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DEGRADATION OF DIESEL OIL BY SOIL BACTERIA IN SHAKE FLASK SYSTEM USING FOOD WASTE AMENDMENTS

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ABSTRACT

Diesel oil contains compounds that can cause harm to humans and the environment. Hence, biodegradation method is an alternative way to reduce the pollution caused by diesel oil. The aim of this study is to determine the diesel oil degradation by soil bacteria amended with food wastes in the flasks system. It also aims to determine the food wastes such as sugarcane bagasse and fishbone to enhance the biodegradation of diesel oil. The degradation analysis was performed in an enrichment culture flask containing soil, diesel oil with the addition of food waste. The degradation analysis was carried out for 42 days at 30°C at 150 rpm. The bacteria was isolated and identified based on colony morphology and biochemical tests. Five potential diesel oil-degrading bacteria were preliminary identified as *Pseudomonas fluorescens, Pseudomonas aeruginosa, Klebsiella* species*, Shewanella putrefaciens* and *Bacillus cereus*. Diesel oil degradation compound was analyzed using Gas Chromatography - Mass Spectrometry. Four compounds namely styrene, ethanol, 2-butoxy, benzene, 1-ethyl-2, 3-dimethyl and benzene 1-ethyl-2, 3-dimethyl showed degradation by bacteria amended with food wastes. The results of this study demonstrate the potential use of food wastes such as sugarcane bagasse and fish bone as substrates for enhancing the remediation of hydrocarbon contaminated soil.

Key words: Degradation, diesel oil, soil bacteria, shake flask

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAH) pollution is one of the most serious problems affecting the world nowadays. The widespread of the PAHs in the environment may cause negative effect on human health such as hamper body defense system, stimulate cancer growth and can cause death (Mumtaz & George, 1996). Analysis done by Agency for Toxic Substances and Disease Registry shows that 17 groups of PAHs have bad consequences on human health (Buha, 2011). Therefore, to reduce the amount of PAHs in the environment, a bioremediation method was developed. Bioremediation is considered as promising environmental friendly treatment to remediate hydrocarbon compounds. It has the capability to reduce the toxic substance to non-toxic substance as the end product. Besides that, the technique is safe and give the complete mineralization of pollutant without affecting the natural ecosystem.

PAHs pollution in the environment can be reduced using PAHs-degrading bacteria. However, bioremediation has some drawbacks such as the low abundance, diversity and activity of indigenous hydrocarbon degrading bacteria and also their slow growth rates. Therefore, food waste composting was developed as a cost-effective and highly efficient tool in biodegradation of hydrocarbon pollution site. This is one of the methods by adding valuable nutrient contain in the food waste such as nitrogen, phosphorus, and carbon to their environment and to enhance the rate of PAHs degradation. Besides increasing PAH decomposition rate, the food waste accumulation in the environment can be reduced. The objective of this study is to determine the diesel oil degradation by soil bacteria with amended with food wastes in the flasks system. It is also to determine the potential food wastes such as

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sugarcane bagasse and fishbone for enhanced biodegrading of diesel oil.

MATERIALS AND METHODS

Sample Collection

The soil sample was collected from diesel oil contaminated soil at motorcycle service in Kuala Terengganu, Malaysia. The soil sample was collected from 10 cm subsurface of the soil using a sterile spatula. The temperature and pH of the soil was measured. Diesel oil was collected at a petrol station area in Kuala Terengganu. Food wastes such as sugarcane bagasse and fishbone were collected from canteens at Universiti Malaysia Terengganu. The food wastes were dried at 90°C and blended to make a powder.

Enrichment of diesel oil-degrading bacteria

The enrichment culture (EC) composed of 22.5 g of soil sample and 15 ml of diesel oil. Then, flasks C and C1 were filled with 112.5 ml of mineral salt medium (MSM) whereas the other flasks were filled with 105 ml of MSM. MSM consisted of the following composition: 0.2 gL^{-1} of MgSO, 0.02 gL^{-1} of CaCl, 1.0 gL^{-1} of KHPO, 1.0 gL^{-1} of KHPO, 1.0 gL^{-1} of NHNO and 0.05 gL^{-1} FeCl. The flasks were then left undisturbed for 2 days. After 2 days of incubation, 7.5 gL^{-1} of sugarcane bagasse powder was added into flasks A and A1 while the same amount of fishbone powder was added into flasks B and B1. Flasks C and C1 served as control. The enrichment culture was shaken at 150 rpm (Abioye *et al*., 2010).

Diesel oil extraction from medium

The diesel oil was extracted at every 7 days intervals for 42 days. The enrichment culture (EC) was suspended with n-hexane (1:1 ratio). The suspension was homogenized for 1 hour and then centrifuged at 150 rpm for 5 minutes. The upper layer of supernatant obtained was resuspended with 1:1 ratio of supernatant to n-hexane. The funnel was shaken gently by inverting. The upper phase was collected and evaporated using a rotary evaporator. The diesel oil extracted was stored at 4°C for further analysis.

Enumeration of diesel oil-degrading bacteria

EC was collected every 7 days for the enumeration of diesel oil- degrading bacteria. The EC was serially diluted and 0.1 ml of diluted sample was transferred to nutrient agar (NA) plates using the spread plate method. The CFU/ml of sample was calculated using the formula below:

$$\text{Total number of bacteria} = \frac{\text{number of colonies}}{\text{(dilution factor) x (actual volume plated)}}$$

Screening for diesel oil biodegradation

The extracted diesel oil was diluted to 1 ppm. The diluted samples were filtered using 0.45 µm PTFE membrane filter before injecting into the GC-MS (QP2010 ultra). The analysis conditions for the GC-MS are shown in Table 1.

The degradation percentage of the diesel oil compound was calculated using the modified formula (Deng *et al*., 2014):

Table 1. Analysis condition of GC-MS

GC-MS	GCMS-QP2010 ultra
[GC]	
Column	Rtx–5MS 0.25 x 30 m df=0.25 µm
Inlet mode	Splitless
Vaporizing chamber temperature	
Column oven temperature	50°C (1 min) →300°C (5 min)
Carrier gas	Helium
Control mode	Constant linear velocity 35.5 cm/sec
High-pressure injection	150kPa (1.00min)
Purge flow rate	3.0 mL/min
Injection rate	1 µl
[MS]	
Interface temperature	300°C
Ion source temperature	200°C
Data sampling time	1.00 min
Measurement mode	Scan
Mass range	m/z 50-600
Event time	0.05 sec

$$DR = \frac{W1 - W5}{W1} x\ 100\%$$

Where:
DR = Diesel oil degradation rate
W1 = Diesel-oil Concentration in Week 1
W5 = Diesel-oil Concentration in Week 5

Identification of diesel oil-degrading bacteria

The diluted samples were transferred to oil agar (OA) and incubated for 24 hours at 30°C. The bacterial colonies with the clear clearing zones on OA were isolated and sub cultured on nutrient agar. Further sub-culturing of bacteria was conducted until pure isolates was were obtained. The isolated bacteria were identified using Gram staining technique and the cell morphology of bacterium was recorded. The bacterial species was further identified using BBL Crystal Identification Kit (Fisher Scientific, USA) as per the manufacturer's instructions. A single pure bacteria colony was suspended in an inoculum fluid tube and then filled into the target area of the base. The inoculum was rolled gently along the tracks and the panel, closed with a lid and incubated at 37°C. The isolated bacterium was identified using BBL Crystal MIND.

RESULTS

Effect of the addition of food waste into the flask system on the growth of bacteria

The bacterial growth curves for all the flasks show the same pattern, which steadily increase and then gradually decrease, after reaching their respective peaks (Figure 1). The flask containing sugarcane bagasse (SB) show the highest growth rate at week 3 compared to the flask system containing fishbone (FB). All the flask systems show approximately the same viable cell count at week 1 and week 2. The bacterial count of the flask system containing SB slightly increases from 2.89 x 10^9 CFU/ml at week 1 to 1.58 x 10^{11} CFU/ml at week 3 and the bacterial count of the flask system without any food waste increases from 2.70 x 10^9 CFU/ml at week 1 to 4.95 x 10^{10} CFU/ml at week 3. On the other hand, the bacterial count of the flask system containing fishbone slightly increases from 2.81 x 10^9 CFU/ml at week 1 to 9.55 x 10^{11} CFU/ml at week 3 (Figure 1).

Effect of addition of food waste on pH value in flasks system

The pH of the shake flask containing fish bone and sugarcane bagasse were dropped at week 5 to pH 5.75 and 5.96 respectively, whereas pH for control did not show significant changes during the incubation period (Figure 2).

Identification of diesel oil- degrading bacteria

Physiological characteristics in Table 2 shows that there were four Gram-negative bacteria and one Gram-positive bacterium that had been identified. Isolate 2 and Isolate 3 showed negative result for oxidase test while all isolates showed negative result for indole test. All of the isolated bacteria are white in colour with a circular form. These isolated bacteria had an entire margin and bacillus shape. The elevation of Isolate 2, Isolate 3, Isolate 4, and Isolate 5 are raised, whereas Isolate 1 is convex.

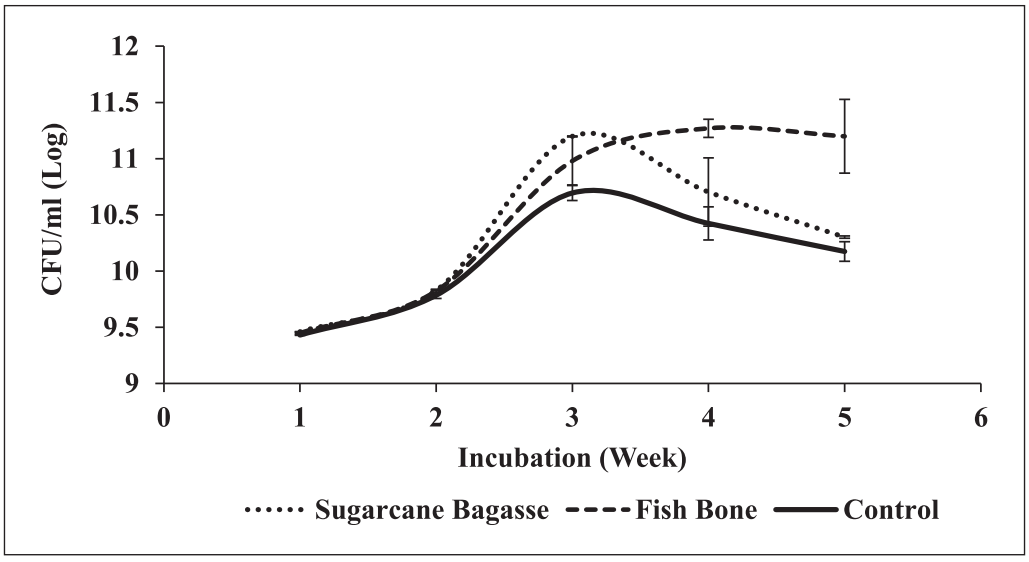

Fig. 1. Bacteria count (CFU/ml) in the flask system containing sugarcane bagasse, fishbone and control after 5 weeks of incubation.

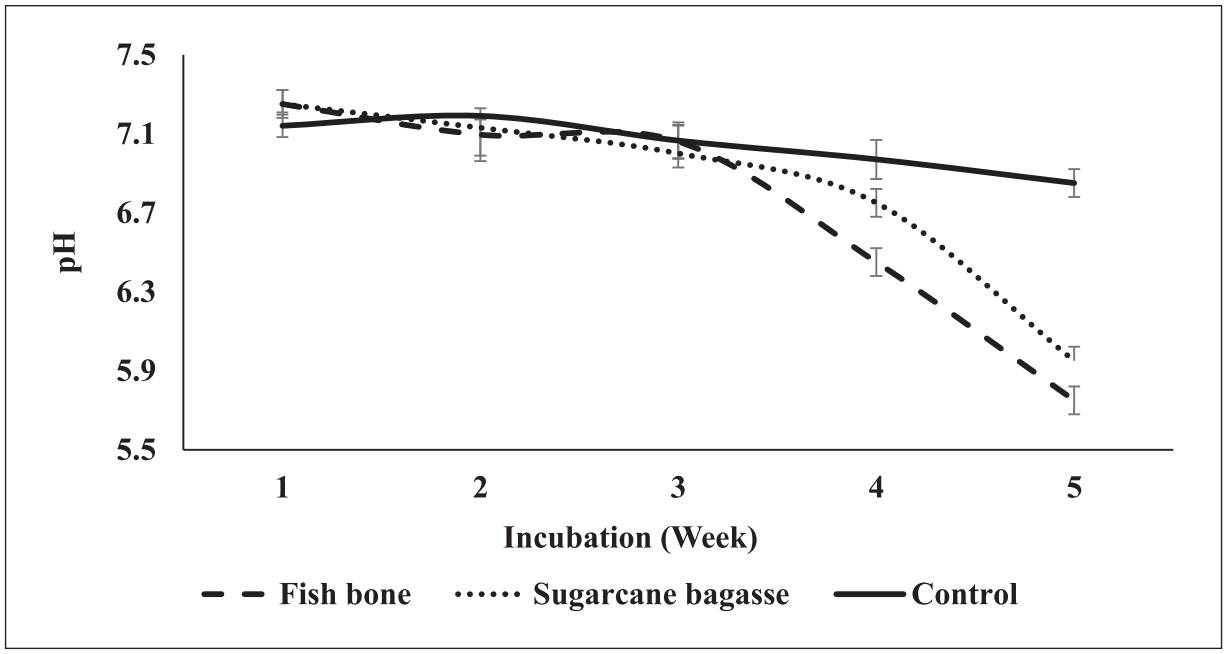

Fig. 2. pH value in the flask system containing sugarcane bagasse, fishbone and control after 5 weeks of incubation.

Table 2. Colony morphology and biochemical activities of isolated diesel oil -degrading bacteria

Characteristics	Gram-stain	Oxidase test	Indole test	Colony Morphology				
				Color	Form	Margin	Elevation	Shape
Isolate 1	–	+	–	White	Circular	Entire	Convex	Bacillus
Isolate 2	–	–	–	White	Circular	Entire	Raised	Bacillus
Isolate 3	–	–	–	White	Circular	Entire	Raised	Bacillus
Isolate 4	–	+	–	White	Circular	Entire	Raised	Bacillus
Isolate 5	+	ND	ND	White	Circular	Entire	Raised	Bacillus

– = negative result; + = positive result; ND – Not Detected.

Table 3 shows the isolated bacteria identified using the BBL Crystal Identification Kit. It shows that, five potential diesel oil-degrading bacteria known as *Pseudomonas fluorescens, Klebsiella* species, *Shewanella putrefaciens, Pseudomonas aeruginosa*, and *Bacillus cereus* has been preliminarily identified.

Effect of the addition of food waste on the degradation rate of the diesel oil compounds

In this study, it was demonstrated that the flask containing sugarcane bagasse shows the highest degradation rate for compounds styrene and ethanol, 2-butoxy but has the lowest degradation rate for benzene, 1-ethyl-2, 3-dimethyl. However, the flask containing fishbone shows the highest degradation rate for compound benzene, 1-ethyl-2, 3-dimethyl and the lowest degradation rate for compound styrene.

Table 3. Diesel oil -degrading bacteria preliminary identified by BBL Crystal Identification Kit

Isolated bacteria	Bacteria Identification
1	*Pseudomonas fluorescens*
2	*Klebsiella* sp.
3	*Shewanella putrefaciens*
4	*Pseudomonas aeruginosa*
5	*Bacillus cereus*

DISCUSSION

According to Figure 1, the counts of diesel oil-degrading bacteria shows a normal bacterial growth curve, which consists of four phases. It begins with the lag phase, followed by the log phase, stationary phase and it ends with a decline phase. During week

1 and week 2 of incubation, the bacteria are trying to adapt to the new environment. Then, the bacterial growth was increased exponentially in week 3 as it had been adapted to the environment. At this stage, bacterial cells are most metabolically active and are able to utilize the diesel oil in the medium. However, the bacterial growth was decreased at week 4 and week 5 due to the limited nutrient supply, which is a decrement in diesel oil concentration and accumulation of toxic wastes produced by the bacteria in the medium. Unlike the bacteria in the flask system containing sugarcane bagasse and fishbone, bacteria in the flask system without food waste grew slower because there was no additional nutrient compared to flask system containing food waste. This finding was supported by Agarry (2018), who stated that amendments of organic and inorganic nutrients might enhance microbial growth, which can promote the efficiency of hydrocarbon degradation.

The amendments of food waste in the enrichment medium promoted the growth of the bacteria population. This is because the sugarcane bagasse and fishbone provide valuable nutrients for the bacteria to grow (Ibrahim *et al.,* 2016). In terms of nutrient composition, sugarcane bagasse is made up of 40% cellulose, 35% hemicellulose and 15% lignin, which contain a carbon source for bacteria to get energy (Alves Da Costa *et al.,* 2015). Fishbone also has a high content of natural calcium and phosphorus (Malde *et al*., 2010). Therefore, with the amendments of the food waste in the enrichment medium, it may enhance the biological activity of bacteria, which is a necessary nutrient for biodegradation activities by the bacteria. Normally, the efficiency for bacteria in undergoing biodegradation is affected by the conditions that are favorable for the bacteria. In this study, organic matter content from food waste supplies enough nutrients for the bacteria to grow. Therefore, it may contribute to the efficiency of diesel oil degradation as well as some minerals needed for enhancing the biodegradation activities (Das & Chandran, 2011).

Based on Figure 2, the graph shows a slight decrease in pH after week 3 because the bacteria had reached their decline phase. This stage is attained due to the depletion of nutrients and accumulation of metabolic waste products by the bacteria in the media. This condition facilitates the bacteria to move on to the death phase. Besides, bacteria will utilize the compounds in the diesel fuel as a carbon source for growth. Thus, it will produce metabolite byproducts after utilizing the compound. This metabolite byproduct, metabolic wastes, dead bacteria and toxic materials will cause the environment to be acidic.

In this study, the isolated diesel oil -degrading bacteria were preliminary identified are *Pseudomonas fluorescens*, *Klebsiella* species, *Shewanella putrefaciens, Pseudomonas aeruginosa* and *Bacillus cereus*. These identified bacteria have utilized the compounds in diesel oil as sole source of carbon and energy source for growth. Potential oil degraders such as *Pseudomonas fluorescens* have shown the properties of high biodegradability of an aliphatic hydrocarbon and low as well as slow biodegradation of aromatic hydrocarbon (Sepic *et al.*, 1997). Apart from that, *Pseudomonas aeruginosa* is well-known for its degradation ability. Various studies showed that *P. aeruginosa* is able to degrade crude oil and oil sludge approximately by 70% (Zhang *et al*., 2005; Das & Chandran, 2011; Al-Wasify & Hamed, 2014). *Klebsiella* species shows ability to degrade toluene, xylene, naphthalene, and nonane (Rodrigues *et al*., 2009). It is believed that *Klebsiella* species isolated in this study also have the capability to degrade diesel oil. However, *Shewanella putrefaciens* have been reported as crude oil degrader in coastal seawater and sea ice (Das & Das, 2015). *Bacillus cereus* is a Gram-positive bacterium that is widely distributed environmentally. *Bacillus* species have been reported to be capable of utilizing phenanthrene, anthracene, and pyrene as carbon sources (Dou *et al.,* 2010; Fazilah *et al*., 2016; Rao *et al*., 2015).

The percentage of diesel oil biodegradation in the three flask systems is shown in Figure 3. The degradation rate of styrene, ethanol-2-butoxy, and benzene, 1-ethyl-2, 3-dimethyl for flask system without food wastes is 5%, 10.7% and 9.5% respectively. For the flask system containing sugarcane bagasse, the degradation rate of styrene, ethanol-2-butoxy, and benzene, 1-ethyl-2, 3-dimethyl is 12.8%, 18.4%, and 0.1% respectively. While, for the degradation rates of flask system containing fishbone for styrene, ethanol-2-butoxy, and benzene, 1-ethyl-2, 3-dimethyl is 4.2%, 14.1%, and 13.9% respectively. The flask system containing the food waste has increased the degradation rate of styrene, ethanol-2-butoxy, and benzene, 1-ethyl-2, 3-dimethyl. However, not all the compounds show the same variations. For styrene, the flask system containing sugarcane bagasse enhances the degradation of styrene by 8.6%, but the flask system containing the fishbone has decreased the degradation rate of styrene by 0.8%. This is due to the concentration of nutrients such as calcium and phosphorus provided by the fishbone is too high for *Pseudomonas fluorescens,* which eventually inhibits the degradation of the styrene (Beltrametti *et al*., 1997; Das & Chandran, 2011; Lang *et al.,* 2016). The degradation rate of ethanol, 2-butoxy for the flask system containing food waste is higher than the flask system without the food waste. This shows that sugarcane bagasse and fishbone have enhanced the degradation rate of

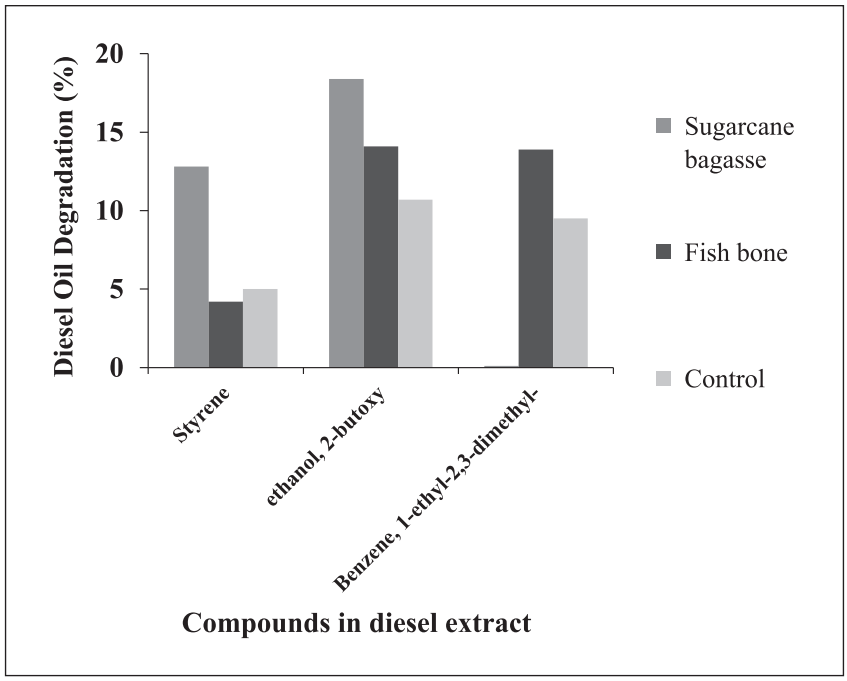

Fig. 3. Degradation percentage (%) of the compounds in diesel oil (styrene, ethanol, 2-butoxy, and benzene, 1-ethyl-2, 3-dimethyl-) in the flask containing sugarcane bagasse and fishbone and flask without food waste (control).

ethanol, 2-butoxy by 7.7% and 3.4% respectively. Then, the degradation rate of benzene, 1-ethyl-2, 3-dimethyl in the flask system containing sugarcane bagasse is inhibited. However, the flask system containing fishbone shows enhancement of 4.4% for the degradation rate of benzene, 1-ethyl-2, 3-dimethyl. This is because the sugarcane bagasse powder is easily absorbed by the bacteria responsible for degrading benzene, 1-ethyl-2, 3-dimethyl. Hence, it becomes the competitor for the bacteria to utilize it as a carbon source for growth.

CONCLUSION

The results of this study shows the addition of food waste to the flask system enhanced the growth rate of bacteria and increased the degradation rate of the compounds in diesel oil. Four compounds such as styrene, ethanol, 2-butoxy, benzene, 1-ethyl-2, 3-dimethyl and benzene 1-ethyl-2, 3-dimethyl showed degradation by bacteria with amended with food wastes. Five potential diesel oil-degrading bacteria have been identified as *Pseudomonas fluorescens, Pseudomonas aeruginosa, Shewanella putrefacians, Klebsiella* species and *Bacillus cereus.* These preliminary results highlighted the potential application of sugarcane bagasse and fish bone for enhancing the biodegradation of diesel oil contaminated soil.

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