VOL. 11, NO. 11, JUNE 2016 ISSN 1819-6608
ARPN Journal of Engineering and Applied Sciences

www.arpnjournals.com

SURVIVAL OF PSEUDOMONAS PUTIDA AS BIOREMEDIATION AGENT IN SOIL CONTAMINATED WITH BIODIESEL-DIESEL MIXTURE (B50) BLENDS

Norshuhaila Mohamed Sunar[1], Quin anak Emparan[2], Paran anak Gani[2] and Abdul Mutalib Leman[3]
[1]Department of Chemical Engineering Technology; University Tun Hussein Onn Malaysia (UTHM); Parit Raja, Batu Pahat, Johor, Malaysia
[2]Department of Water and Environmental Engineering; University Tun Hussein Onn Malaysia (UTHM); Parit Raja, Batu Pahat, Johor, Malaysia
[3]Department of Mechanical Engineering Technology; University Tun Hussein Onn Malaysia (UTHM); Parit Raja, Batu Pahat, Johor, Malaysia
E-Mail: shuhaila@uthm.edu.my.

ABSTRACT

The commercialization of biodiesel and their diesel blends on the market of many countries would result towards environmental damages due to accidental spills such as leakage. This study aims to investigate the survival of Pseudomonas putida as bioremediation agent in soil contaminated with biodiesel/diesel blends (B50). The spillage stimulation of B50 was conducted at laboratory scale for 24 days of treatment time. The experimental results show that introduction of biodiesel/ diesel blends into the soil induced a reduction in growth of Pseudomonas putida in relation to control values. The Pseudomonas putida growth was influenced by with or without biodiesel/diesel content. The growth of Pseudomonas putida gained from contaminated soil was higher in control sample followed by B50. These findings enhance our understanding that Pseudomonas putida is sensitive to any ecosystem perturbation, and measurement of their colonies may serves as indicators of soil pollution and soil health.

Keywords: soil bioremediation, pseudomonas putida, biodiesel, and diesel.

1. INTRODUCTION

The increasing use of diesel oil in diesel engines of cars, industrial truck, and generators has lead to an increased demand for diesel oil [1].The increase for diesel demand and consumption will make petroleum reserves decreasing day by day. Due to the depletion of petroleum reserves in the world alternatives fuels have attracted increased attention [2, 3]. Recently, biodiesel fuels have attracted increasing attention worldwide as blending components or direct replacements for diesel fuel in conventional diesel engines. It can be legally blended and used in many different proportions such as B2 (blend 2% biodiesel + 98% diesel), B5 (blend 5% biodiesel + 95% diesel), B20 (blend 20% biodiesel + 80% diesel) and B100 (100% biodiesel). Since the early 21st century onwards, other states such as Australia, Brazil, Canada, Germany, China, South Korea and also Malaysia are also beginning to mandate that all diesel fuel sold at the petrol stations should at least contain 2% of biodiesel [4]. These benefits are good evident that pure biodiesel and their diesel blends will be used in the energy sector attending to transport vehicles driven by conventional diesel engines in the near future; however this means new threats to the environment in case of their spillage. The commercialization of pure biodiesel their diesel blends on market of many countries can cause environmental damage due to spills. Diesel spills usually take place during manufacturing, storage and transportation. Major spills such as pipeline, tanker or storage tank accidents create an acute problem of pollution. On the other hand, continuous low-level inputs are rarely noticed and may pose a serious threat to the environment as contamination accumulates.

Diesel is considered harmful and possibly carcinogenic to humans and it contains polyaromatic hydrocarbons that create a risk for human health because of their carcinogenic, mutagenic, and teratogenic properties [5]. Besides that, oil hydrocarbons disrupt ecosystem function such as respiration and the nitrogen cycle [6]. In addition, oils contain ingredients that are toxic to flora and fauna as well as to human health [7]. Moreover, oil damages and destroys infrastructure and contaminates landscape. Furthermore, diesel fuels have been observed to cause skin irritation and tumorigenic responses in mice especially if the fuel contains cracked material [8]. Diesel causes eye and skin irritation in human, but otherwise its effects on human are considered to be poorly investigated [9].

Biodiesel is composed of methyl or ethyl esters of long chain fatty acids with low structural complexity as oleate, palmitate, estearate, linoleate, myristate, laureate and linolenate derived from a variety of vegetable oil sources such as palm oil, soybean, peanut, coconut, sun-flower, cotton, babassu and castor oil and also from animal fats [10]. The biggest advantage of biodiesel as diesel fuel is environmentally friendliness that it has over petroleum diesel. The other advantages of biodiesel are its portability, ready availability, renewability, lower sulfur and phosphorus as well as aromatic hydrocarbons [11]. The contaminated areas can be cleaned-up by the emerging science and technology of bioremediation. It is a technique based on the action of microorganisms, in which hazardous contaminants will be turned into non toxic substances such as carbon dioxide (CO_2), water (H_2O) and biomass [10]. Potentially, bioremediation technology is

ARPN Journal of Engineering and Applied Sciences

cost-effective and environmentally friendly in comparison with physical-chemical treatments. Increasing attention has been paid to bacteria, which act as efficient tools in the bioaugmentation process. Often bacteria belonging to the genera Pseudomonas, Acinetobactor, Coryneform, Flavobacterium, and Burkholderia were used [12]. Natural soil bacteria can present in dormant or slow-growing state, but when stimulated by optimum environmental conditions, they multiply rapidly and then adapt to new environment [13].

Pseudomonas putida (P. putida) is a gram-negative, chemoorganotropic, aerobe obligate and aerobically respiratory metabolisms. They are straight or curved rods with dimensions in range size between 0.5 and 1.0 µm × 1.5 - 4.0 µm [14]. The temperature range of their growth is 0-42ºC and the optimum temperature is 35ºC [14]. This bacterium lives saprophytic ally in soil and water. P. putida is tolerant to xenobiotics and play a vital role in the treatment of petroleum-contaminated soil [15]. It occurs in various environmental niches because of its metabolic versatility and low nutritional requirement [16]. Recently, many studies on degradation of hydrocarbon by bacterium consortia, including P. putida have been carried out because of its high capability to degrade recalcitrant substances and inhibiting xenobiotics. In this sense, it can adapt to diverse substrates and posses some catabolic pathways capable of acting on recalcitrant substances [17]. The objective of the present work paper is to investigate the survival of P. putida as bioremediation agent in soil contaminated with biodiesel/diesel blends (B50). The contribution of this study can be capitalized as necessary tools for bioremediation of contaminated soil.

2. METHODS AND MATERIALS

a) Biodiesel and diesel

The biodiesel is palm-based biodiesel which produced by transesterification with methanol were obtained from Biodiesel Pilot Plant, Faculty of Engineering Technology, Universiti Tun Hussein Onn Malaysia (UTHM). Petroleum diesel oil (EN 590: 2004) was purchased from commercial diesel pump station at Parit Raja, Johor.

b) Soil

The soil samples were taken from campus area. The soil samples were collected in the range of 7-8 kg from the surface of 5-10 cm deep layer of soil.

c) Pseudomonas Putida Broth culture

Pure Pseudomonas putida ATCC 49128 samples were purchased from United States of America (USA) in dry culti-loop form. They were stored in chiller at temperature of 4 ºC prior to cultivation procedure. Approximately 16 g of medium (nutrient broth) was filled into Erlenmeyer flask containing distilled water. The mixtures were stirred gently and then dissolved by heating on hot plate at temperature of 100 ºC for about 2 hours until complete dissolution. After that, the medium was sterilized in autoclave at temperature of 121 ºC for about 15 minutes. It was then allowed to cool down for a few minutes until its temperature drop in between 35ºC to 37 ºC. One loops shaft was removed from the handle straight into the medium according to the manufacturer's instruction. The mixtures were then stirred until homogeneous and keep in the chiller at 4 ºC for 48 hours prior to further inoculation procedures.

d) Soil-Biodiesel/Diesel and Pseudomonas Putida mixture

Spill simulations with biodiesel/diesel blends in soil were carried out in accordance with Taylor [18], with modification. A 50% of biodiesel (250 mL) and 50% of diesel (250 mL) was added into container containing 1000 g of soil for sample B50. The contaminated soil samples were inoculated with approximately 200 mL of 1×10^6 cfu/mL of P. putida broth culture and placed in dark condition. As a control sample another soil was prepared and then inoculated with P. putida without addition of both biodiesel and diesel. The day when the soil was mixed with biodiesel, diesel and their blends as well as P. putida was considered as Day 0. The growth of P. putida was enumerated at interval of three days for up to Day 24 of bioremediation period (Day 0, 3, 6, 9, 12, 15, 18, 21 and 24). All measurements were made in three replications.

e) Enumeration of Pseudomonas Putida

Approximately 25 g of inoculated soil samples were dissolved in 500 mL Erlenmeyer flask containing 225 mL of sterilized distilled water. The flask was shaken on orbital digital shaker with shaking speed of 200 rpm for approximately 30 minutes. After leave for sedimentation time approximately for 2 hours, then 1 mL of supernatant from the flask was placed into seven sterile dilution bottles each containing 9 mL of sterile distilled water in triplicates. Hereafter, 1 mL of the previous dilution was added into 9 mL of sterile water in other dilution bottles. Next, each sample volume from dilution bottles was passed through a membrane filter under partial vacuum. The filter papers were placed onto warm CHROM Agar Pseudomonas media agar. The CHROM Agar Pseudomonas media agar was prepared according to manufacturer's instruction. Inoculated media agar has been incubated at temperature of 37 ºC in incubator. After 24 hours of incubation the colonies of P. putida were counted.

3. RESULTS AND DISCUSSIONS

Biodiesel/diesel blends spillage into soil inhibited growth of P. putida as shown in Figure-1. The survival of P. putida in sample B50 was lower than control. Total 24 days of treatment characterized by fluctuation, e.g. repeated increase and decrease survival trend in sample B50. This is possibly due to sample B50 intermingle to provide nutrients such as total organic carbon, total nitrogen, phosphorus and sulfur to P. putida. The fuels affect cell metabolism of various group of microorganisms

ARPN Journal of Engineering and Applied Sciences

including P. putida. The survival of P. putida in B50 was lower than control. Changes in soil properties due to contamination with petroleum-derived substances can lead to water and oxygen deficits as well as to shortage of available forms of nitrogen and phosphorus [19].

The finding is consistent with findings of past studies by Wyszokowski [20] which reported that the addition of diesel oil to the soil led to a significant reduction of organic carbon content of the soil.

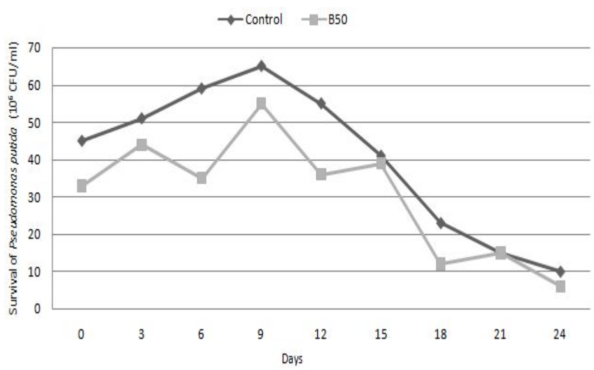

Figure-1. The survival of *P. putida* (10^6 CFU/mL) at pH 6.8-7.8; temperature is 27°C in sample of B50 and control.

Since the petroleum-products are known to reduce nutrients availability [21], therefore the reduction of survival of P. putida by petroleum products in sample B50 was observed; compared to control. From this study, it was found that an amount of introduced biodiesel/diesel blends and time significantly affected the survival of P. putida in the both examined soils as summarized in Table-1.

Table-1. The summary of *P. putida* colonies measured in contaminated soil with B50 biodiesel/diesel blend.

Sample	P. putida growth (CFU/ml) at different bioremediation period (Day) (n=3)								
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24
B50	3.3 $\times 10^7$	4.4 $\times 10^7$	3.5 $\times 10^7$	5.5 $\times 10^7$	3.6 $\times 10^7$	3.9 $\times 10^7$	1.2 $\times 10^7$	1.5 $\times 10^7$	6.0 $\times 10^6$
Control	4.5 $\times 10^7$	5.1 $\times 10^7$	5.9 $\times 10^7$	6.5 $\times 10^7$	5.5 $\times 10^7$	4.1 $\times 10^7$	2.3 $\times 10^7$	1.5 $\times 10^7$	1.0 $\times 10^7$

The P. putida colonies was slightly increased from Day 0 (4.5x 10^7 CFU/ml) to Day 9 (6.5x 10^7 CFU/ml) for control sample. Nutrients from native soil act as carbon source may lead to the enhancement of P. putida growth. However, starts on Day 9, the amount of colonies P. putida starts to deplete until Day 24. The depletion colony P. putida might be due to the availability of nutrient in the sample that become low over time of treatment period once the bacteria has used them as source of carbon and energy [21]. The same occurred to the survival of P. putida in sample B50 was increased at particular day due to additional carbon source offered from biodiesel/diesel blends which acts as their source of carbon may lead to increase of P. putida growth [4]. However, after Day 9, the amount of colony P. putida starts to depletion until Day 24. The depletion colony P. putida might be due to the availability of nutrient in the sample that become low over time of treatment period once the bacteria has used them as source of carbon and energy [4]. This pattern of P. putida growth was also proven by Schleicher [22] who verified that microbial will be rose up from the beginning but will decreased throughout bioremediation period; however it also depends on fuel composition too. It can be concluded that the increase in number of days in bioremediation period reduced the bacterial viability. Nevertheless, the amount of P. putida is still high which is at 10^6 CFU/ml even until up to Day 24. This indicated that the P. putida survival is high. The P. putida grows well in simple nutrient demand. Meanwhile for control sample, the growth of bacteria was elevated higher in control sample than B50 until Day 24.

Diesel oil hydrocarbons may favor survival of microorganisms including P. putida in which they are act as a valuable source of carbon and energy or induce inhibition of P. putida colonies and activity [23], depending on amount and types of contamination [24-28]. Even though the survival of P. putida in sample B50 was increased in the 9 days of treatment period however, their growth was still lower compared to control. This means that the fuels inhibited the growth of P. putida in the soil.

4. CONCLUSIONS

The most obvious finding to emerge from this study is biodiesel/diesel blends (B50) influenced the survival of P. putida as bioremediation agent. Notably, this research has thrown up many questions in need of further investigation. Further work needs to be done to establish whether at different ratio blends of biodiesel/diesel contamination possibly affect the survival of P. putida as bioremediation agent.

ACKNOWLEDGEMENT

We are grateful to Research Acculturation Grant Scheme (Vote R002) and Multidisciplinary Research Grant (MDR) from Research & Innovation Centre UTHM for financial supports of this work.

REFERENCES

[1] Ogbo, E.M. 2009. Effect of diesel fuel contamination on seed germination of four crop plants-Arachis hypogea, Vigna unguiculata, Sorghum bicolor and Zea mays., Afr. J. Biotechnol. 8(2), 250-253.

[2] Leung, D.Y.C., Koo, B.C.P., Guo, Y. 2006. Degradation of biodiesel under different conditions, Biores. Technol. 97, 250.

ARPN Journal of Engineering and Applied Sciences

[3] Knothe, G. 2005. The biodiesel handbook, AOCS Press, Champaign, Illinois.

[4] N. M. Sunar, Q. Emparan, A. T. A. Karim, S. F. M. Noor, M. Maslan, F. Mustafa, N. Khaled, The effectiveness of Pseudomonas putida 49128 as biodegradable agent in biodiesel soil contamination, in Proc. INCIEC, 2014, Springer Singapore, (Part VIII), pp. 817- 823.

[5] Grant, R. J., Muckian, L.M., Clipson, N. J. W. and Doyle, E. M. 2007. Microbial community changes during the bioremediation of creosote-contaminated soil, Letters in Applied Microbiology, 44, 293-300.

[6] Schafer, A. N., Snape, I., and Siciliano, S.D. 2009. Influence of Liquid Water and Soil Temperature on Petroleum Hydrocarbon Toxicity in Antarctic Soil, Environmental Toxicology and Chemistry, 28, 1409-1415.

[7] Van Gestel, C. A. M., Van der Waarde, J. J., Derksen, J.M., Van der Hoek, E., Veul, M. F. X. W., Bouwens, S., Rusch, B., Kronenburg, R., and Stokman, G. N. M. 2001. The use of acute and chronic bioassays to determine the ecological risk and bioremediation efficiency of oil-polluted soils, Environmental Toxicology and Chemistry, 20,1438-1449.

[8] Nessel, C. S. 1999. A comprehensive evaluation of the carcinogenic potential of middle distillate fuels, Drug and Chemical Toxicology, 22, 165180.

[9] Muzyka, V., Bogovski, S., Viitak, A. and Veidebaum, T. 2002. Alterations of heme metabolism in lymphocytes and metal content in blood plasma as markers of diesel fuels effects on human organism, Science of the Total Environment, 286, 73-81.

[10] Mariano, A.P., Tomasella, R.C., Oliveira, D.L.M., and Angelis, J.C. 2008. Biodegradability of diesel and biodiesel blends, African Journal of Biotechnology, 7, 1323-1328.

[11] Demirbas, A. 2008. New liquid biofuels from vegetable oils via catalytic pyrolysis, Energy Educ SciTechnol., 21, 1-59.

[12] Wang, S.Y., and Vipulanandan, C. 2001. Biodegradation of naphthalene contaminated soils in slurry bioreactor, J. Environ. Eng., 127, 748-754.

[13] N. M., Sunar, Emparan, Q, Karim, A. T. A. , Noor, S. F. M, Maslan, M., Mustafa, F., and Khaled, N. 2014. The effectiveness of bioremediation treatment for diesel-soil contamination, Advanced Materials Research, 845, 817-823.

[14] Kucerova, R. 2006. Application of Pseudomonas putida and Rhodococcus spp. by biodegradation of PAHs, PCBs and NEL soil samples from the hazardous waste dump in Pozdatky, Rud. Geol. Naf. Zb., 18, 97-101.

[15] Haritash, A.K., and Kaushik, C.P. 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons PAHs): a review, J. Hazard. Matter, 169, 1-15.

[16] Timmis, K. 2002. Pseudomonas putida: a cosmopolitan opportunist par excellence, Environ. Microbiol, 4,779-781.

[17] Ignacio, P. C., Judith, B., Katrin, D., Vitor, M.D.S., and Christoph, W. 2012. Industrial biotechnology of Pseudomonas putida and related species, Appl. Micro. Biotechnol, 93, 2279-2290.

[18] Taylor, L.T., and Jones, D.M. 2001. Bioremediation of coal tar PAH in soil using biodiesel, Chemosphere, 44, 1131–1136.

[19] Wyszkowska, J., and Kucharski, J. 2000. Biochemical Properties of soil contaminated by petrol., Polish J.Environ. Studies, 9(6), 476-485.

[20] Wyszkowski, M., and Ziolkowska, A. 2008. Effect of Petrol and Diesel oil on content of organic carbon and mineral components in soil, American Eurasian J. Sust. Agric., 2(1), 54-60.

[21] Agbogidi, O.M., Eruotor, P.G., Akparobi, S.O., and Nnaji, G.U., 2007, Evaluation of crude oil contaminated soil on the mineral nutrient elements of maize (*Zea maize* L.), J. Agron., 6(1), 188-193.

[22] Schleicher, T., Werkmeister, R., Russ, W., and Piroff, R. M. 2009. Microbiological stability of biodiesel-diesel mixture, Bioresour. Technol., 100, 724-730.

[23] Mrozik, A., Piotrowska-Seget, B., and Labuzek, S. 2003. Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons, Polish J. Environ. Stud., 12, 15-25.

[24] N. M. Sunar, Q. A. Emparan, A. T. A. Karim, S. F. M. Noor, M. Maslan, F.Mustafa. 2014. Characteristics of polycyclic aromatic hydrocarbons emissions of palm biodiesel blend, Materials Research Innovations. Volume 18, Issue S6, pp. 6-43.

[25] N. M. Sunar, Q. Emparan, A. T. A. Karim, M. Maslan, F. Mustafa, N. Khaled. 2015. Bioremediation of biodiesel/diesel blend (B50) in soil contamination by using Pseudomonas Putida, Applied Mechanics and Materials Vol. 699, pp. 1012-1019.

[26] N. M. Sunar, Q. A. Emparan, A. T. A. Karim, S. F. M. Noor, M. Maslan, F.Mustafa. 2014. Survival of

Pseudomonas putida for biodiesel blend (B5) in soil bioremediation, Applied Mechanics and Materials Vol. 567, pp. 139-143.

[27] Q. Emparan, N. M. Sunar, P. Gani, E. C. Ming, U.K. Parjo. 2015. Survival of Pseudomonas putida for biodiesel blend (B20) in soil bioremediation. Applied Mechanics and Materials. Vols 773-774, pp. 1324-1329.

[28] N. M. Sunar, Q. Emparan, A. T. A. Karim, S. F. M. Noor, M. Maslan, F. Mustafa, N. Khaled. 2014. Bioremediation of bio-fuel-soil contamination by using Pseudomonas putida, Advanced Materials Research Vol. 845, pp. 138-145.