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EFFECT OF AZOTOBACTER SP. AND PSEUDOMONAS SP. AS A BIOSTIMULANT IN PETROLLEUM WASTE USING SORGHUM PLANT (Shorgum bicolor L.)

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ABSTRACT

Inoculation of Azotobacter sp. and Pseudomononas sp. as a biostimulant agent is an appropriate strategy to be carried out to improve the phytoremediation performance of petroleum waste. Azotobacter sp. can produce phytohormones and fix N2, while Pseudomonas sp. are phosphate solubilizing bacteria. The study aims to examine the potential of Azotobacter sp. and Pseudomonas sp. in increasing the biodegradation of petroleum hydrocarbons, in the Phytoremediation system of Sorghum (Sorghum bicolor L.). The research design used was a factorial randomized block design, which consisted of 2 factors, namely the treatment of Azotobacter sp., which consists of 4 levels and treatment of Pseudomonas sp. which consists of 4 levels. The results showed that there was an interaction between Azotobacter sp. and Pseudomonas sp. on the efficiency of hydrocarbon biodegradation, but there was no interaction to the population of Azotobacter sp. and Pseudomonas sp., and growth of sorghum plants. Treatment of 2% Azotobacter sp. + 3% Pseudomonas sp. and treatment of 3% Azotobacter sp. + 3% Pseudomonas sp showed the value of biodegradation efficiency which tends to be higher than other treatments. Aplication of the two isolates was not able to increase the population of Azotobacter spp, Pseudomonas spp. and the height of sorghum, but can act as a biostimulant to significantly increase the biodegradation process of petroleum hydrocarbons.

Keywords: Azotobacter sp., , phytoremediation, petroleum hydrocarbon, Pseudomonas sp, shorgum.

1. INTRODUCTION

Petrolleum is a very important energy source until this decade for the industrial and household sectors. The development of the oil industry sector in addition to having a positive impact also has a side effect, namely the occurrence of environmental pollution, is the presence of soil contaminated with petroleum [7], and difficult to decompose which can cause damage to ecosystems and disrupt the water cycle.

Pollutant compounds that enter the environment naturally can be degraded through biological and chemical processes. However, often the burden of pollution in the environment is greater than the rate at which the pollutant degrades naturally. As a result, pollutants will accumulate in the water and soil environment so that human intervention with existing technology is needed to overcome the pollution [11]. One of alternative technology to overcome the environment polluted by petroleum is the phytoremediation technique, which is a technology that is environmentally friendly, effective and economical. The main biological agent in the

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phytoremediation method is to utilize plants supported by microbial activity such as bacteria and fungi which aims to remove and improve the condition of soil, sludge, ponds, rivers from contaminants [9].

In this study, the plant selected as a phytoremediation agent was sorghum (*Sorghum bicolor* L.) combined with the bacterium *Azotobacter* sp. and *Pseudomonas* sp. Sorghum plants have features, among others, are more resistant to drought and grow in almost all types of soil, are relatively more adaptable to a wide range of ecological conditions and can adapt to conditions that are not suitable [13], have an extensive root system and spread. so that it can function as a potential phytoremediation agent [17]. However, the use of plants in the phytoremediation process is generally less able to adapt to an environment containing pollutants with high levels of toxicity, so that they can only degrade about less than 5% of toxic waste [18]. To support the phytoremediation process, it is necessary to add bacterial inoculants as biostimulants that can support the degradation process of petroleum waste and the growth of phytoremediator plants.

Azotobacter sp. is a non-sombiotic bacterium that has the ability to fix nitrogen for supplaying avalaiiblity N in the soil [10]; [8]. The characteristics of the genus *Azotobacter* sp. can produce extracellular compounds in high quantities. The characteristics of the resulting extracellular products are diverse in structure and constituent components, and function as biosurfactants [16]. The Azotobacter genus can also excrete biostimulant compounds [4], such as thiamine, riboflavin, pyridoxine, cyanocobalamin, nicotine, indole acetic acid, gibberellins and pantothenic acid [14].

Another bacterium that can have a positive impact on plant growth is Pseudomonas aeruginosa. The ability of P. aeruginosa and to produce Rhamnolipid biosurfactants. Rhamnolipid production is related to the presence of nitrogen which plays a role in biosurfactant synthesis [3] The synergism of the use of the two bacterial inoculants is aimed at improving the performance of the phytoremediation system of soil contaminated with petroleum waste. Potential use of Azotobacter sp. is a producer of N sources, because this genus is able to fix N free from the air into N in a form that can be utilized by other bacteria and plants, while Pseudomonas sp. as bacteria capable of dissolving bound P into P that is available to plants [5]. Thus, if the two bacteria are applied in appropriate dose in the phytoremediation system will be able to improve the phytoremediation performance while reducing the use of N fertilizer and making the use of P fertilizer more effective.

The purpose of this research is to examine the effect of *Azotobacter* sp and *Pseudmonas* sp in improving performance of hydrocarbon biodegradation of waste petroleum in a phytoremediation system using sorghum (*Shorgum bicolor* L.) plants. An investigation was conducted on the efficiency of hydrocarbon biodegradation, the population of *Pseudomonas* spp. and *Azotobacter* spp., and growth of sorghum plants.

2. MATERIAL AND METHOD

2.1 The research design

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The research design used a factorial randomized block design (RBD).
Factor I: treatment of *Azotobacter* sp. (A)

a_0 = without *Azotobacter* sp.
a_1 = *Azotobacter* sp. 1%
a_2 = *Azotobacter* sp. 2%
a_3 = *Azotobacter* sp. 3%

Factor II: treatment of *Pseudomonas* sp. (B)

b0 = without Pseudomonas sp.
b_1 = *Pseudomonas* sp.1%
b_2 = *Pseudomonas* sp. 2%
b_3 = *Pseudomonas* sp. 3%

2.2 Initial analysis of experimental soil and production of isolat culture

Soil samples as much as 500 mg were analyzed for physical and chemical properties of the soil, soil samples were taken compositely at a depth of 0-20 cm from the soil surface.

Inoculant production of *Azotobacter* sp. was carried out using liquid mineral media containing 2% molasses while *Pseudomonas* sp. using Pikovskaya liquid medium. As much as 10% pure culture was inoculated into the reactor containing each liquid medium for *Azotobacter* sp. and *Pseudomonas* sp. then shaken at 100 rpm for 72 hours at room temperature and pure culture Pseudomonas sp. and Azotobacter sp. harvested.

2.3 Preparation of Sorghum seed and petroleum contaminated soil

Sorghum seed preparation and planting Soil for planting media is the order Inceptisols taken compositely. The media used as a growing medium for sorghum was a mixture of Inceptisols mineral soil with compost as basic fertilizer at a dose of 25 g per 10 kg ha^{-1} of soil into each polybag, then incubated for 1 week. The dose of fertilizer used is of the recommended dose of the Ministry of Agriculture for sorghum or cereal crops, namely, 0.5 g pot^{-1} urea fertilizer (equivalent to 100 kg ha^{-1}), SP-36 fertilizer 0.25 g pot^{-1} (equivalent to 50 kg ha^{-1}), and 0.125 g pot^{-1} KCl fertilizer (equivalent to 25 kg ha^{-1}). Urea fertilizer application was given twice during the growing season with a dose of 0.25 g at the time of planting. planting sorghum and one month after planting as much as 0.25 g.

2.4 Inoculation of *Azotobacter* sp. and *Pseudomonas* sp. and observation research

The petroleum hydrocarbons used were 5% TPH (Total Petroleum Hydrocarbon) per 10 kg of soil media in microcosms which were mixed directly with each treatment of *Azotobacter* sp., *Pseudomonas* sp. Then, it is mixed homogeneously and used as a planting medium that already contains compost. Furthermore, sorghum planting and plant maintenance and observations until the final vegetative. Observations were carried out every fourteen days by analyzing the concentration of Total Petroleum Hydrocarbon (TPH), TPH concentration analysis was carried out using the n-hexane gravimetric method. At the end of the study, the calculation of the efficiency of hydrocarbon biodegradation was carried out. Population analysis of *Azotobacter* sp. and *Pseudomonas* sp. using the Total Plate Count (TPC) serial dilution method. Observation of

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the growth of sorghum in the vegetative phase was carried out by measuring the increase of plant height every fourteen days from the beginning of planting sorghum seeds until the maximum vegetative phase.

3 RESULT AND DISCUSSION

3.1 Characteristics of initial inceptisol of the experiment.

The soil used in this experiment was Inceptisols taken in the topsoil layer with a depth of 0-20 cm around Ciparanje Jatinangor Land. Results of initial soil analysis represented in Table 1.

Inceptisols in this study had a slightly acidic soil reaction (5.98), low CEC (15.90 cmol kg-1), moderate C-Organic (2.29 %), low total N (0.16%) and very low potential P2O5. The content of exchangeable cations was low. The inial soil analysis data of Inceptisols has a high dusty clay content compared to sand. This indicates that soils containing higher dusty clay have higher total pores so that the presence of water and air is available in the soil [6] with the acidity level of the soil tends to be acidic.

Table 1. Characteristics of Inceptisols at the beginning of the experiment

No	Parameters	Unit	value	Criteria*)
1.	pH H_2O (1 : 2,5)		5,98	slightly acid
2.	pH KCl 1 N (1 : 2,5)		5,24	-
3.	C-Organic	%	2,29	medium
4.	N-total	%	0,16	low
5.	C/N rasio		14,31	medium
6.	P_2O_5 Bray I	Ppm P	4,75	low
7.	P_2O_5 HCl 25%	mg 100 g^{-1}	56,28	high
8.	K_2O HCl 25%	mg 100 g^{-1}	21,05	medium
9.	Al-dd	cmol kg^{-1}	0,10	-
	H-dd	cmol kg^{-1}	0,41	-
10.	CEC	cmol kg^{-1}	15,90	low
11.	Texture:			
12.	Pasir (%)	%	7	dusty clay
13.	Debu (%)	%	40	dusty clay
	Liat (%)	%	53	dusty clay

3.2 The efficiency level of hydrocarbon biodegradation due to inoculation of *Azotobacter* sp. and *Pseudomonas* sp.

The level of hydrocarbon biodegradation due to treatment is shown in table 2., which is represented by the value of biodegradation efficiency describing the phytoremediation performance.

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Table 2. Effect of *Azotobacter* sp. and *Pseudomonas* sp. in Petroleum Hydrocarbon Phytoremediation Process on Hydrocarbon Biodegradation Efficiency (%) at week 8.

Azotobacter sp.	*Pseudomonas* sp.			
	0% (b_0)	1% (b_1)	2% (b_2)	3% (b_3)
0% (a_0)	43.71 (a) A	59.71 (b) A	55.41 (ab) A	51.6 (a) A
1%(a_1)	40.80 (a) A	57.70 (b) A	53.76 (ab) A	53.73 (a) A
2%(a_2)	29.68 (a) A	42.47 (ab) A	38.98 (a) A	61.15 (a) B
3%(a_3)	42.30 (a) A	34.65 (a) A	63.63 (b) B	68.58 (a) B

Note: Numbers followed by the same letter are not significantly different at the 5% Duncan test level. Unit of biodegradation efficiency in %. Letters without brackets are read horizontally and letters in brackets are read vertically ($p < 0.05$).

The results of the investigation showed that there was an interaction between *Azotobacter* sp. with *Pseudomonas* sp. on the level of efficiency of hydrocarbon biodegradation.

Application of 3% dose of Azotobacter sp. plus 3% *Pseudomonas* sp. and 3% *Azotobacter* sp. added by 2% *Peudomonas* sp. was able significantly ($p < 0.05$) increasing the efficiency of biodegradation compared to other treatments. However , that two treatments were not significantly different. Treatment of the dose of 2% *Azotobacter* sp. added by 3% *Pseudomonas* sp. also showed a significant increase ($p < 0.05$) compared to the 2% *Azotobacter* sp. which added 0%, 1% and 2% Pseudomonas sp. application. The level of biodegradation efficiency in the treatment of 3% *Pseudomonas* sp. and 3% Azotobacter sp. showed the biodegradation value tend highest compared to other treatments.

The results of the investigation showed that the addition of 3% *Azotobacter* sp. effectively increase the level of hydrocarbon biodegradation. This is due to that *Azotobacter* sp. capable of producing extracellular which functions as a biosurfactant. Biosurfactant is an extracellular product of *Azotobacter* sp. which was able to increase the availability of hydrocarbons as substrates for degrading microorganisms by increasing the emulsification of hydrocarbons, so that they can be readily available as substrates for *Pseudomonas* sp. As stated by [15] and [16] that *Azotobacter chroococum* can produce potential biosurfactants to significantly increase the biodegradation of petroleum hydrocarbons. The higher the population of *Azotobacter* sp. the higher the biosurfactant produced so that the level of solubility of petroleum hydrocarbons is high and easily degraded by petrobacter indigenous or *Pseudomonas* sp.

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3.3 The population of *Azotobacter* spp.

The population of *Azotobacter* sp. observed was the final population of *Azotobacter* sp. at 8 weeks after planting. The result of the final population analysis of *Azotobacter* sp. (Table 3.) showed that there is no interaction between the application of *Azotobacter* sp. and *Pseudomonas* sp. during the phytoremediation process of petroleum hydrocarbons. The result of the investigation of the population of *Azotobacter* sp. displayed by ranking.

Treatment of *Azotobacter* sp.1% added by 3% *Pseudomonas* sp. resulted in the density of *Azotobacter* spp. in soil which tends to be the highest compared to other treatments, although not statistically significant. The growth of *Azotobacter* spp. in soil based on statistical tests showed that it did not increase significantly due to the availability of substrates for *Azotobacter* spp. which is limited, this shows that *Azotobacter* sp. is not able to use hydrocarbons as its substrate. The cell regeneration of *Azotobacter* spp. process showed slow down, so that there is no significant increase in its growth.

Table 3. Effect of *Azotobacter* sp. and *Pseudomonas* sp. to the Final Population of *Azotobacter* sp. on the Petroleum Hydrocarbon biodegradation process on the 8th week. after planting

No Rank	Treatment	*Azotobacter* spp. dencity (10^8 CFU/g)
1	a_1b_3 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 3%)	6.03
2	a_0b_1 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 1%)	5.58
3	a_3b_2 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 2%)	5.28
4	a_3b_0 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 0%)	5.15
5	a_1b_0 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 0%)	4.53
6	a_1b_2 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 2%)	4.41
7	a_0b_0 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 0%)	4.41
8	a_1b_1 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 1%)	4.30
9	a_2b_3 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 3)%	4.18
10	a_2b_0 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 0%)	4.18
11	a_3b_0 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 0%)	4.15
12	a_2b_2 (*Azotobacter* sp. 2% + *Pseudomonas* sp.	4.13

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	2%)	
13	a_0b_3 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 3%)	4.11
14	a_2b_1 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 1%)	3.51
15	a_3b_1 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 1%)	3.01
16	a_0b_2 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 2%)	0.07

Based on the concept of hydrocarbon biodegradation, *Pseudomonas* sp. is including bacteria that can degrade hydrocarbon compounds effectively and is able to produce intermediate compounds that can be used as substrates by *Azotobacter* sp. and *Azotobacter* sp. can produce biostimulant compounds and produce N nutrition. Meanwhile, the result of this study showed that the growth of the *Azotobacter* spp did not increase, this is assumed that *Pseudomonas* sp. in this research was not effectively in degrading hydrocarbons circumstance, so that *Azotobacter* sp could not obtain intermediates substances as its substrates. Also, this might be showing that stimulation of root exudates in the rhizosphere does not always cause encrease contaminant degradation, because it is possible that the rhizosphere microorganism population increases but the degrading hydrocarbon activity by microorganism decreases [2]. A research reported by [19] showed that the presence of petroleum hydrocarbons can reduce the number of bacterial species in a group, although the number of individual species increases with the amount of petroleum waste. Also due to the pH conditions of the experimental soil which tend to be acidic, resulting the metabolic activity of *Azotobacter* spp. in the soil was inhibited, leading in the growth of *Azotobacater* spp. during the hydrocarbon degradation process was not signifficant increasing

3.4 The population of *Pseudomonas* sp.

The results of the investigation of the final population of *Pseudomonas* sp. shown in Table 4.

The results of statistical analysis showed that there was no interaction between *Azotobacter* sp. and *Pseudomonas* sp.application to the final population of *Pseudomonas* spp. on the phytoremediation system by sorghum plants. Based on the ranking results in Table 4, it can be seen that the treatment 2% *Azotobacter* sp. + 0% *Pseudomonas* sp. had the highest population value of 20.28. 10^8 x cfu/g compared to other treatments. The addition of *Azotobacter* sp. and *Pseudomonas* sp. had no effect in increasing the growth of *Pseudomonas* spp., this was due to the inhibition caused by complex mechanisms, including the possibility of antagonistic effects caused by *Azotobacter* sp. to *Pseudomonas* sp. population. This resulted in the growth rate of *Pseudomonas* sp. inhibited. Another factor that cause of a low rhizosphere effect phenomenon. The rhizosphere effect of the sorghum plant was not maximal in excreting exudate which acts as a stimulant compound and important nutrients for supporting the growth of *Pseudomonas* sp. and *Azotobacter* sp. It can be seen by observations that the sorghum plants showed stunted growth due to the toxicity conditions caused by petroleum hydrocarbons in the soil and resulting the root exudate production process was also to be inhibited.

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Table 4. Effect of *Azotobacter* sp. and *Pseudomonas* sp. on the Final Population of *Pseudomonas* spp. on the Petroleum Hydrocarbon Phytoremediation Process on 8th week.

No Rank	Treatment	*Pseudomonas* spp. dencity (10^8 CFU/g)
1	a_2b_0 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 0%)	20.28
2	a_3b_1 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 1%)	12.78
3	a_0b_2 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 2%)	12.51
4	a_1b_3 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 3%)	11.58
5	a_3b_2 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 2%)	11.33
6	a_2b_2 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 2%)	10.10
7	a_2b_1 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 1%)	9.61
8	a_1b_0 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 0%)	9.45
9	a_1b_1 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 1%)	9.23
10	a_1b_2 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 2%)	8.31
11	a_3b_3 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 3%)	8.21
12	a_2b_3 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 3%)	8.13
13	a_0b_1 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 1%)	7.78
14	a_3b_0 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 0%)	7.11
15	a_0b_3 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 3%)	6.36
16	a_0b_0 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 0%)	5.70

Root exudates excreted by plant are alcohol, sugar, and organic acid which can provide sufficient energy and carbon for 10^8-10^9 microbial cells per gram of soil in the rhizosphere. The presence of this root exudate causes the microbial population to be 5-100 times increase. Increasing the number of microbial populations is known as the Rhizosphere Effect (ER) [1]. But the results of this study that the exudate produced by the sorghum plant was not able to increase the population

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of *Pseudomonas* sp. as a hydrocarbon-degrading bacterium. As according to the statement of Pivetz et al., [12] that the stimulation of rhizosphere does not always cause the degradation of contaminants increase. It because the rhizosphere microorganisms.

3.5 Increasing of Sorghum Plant Height

The plant height observed during 8 WAP represnted on Table 5. The results of statistical analysis showed that there was no interaction between the application of *Azotobacter* sp. and *Pseudomonas* sp. on the phytoremediation process of petroleum hydrocarbons on plant height increase.

Table 5. Effect of *Azotobacter* sp. and *Pseudomonas* sp. on the increasing of Sorghum Plant Height in the Petroleum Hydrocarbon Phytoremediation Process on the 8th week.

No Rank	Treatment	Increasig in plant height (cm)
1	a_0b_2 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 2%)	7,13
2	a_0b_0 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 0%)	5,73
3	a_2b_2 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 1%)	3,70
4	a_1b_0 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 0%)	2,43
5	a_3b_1 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 1%)	2,20
6	a_1b_3 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 3%)	2,20
7	a_2b_0 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 0%)	2,17
8	a_3b_0 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 0%)	2,03
9	a_1b_2 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 2%)	1,90
10	a_0b_3 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 3%)	1,87
11	a_0b_1 (*Azotobacter* sp. 0% + *Pseudomonas* sp. 1%)	1,70
12	a_3b_2 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 2%)	1,60
13	a_1b_1 (*Azotobacter* sp. 1% + *Pseudomonas* sp. 1%)	1,27
14	a_2b_1 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 1%)	1,23

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15	a_2b_3 (*Azotobacter* sp. 2% + *Pseudomonas* sp. 3%)	0,60
16	a_3b_3 (*Azotobacter* sp. 3% + *Pseudomonas* sp. 3%)	0,50

Application of *Azotobacter* sp. and *Pseudomonas* sp. did not increase the plant height of Sorghum significantly, this was because the population of *Azotobacter* sp and *Pseudomonas* did not grow optimally on media contaminated with petroleum hydrocarbons, so that the adaptability of *Azotobacte*r sp. and *Pseudomonas* sp. decreased, which resulted in not providing a significant role as a biological agent that increases the growth of sorghum plants. The plant height in the study was lower than the plant height in the description of plants that grew normally, which was 90 cm. The stunted growth of sorghum plants occurs due to the negative impact of petroleum hydrocarbon contamination, where petroleum hydrocarbon reduced the level of nutrient absorption and water flow by the roots of the sorghum plant. The inhibition of the growth of sorghum plant height was also caused by the pH of the experimental soil which tended to be acidic.

4. CONCLUSION

Application of *Pseudomonas* sp. 2% - 3% added *Azotobacter* sp 2% - 3% can increase the efficiency of hydrocarbon biodegradation significantly in the phytoremediation system using sorghum plant, but both of bacteria were unable increasing the growth of sorghum as a phytoremediator plant. The population density of *Azotobacter* spp. and *Pseudomonas* spp. during the hydrocarbon degradation process took place increased but not significant. The pH conditions which tend to be acidic also suppress the activity and growth of *Azotobacter* sp. and *Pseudomonas* sp. Sorghum plants was not able to adapt optimally to toxic stress condition couse of petroleum hydrocarbons existing, by showing stunted growth. Both of bacteria tend to act as biostimulants in the hydrocarbon biodegradation process but do not play a role in increasing the growth of sorghum plants.

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