BIOREMEDIATION BY SINGLE CULTURE OF PSEUDOMONAS PUTIDA IN PETROLEUM CONTAMINATED SOIL

M. A. NOGO OLE, A. TUHULOULA, S.R. JULIASTUTI

[1,2,3]Department of Chemical Engineering Institut Teknologi Sepuluh Nopember, Surabaya 60111Indonesia
E-mail: julizainul@gmail.com

Abstract- Land contamination by petroleum exploration, production and discharge of waste causing serious damage to the ecosystem of the environment, human and animal. Biodegradation (bioremediation) processes have difficulty focused due to the complexity of the hydrocarbons that was adsorbed by the soil. The objective of this research was to determine the efficiency of bioremediation as a function of bacterial concentration. A method that was developed in bioremediation of petroleum-contaminated soil in addition to in-situ bioremediation was the ex-situ bioremediation with slurry phase bioreactor. The mixture of soil and water were fed in the bioreactor. The aerobic bacteria used in biodegradation of benzene, toluene, and xylene (BTX) process were Pseudomonas putida. The variables that would be measured were BTX concentration; bacterial population and operating conditions (temperature, pH and dissolved oxygen). Residues of petroleum hydrocarbon (BTX) were measured by gas chromatography method. Process was identified during 56 days in 3 bioreactors in parallel. Reactor was added by 12,5% (v/v) (A), 15% (v/v) (B)and 17,5% (v/v) (C) Pseudomonas putida bacteria. The bioreactors were agitated and aerated during bioremediation process. After 56 days, this research resulted in total degradation of BTX in reactor A reached 91,0400%; reactor B 91,1489%; and reactor C 97.6726%.

Keywords- Pseudomonas putida; bioremediation; Benzene; Toluene; Xylene; slurry bioreactor

I. INTRODUCTION

Land contamination by hydrocarbon can be affected by petroleum exploration. Based on data on 2013, for a drilling wheel contaminated area reached 0.5 – 2.1 m^2 [1]. When petroleum hydrocarbons released into the environment, parts of contaminated soil are dehydrated oil sludge, separated from the mixture of oil, water and soil. Most of the oil sludge is piled up outdoor next to the production site without any treatment and poses serious environmental problems.

The hydrocarbons in the sludge penetrate from the top soil into the subsoil slowly, presenting direct risk of contamination to subsoil and groundwater. On the other hand, the light hydrocarbons in the oil sludge vaporize, leaving behind a layer of oil-containing dust of soil which blows upwards to pollute the air. These contaminations of soil, water and air pose serious risk for the environment and human population [2].

The mono aromatic hydrocarbons, abbreviated BTEX which stand for benzene, toluene, ethyl benzene and three xylene isomers are highly soluble and volatile toxic substances, thus forming one of the main groundwater and health-risk contaminant group. BTEX enter the environment primarily through processes associated with gasoline and petroleum fuels, leakage of underground petroleum storage tanks, and spills at petroleum walls.

BTEX are highly receptive to microbial attack and the degradation mostly occurs under aerobic condition. [3] [4]. The final value of the processing of petroleum waste were listed in Environment Minister's Decision no. 128 (2003), as shown in table 1.

Table 1. Final value of the processing of petroleum waste [5]

Parameter	unit	Final value (after treatment)
sludge waste analyze*)		
1. pH		6 – 9
2. TPH	(µg/g)	10.000
3. Benzene	(µg/g)	1
4. Toluene	(µg/g)	10
5. Ethylbenzene	(µg/g)	10
6. Xylene	(µg/g)	10
7. Total PAH	(µg/g)	10

Bioremediation is an option that offers the possibility to destroy various contaminants using natural biological activity. As such, it uses relatively low cost, low technology techniques, which generally have a high pubic acceptance and can often be carried out on site [6]. Moisture content of soil, microbial population, nutrient availability, soil type, salinity and oxygen transport in soil are among the factors affecting the process of bioremediation [7]. Besides that, biodegradation happened by the presence of microorganism. Microorganism such as bacteria, fungi and algae could help bioremediation process. The main requirement on bioremediation process is the availability of carbon source for microorganism. On the aerobic bioremediation, microorganism requires oxygen supply. BTEX degraders detected in soil include Alcaligenes, Arthrobacter, Acidovorax, Agrobacterium, Aquasprilium, Brevibacterium, Bradyrhiobium, Variovorax [4], Pseudomonas sp., Bacillus sp., B. stereothermophilus, Vibrio sp., Nocardia sp., Corynebacterium sp., Achromobacter sp [8].

Pseudomonas putida was aerobic bacteria with shaped rod and 2 – 4 µm long and has flagella. This bacteria could live in normal condition and temperature 25 – 30°C [9]. Pseudomonas putida is gram negative bacterium able to metabolize BTEX and other aromatics as the only carbon and energy source. The strain demonstrates a diverse metabolism, and it is non-pathogenic compared to other species [4]. Based on study about Pseudomonas putida that has been done by Robledo-Ortíz et al in 2011, this bacterial could degraded benzene, toluene and o-xylene in a batch reactor at 30°C and pH 7for 6-14 hour [10].

II. DETAILS EXPERIMENTAL

2.1. Sampling and soil treatment

Soil samples were obtained from oil drilling sites by Pertamina-Petrocina East Java (PPEJ), Tuban, East Java, Indonesia. Soil was prepared by separating leaves, rocks and other large material and soil was removed from contaminated area (ex situ bioremediation).

The soil samples were sterilized to make sure there were not indigenous bacteria culture inside the soil. We hope only Pseudomonas putida culture inside bioreactors.

Bioremediation process was implemented in Wastewater Treatment Laboratory, Chemical Engineering Department, Institut Teknologi Sepuluh Nopember Surabaya. Soil slurry was made by mixing the soil with water at 20:80 ratio.

2.2. Preparation of medium and Pseudomonas putida

Liquid medium was prepared by mixing 8 grams of nutrient broth with 3 grams of glucose into 1 liter of boiling water. After sterilization by autoclave at 121°C, media was cooled until 28°C. Local strain of Pseudomonas putida was moved from agar medium to liquid medium in laminar flow. Bacteria and media were inoculated inside incubator shaker at 30°C and 90 rpm. Bacteria were counted by total plate count (TPC) method and after 36 hours, total bacteria were 9×10^8 CFU.

2.3. Soil Bioremediation

Bioremediation was performed in slurry phase bioreactor (figure 1) with agitation 100 rpm and aeration. Dissolved oxygen must be more than 2 mg/L and pressure 1 atm. Temperature was between 20 – 30°C and pH was between range 6,5 – 8.

5 liter of soil slurry inside bioreactor was aerated for an hour to keep the system aerobic. There were 3 bioreactors, bioreactor (bioreactor A, B and C). Media contained with Pseudomonas putida was added to bioreactor with concentration 12,5% (A); 15% (B); and 17,5% (C) (v/v). Bioremediation process occurred for 8 weeks.

Every 14 days, soil samples taken from bioreactors and extracted with n-hexane for 10-16 hours. Oil separated from solvent with vacuum evaporator for 7 – 10 minutes.

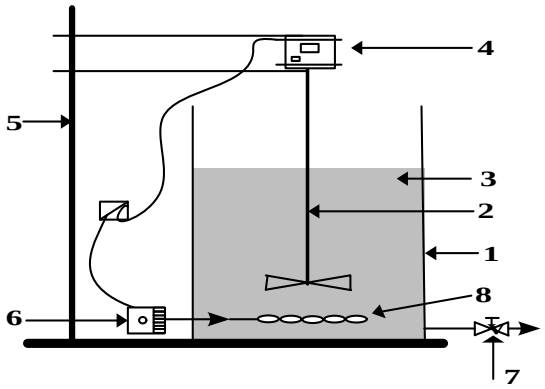


Figure 1. Slurry Bioreactor

1. Bioreactor
2. Impeller
3. Soil slurry
4. Impeller motor
5. Statip and klemp
6. Aerator
7. Valve
8. Spurger

2.4. Temperature, pH and Dissolved Oxygen

Temperature and dissolved oxygen were measured by DO meter Crison OXI 45 P and pH of slurry inside bioreactors was measured by pH meter Inolab WTW 7110. Temperature, pH and DO were measured three times a day for 56 days to make sure that bioreactor operation conditions was between the range

2.5. Total Cell Counts

Total cells counts were determined during the degradation of hydrocarbons. The counting method was counting chamber. 1 ml of slurry was diluted by 9 ml aquadest. Dilution was put in haemacytometer. Cell counts were observed and counted under microscope. Cell counts were done three times for a week. The result was plotted in graphic, compared with time and decreased of BTX.

2.6. BTX Analysis

Oil from soil extraction then analyzed by gas chromatography (GC) at Pusat Studi Lingkungan, Surabaya University, by HP6890 GC method and helium as gas carrier at 275°C and 16.26 psi.

III. RESULTS AND DISCUSSION

3.1. Soil Characteristic

Table 2. Soil Characteristic from oil mining sites at PPEJ Tuban

Parameter	Characteristic
color	brown
pH	9,101
temperature	28°C
Concentration of BTX:	
Benzene	26,440 µg/g
Toluene	121,000 µg/g
Xylene	129,000 µg/g
Concentration of PAH:	
Napphthalene	115,646 µg/g
Fluorene	30,272 µg/g
Anthrancene	101,183 µg/g
Fluoranthene	9,691 µg/g
Pyrene	18,258 µg/g
Chrysene	24,476 µg/g

Based on Law No. 128 of 2003, BTX in soil were too much and the concentration was above the limit that was set by government. Therefore the soil needs to be

treated until it eligible to be released into environment.

3.2. Effect of Pseudomonas putida

The addition of bacteria on the oil spill accelerate the process degradation of petroleum hydrocarbon [11]. Addition of Pseudomonas putida was supposed to decrease the hydrocarbons (BTX) that was contained in the soil.

The results of the chemical analysis related to the BTX concentration in three bioreactors were shown in table 3. Chemical analyses after biodegradation showed decreased BTX amounts in all reactors. The reduction of BTX was exclusively caused by the consumption of the mixture as the only carbon (C) source for Pseudomonas putida.

Table 3. a. Result of BTX degradation by 12.5% Pseudomonas putida

Hari	BTX concentration (µg/g)			
	12.5% *Pseudomonas putida*			
	B	T	X	total
0	26.44	121	129	276.44
14	24.24	29.07	13.38	66.69
28	12.39	10.65	10.84	33.88
42	10.57	4.091	10.56	25.221
56	10.36	3.989	10.42	24.769

Table 3. b. Result of BTX degradation by 15.0% Pseudomonas putida

Hari	BTX concentration (µg/g)			
	15.0% *Pseudomonas putida*			
	B	T	X	total
0	26.44	121	129	276.44
14	19.71	40.38	32.71	92.8
28	10.97	15.64	21.71	48.32
42	9.03	9.78	8.017	26.827
56	8.15	8.42	7.898	24.468

Table 3. c. Result of BTX degradation by 17.5% Pseudomonas putida

Hari	BTX concentration(µg/g)			
	17.5% *Pseudomonas putida*			
	B	T	X	total
0	26.44	121	129	276.44
14	12.17	2.405	13.59	28.165
28	1.175	2.193	8.351	11.719
42	1.032	0.7235	5.176	6.9315
56	0.925	0.703	4.806	6.4338

Concentration BTX decreased day by day for all bioreactors. It showed that bacteria in polluted soil could decrease BTX if supported by operating condition, agitation and aeration. Table 3 showed that concentration of bacteria affected to BTX concentration. In bioreactor A, with addition of 12.5% Pseudomonas putida, at the end of process the total BTX was decreased from 276.44µg/g become 24.769µg/g. In bioreactor B with addition of 15% Pseudomonas putida, decreasing of BTX from 276.44µg/g total BTX at the first week, become 24.468µg/g at the end of process. In bioreactor C, decreasing of BTX becomes significant, from 276.44µg/g total BTX becomes 6.4338µg/g total BTX after 56 days.

From the data in the table above could be seen that the decrease in the concentration of BTX occurs fastest on day 0 to day 7. Further reduction continued BTX reduced but not as much as in the first week. According to Leahy and Colwell (1990) [12], the growth of bacteria in the first week called adaptation phase, which will occur three mechanisms, were the induction or secretion of enzymes specific, occurring genetic changes that impact on the ability of bacterial metabolism, resulting in the increased ability of the organism to reduce certain components.

Leahy and Colwell (1990) explained that biodegradation rates have been shown to be highest to the saturates, followed by the light aromatics (monoaromatics hydrocarbons), with high molecular weight aromatics and polar compounds[11]. This makes the bacteria in the adaptation phase prefer to reduce alkenes and BTX first.

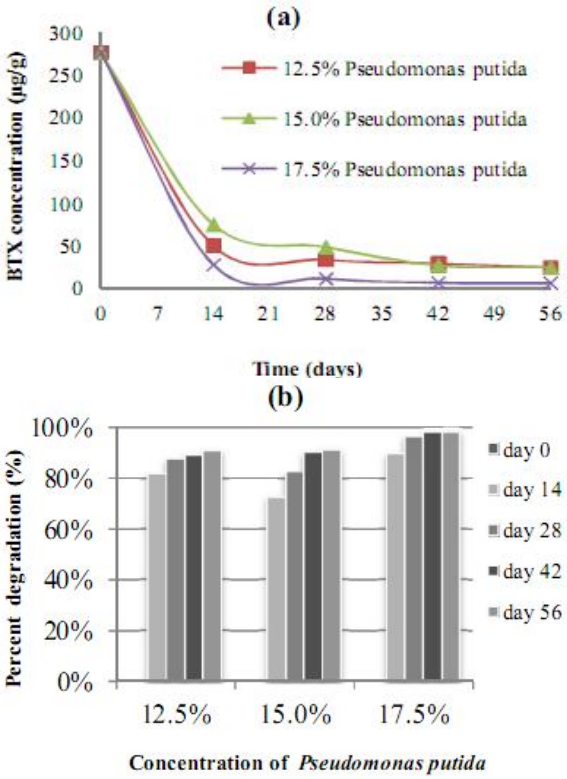


Figure 2. Effect of Pseudomonas putida in bioremediation process (a) decrease of BTX and (b) percent degradation of BTX

Figure 2 (a) showed the decrease of BTX in all bioreactor. Rapid decreases are happened at the first seven days. BTX was mono aromatics hydrocarbon component that was easier to be degraded compared with poly aromatics compounds. In contaminated

soil, besides mono aromatics, there were also poly aromatics too. At first 14 days, bacteria were in adaptation phase, bacterium tends to attack the carbon chain from mono aromatics compounds. When the BTX levels have decreased and bacteria enter the log phase, then the other hydrocarbon compounds reduced. As shown in figure 2 (a), after 28 days decreasing of BTX becoming more slowly but still continue until reach the lowest concentration of BTX at the 56th day.

As shown in figure 2 (b), percent degradation increase day by day, and at the end of process, percent degradation reached 91.04% for bioreactor with 12,5% Pseudomonas putida; 93.14% for bioreactor with 15,0% Pseudomonas putida and 97.67% for bioreactor with 17,5% Pseudomonas putida. The best result was reached by bioreactor with addition of 17,5% Pseudomonas putida.

3.3. Effect of bacteria population

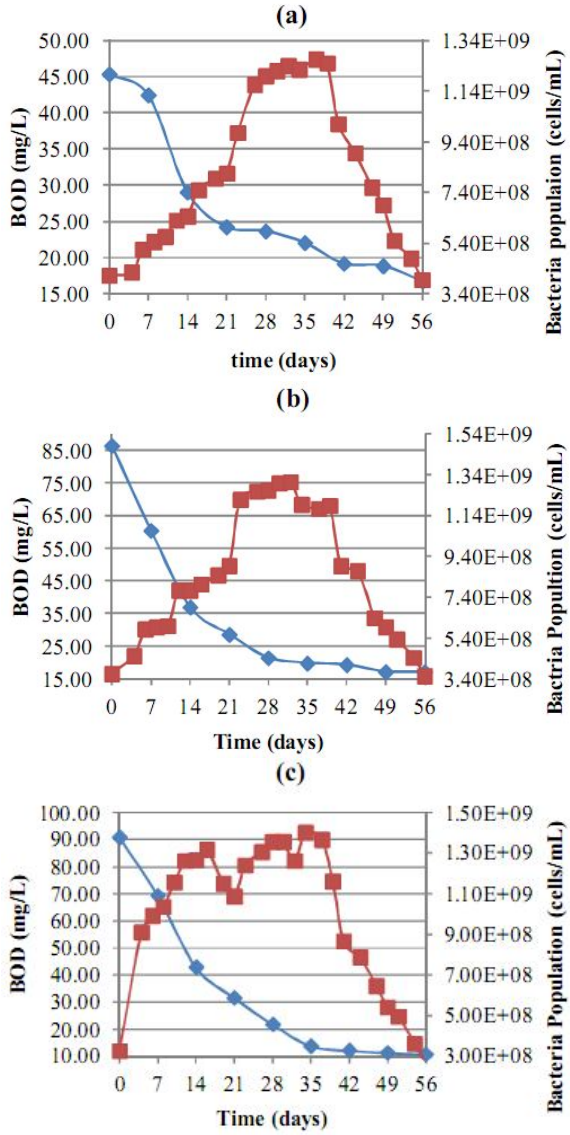


Figure 3. Correlation between BOD and bacterial population in bioreactors with addition of (a) 12,5% Pseudomonas putida; (b) 15,0% Pseudomonas putida; and (c) 17,5% Pseudomonas putida

Figure 3 shows the correlation between substrate inside bioreactors (measured with BOD) and bacterial population in three bioreactors. Bacteria use petroleum hydrocarbon as carbon source to do their metabolism and to reproduce. Figure 3 (a) showed that in bioreactor A, BOD decreased slowly. Bacterial population increased slowly and raised the stationer phase after 21 days. Similar thing happened ini bioreactor B, as shown in figure 3 (b), bacterial population increased rapidly from the 14th day and reached stationer phase after 21 days. At the same time, BOD decreased. It showed that the bacterial use substrate inside reactors to do their metabolism and reproduce. Figure 3 (c) showed that bacterial population increased rapidly since the beginning of the process along with the decreasing of substrate concentration (BOD). After 35 days, BOD becomes constant and the bacterial population decreased. This is happened because of the substrate was reduced and the bacteria was entering the death phase. At this phase, reduction of BTX still continued but the decreasing was not significant. That makes the biodegradation process was stopped after 56 days.

CONCLUSION

From the research that has been done, it could be concluded as follows:

1. Soil drilling containing petroleum hydrocarbons above the specified threshold required further treatment before being released into the environment.
2. Bacterial concentration affects the rate of BTX degradation. Best result of BTX reduction occurred in 17.5% of Pseudomonas putida addition with the ability to degrade total 97.67% of the initial level.
3. From overall data, the best result is 17.5% bacteria addition to degrade BTX from contaminated soil.

ACKNOWLEDGMENTS

We would like to say thank you for Chemical Engineering Departement of Institut Teknologi Sepuluh Nopember Surabaya, especially Wastewater Treatment Laboratory crew. Authors also grateful to the financial support from Lembaga Pengelola Dana Pendidikan (LPDP) of Indonesia.

REFERENCES

[1]. Chun-rong, L., Wen-ke, W., Yu-qing, C., Li-juan Wang. "Bioremediation of Petroleum Contamines-Soil". Presented at The 34th Congress of International Association of Hydro geologists; (2013)

[2]. Yudono, B. Said, M., Hakstege, P., Suryadi, F.X. "Kinetics of Indigenous Isolate Bacteria Bacillus mycoides used for Ex-situ Bioremediation of Petroleum Contaminated Soil in PT. Pertamina Sungai Lilin South Sumatera". J. Sustain. Dev., Vol.2; p 64-71 (2009)

[3]. Andreoni, V. & Gianfreda, L., "Bioremediation and monitoring of aromatic-polluted habitats", Appl. Microbial Biotechnol, Vol. 76; p. 287-308 (2007)
[4]. El-Naas, M.H., Acio, J.A., Telib, A.E.E., "Aerobic Biodegradation: Progresses and Prospects", Journal of Enviromental Engineering 2; p. 1104-1122 (2014)
[5]. Keputusan Menteri Lingkungan Hidup Nomor 128 Tahun 2003, "Tentang Tata Cara Persyaratan Teknis Pengolahan Limbah Minyak Bumi dan Tanah Terkontaminasi oleh Minyak Bumi Secara Biologis". (2003)
[6]. Vidali, M. "Bioremediation. An Overview", Pure Appl.Chem., Vol. 73, No. 7, p. 1163-1172. (2001),
[7]. Haghollahi, A. Fazalelipoor, MH. Schaffie, M. "The Effect of Sool Type on te Bioremediation of Petroleum Contaminated Soils", Journal of Environmental Management; p.197-201 (2016)
[8]. Desai, A., Vyas, P., Applied Microbiology. "Petroleum and Hydrocarbon Microbiology", Departement of Microbiology, M.S University of Baroda. (2006)
[9]. Elomari, M., Coroler, L., Verhille, S. Izard, D., Leclerc, H., (1997), "Pseudomonas monteili sp. nov., Isolated from Clinical Specimens", International Journal of Systematic Bacteriology, Vol. July 1997, p. 846-852
[10]. J.R. Robledo-Ortíz, D.E. Ramírez-Arreola, A.A. Pérez-Fonseca, C. Gómez, O. González-Reynoso, J. Ramos-Quirarte, et al, "Benzene, toluene, and o-xylene degradation by free and immobilized P. putida F1 of postconsumer agave-fiber/polymer foamed composites", International Biodeterioration & Bioegradation 65 (2011) p. 539-546. http://dx.doi.org/10.1016/j.ibiod.2010.12.011
[11]. Nugroho, A. "Bioremediasi Sludge Minyak Bumi dalam Skala Mikrokosmos: Simulasi Sederhana Sebagai Kajian Awal Bioremediasi" Land Treatment, Makara Teknologi, Vol. 10, No. 2; p. 82-89 (2006).
[12]. Leahy, JG., Colwell, RR., "Microbial Degradation of Hydrocarbons in the Environment", Microbiological Reviews Vol. 54, No.3; p. 305-315 (1990).

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