Naphthalene is degraded by bacteria using various enzymes, namely naphthalene dioxygenase, cis-dihydrodiol dehydrogenase, catechol 1,2-dioxygenase or catechol 1,3-dioxygenase. Then produce compounds that can enter the Krebs cycle and produce energy (Travkin and Polyanikova 2021). In this study, with the addition of glucose, it was seen that *Pseudomonas* sp. LBKURCC149 was the most superior isolate in degrading naphthalene by only 13.95% while the other isolates did not reduce the concentration of naphthalene. When compared with no glucose, the proportion of naphthalene degradation was greater (Vanishree et al. 2014). Lee et al. (2005) reported that 2 g/L glucose degradation did not increase phenanthrene even though there was an increase in microbial growth. This is due to increased microbial growth and the production of products in abundant organic acids, causing an increase in media growth. Media that is too acidic reduces microbial activity. The concentration of glucose as co-substrate and the complexity of PAHs can also influence the ability of microorganisms to carry out degradation. Another factor is that microorganisms can degrade simpler to more complex compounds (Kermani et al. 2013). So in this study, glucose was used as the dominant substrate for seven incubation times because of its simpler structure than naphthalene (Lee et al. 2005). The degradation of complex compounds took longer time (Kermani et al. 2013).

The presence of glucose as much as 10 g/L with 7 days incubation as a co-substrate increased PAH degradation by 50% as compared to without glucose. In the presence of glucose normal growth of microorganisms, takes place then when the glucose has started to run out, and these microorganisms must live use PAH substrates. These microorganisms are induced to produce enzymes that can degrade naphthalene (Bourguignon et al. 2014). Naphthalene analysis in isolation with the highest naphthalene degradation was carried out using an HPLC instrument with a reversed phase with an elution gradient system. Based on the analysis of the use of HPLC at a wavelength of 291 nm, two dominant peaks were produced, namely the retention time of 3,4 and 9.4 in the media without glucose. Naphthalene is known to be detected at a retention time of 9.4 while the peak that appears at a retention time of 3.4 is thought to be acetone which is an early discovery of naphthalene. HPLC analysis that supports a decrease in concentration between the control and the analyzed sample. Evidenced by the decrease in peak area in the sample compared to the control. The peak area expresses the concentration of components in the mixture (Hendayana, 2006; Su et al. 2011).

In conclusion, the most effective isolate in degrading naphthalene was *Pseudomonas* sp. LBKURCC149 with a degradation percentage of 13.95% accompanied by an increase in optical density and a decrease in pH in the media. The growth of each isolate was successfully increased in the presence of glucose and the level of naphthalene degradation ability by bacterial isolates. *Pseudomonas* sp. LBKURCC149 has the highest ability to degrade naphthalene by 13.95%, which was confirmed by HPLC chromatogram results where there was a decrease in the concentration of naphthalene after degradation. This study infers that *Pseudomonas* sp. LBKURCC149 strain can help remediate contaminations attributed to naphthalene.

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