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Bioremediation of Biofuel-soil Contamination by Using *Pseudomonas Putida*

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Abstract. The commercialization of biodiesel/diesel blends on the market can cause environmental damages due to spills. This study aims to investigate the effectiveness of *Pseudomonas putida* as oil-biodegradable agent in soil contaminated with biodiesel/diesel blend (B20). The effectiveness on bioremediation have been conducted by examined several physico-chemical tests on biodiesel/diesel-contaminated soil before and after seeding of *P. putida*. The spillage stimulation of B20 was conducted at laboratory scale for 24 days of incubation time. The results show that the bioremediation treatment able to remove up to 82%, 77%, 16%, and 10% of nitrogen, phosphate, sulfate and total organic carbon, respectively. The pH of soil sample was changed from pH 7.45 (Day 0) to 7.25 (Day 24) after the treatment. Meanwhile, the moisture content in the sample has increased from 44.11% (Day 0) to 50.35% (Day 24). All of these results show the good indication of quality improvement of polluted soil after treated with *P. putida*. It is apparent from the acquired results that the application of *P. putida* is suitable as effective microorganism and potentially exploits as useful oil-soil biodegradable agent in polluted soil.

Introduction

Biodiesel is made of a monoalkyl esters of long chain fatty acids (FAME), derived from a renewable lipid feedstock, such as vegetable oil like palm oil, soybean, sunflower, peanut, cotton, coconut, babassu and castor oil) and from animal fat [1,2]. In fact, it can be legally blended and used in many different concentrations such as B2 (2% biodiesel + 98% diesel), B5 (5% biodiesel + 95% diesel), B20 (20% biodiesel + 80% diesel) and B100 (pure biodiesel). However, the most common biodiesel blend is B20, which qualifies for fleet compliance under the Energy Policy Act (EPAct) of 1992 [3]. The use of commercial pure biodiesel fuel and blends with diesel fuel in conventional diesel engines significantly reduces the particulate matter (PM), carbon dioxide (CO_2), carbon monoxide (CO), sulfates, polyaromatic hydrocarbons (PAHs), nitrated polyaromatic hydrocarbons, ozone-forming hydrocarbons and unburned hydrocarbons (HCs) compared to the petrodiesel base fuel [4]. According to EPA [5], a soybean-based biodiesel blends with diesel fuel (B20) has reduced PM emissions by 10%, CO by 11%, HCs by 21% and CO by 15%. In addition to that, biodiesel is renowned because of its environmentally friendliness, portability, ready availability, renewability, higher combustion efficiency, lower sulfur and aromatic carbon, higher cetane number and higher biodegradability [6, 7]. Due to the substantially benefits, the biodiesel fuels have attracted increasing attention worldwide as blending components or direct replacements

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for diesel fuel in conventional diesel engines. In Malaysia, actual engine trials was managed by Malaysian Palm Oil Board (MPOB) on B2, B5 and B10 (10% biodiesel + 90% diesel) of processed liquid palm oil (PLPO)/ petroleum diesel blends). These efforts have been ongoing since 2002 [8]. So far, no technical problems have been reported and the long term effects on the engines are being studied.

As occurs to the diesel fuel, the commercialization of biodiesel or the biodiesel/diesel blend on the market of many countries can cause environmental damages due to spills. The danger associated with ecological threat caused by the infiltration of biodiesel or biodiesel/diesel blends into environment media like soil and ground water during its extraction, transportation, processing and distribution of the fuels [9]. The long term effects of genotoxic compound in contaminated soil can adversely defects the health of many living organisms including human beings, by exposing them through diverse pathways, such as ingestion of plants that uptake soil pollutants and also leaching of compound from contaminated soil to ground and surface water used as drinking water [10]. The contaminated areas can be cleaned-up by the emerging science and technology of bioremediation. According to EPA [11], bioremediation is defined as a managed of spontaneous process in which microbiological process are used to degrade, break down or transform hazardous contaminants to less toxic or nontoxic forms, thereby remedying or removing and eliminating contaminants from environment media. Microorganisms used chemical contaminants as an energy source using their metabolic process throughout the microbiological process. Factors such as nutrient availability, nitrogen, phosphorus, moisture content, pH, temperature of soil matrix are necessary for microbial biodegradation activity and cell growth. However, the excessive amounts inorganic nutrients in soil cause microbial inhibition [12]. These microbiological process reduced hydrocarbon concentration in various types of soils and sediments to levels that no longer pose an unacceptable risk to the environment or human health [13].

Pseudomonas putida is a gram-negative bacteria, chemoorganotropic, aerobe abligae and aerobically respiratory metabolisms. They are straight or curved rods with dimensions range between 0.5 and 1.0 μm × 1.5- 4.0 μm. This bacteria moved by one or more polar-located flagella. These bacteria is arranged commonly individually or in small clusters or chains and grown under aerobic conditions in common substrates, in which they form irregularly large colonies producing water-soluble exopigment (pyocyanine and fluoresceine). The *P. putida* diffuse into the atmosphere and dyes it yellow or blue-green. The temperature range of their growth is 0- 42 °C and the optimum temperature is 35 °C. The enzymatic activity of *Pseudomonas* bacteria is dependent on ecological conditions out of where it was isolated [14].

The *P. putida* is tolerant to a wide range of xenobiotics such as PAHs [15] and play a vital role in the treatment of petroleum contaminated lands. It occurs in various environmental niches because of its metabolic versatility and low nutritional requirement [16]. Recently, the extensive biochemical analysis of *P. putida* has been carried out due to its high capability to degrade recalcitrant substances and inhibiting xenobiotics. This is due to it ability to diverse substrates and possesses some catabolic pathways and capable of acting on recalcitrant substances [17]. Besides that, it also indicates a very high robustness against extreme environmental conditions like high temperature, extreme pH, or the presence of toxins or inhibiting solvents [18].

Recently, more studies have described the capabilities of microorganisms especially *Pseudomonas spp.* to biodegrade petroleum hydrocarbons. *Pseudomonas* spp. is widely known as a model microorganism for studying hydrocarbons degradation. Malik [19] previously tested on the biodegradation of total aliphatic and aromatic with a sample collected from oil contaminated site in Pakistan by means of bacterial consortium (*Pseudomonas, Alcaligens, Psychrobacter, Bacillus, Micricoccus,* and *Staphylococcussp*). The acquired results indicated that total aliphatic and aromatic degradation were up to 94.84% and 93.75% respectively after 24 days of incubation. Meanwhile, Hamed [20] stated the biodegradation of soil that has been exposed to crude petroleum oil by means of bacterial consortium (*Pseudomonas stutzeri* AT3, *Bacillus thuringiensis* AT5, *Bacillus pumilus* AT11 *and Bacillus cereus* AT15. The removal of hydrocarbon is highest by *Pseudomonas stuzeri* AT3 (90%), followed by *Bacillus thuringiensis* AT5 (56.67%), *Bacillus cereus* AT15 (52.33%) and

Bacillus pumilus (50.60%). Contributing to the work of previous investigators, this research deals with *P. putida* alone as oil-biodegradable agent in soil contaminated with biodiesel/diesel blend. Additionally, the effectiveness of *P. putida* to remove selected physico-chemical parameters was investigated.

Materials and Methods

Biodiesel and diesel. The biodiesel from palm-based biodiesel produced by transesterification process with methanol were collected 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. The collected biodiesel and diesel was filled in clean dark bottles made of plastic with a volume of 1500mL for further experimentations.

Soil. The soil samples were taken in campus area. The soil samples were collected in the range of approximately 7-8 kg from the surface of approximately 5-10 cm deep layer of soil. The collected soil samples were then put into the clean dark plastic containers with a volume of 5000mL.

***P. putida* broth culture.** Pure *P. putida* ATCC 49128 were purchased from United States of America (USA) in dry culti-loop form. This *P. putida* samples were stored in chiller at 4°C prior to cultivation procedure. Approximately 16g of medium (nutrient broth) was filled into the 2000mL Erlenmeyer flask containing 2000 mL of distilled water. The mixtures were stirred gently and then dissolved by heating on hot plate at 100 °C for about 2 hours until complete dissolution. After that, the medium was sterilized in autoclave at 121 °C for about 15 minutes. It was then allowed to cool down for a few minutes until its temperature drop in between 35 °C to 37°C. One loop 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°C for 48 hours.

Soil-biodiesel/diesel and *P. putida* mixture. Spill simulations with biodiesel/diesel blends (B20) in soil were carried out in accordance with Taylor [21], with modification. The soil-biodiesel/diesel mixture samples were then inoculated with approximately 200mL of $1.0x10^6$ cfu/mL *P. putida* broth culture and placed in dark condition.

Physico-chemical test. The moisture content of the soil samples were determined by oven-drying method (BS 1377: Part 2: 1990: 3.2), which wet soil samples were weighted and dried in an oven at 105 °C for 24 hours. The dried samples were weighted again once more and final weight was recorded. The moisture content of soil samples was then calculated by percentage of dry soil mass formula. The pH of the soil samples were determined by electrometric method (BS 1377: Part 3: 1990), which gives a direct reading of the direct reading of the pH value of soil suspension in water. Both TOC (total organic count) and TN (total nitrogen) in soil samples were determined by TOC analyzer. The phosphate and sulfate for soil samples were determined by using ion chromatography (IC) analyzer. Preparation of soil samples for testing was conducted according to Jackson [22] prior to analysis.

Results and discussion

The Fig. 1 shows the moisture content of sample B20 for 4 weeks of treatment with *P. putida*. The moisture content of sample B20 was lowest detected on Day 0 which is 44.11% while the highest one on Day 9 at 69.49%. Overall, the moisture content of sample B20 was increased from Day 0 to Day 24 possibly due to the degradation of contaminants in biodiesel/diesel blends by *P. putida* that produced carbon dioxide (CO_2), water (H_20) and biomass as a by-product of bioremediation [23]. The moisture content of sample was within the range of soil standard guidelines (40-60%) except on Day 9 and Day 12. According to Walworth [12], moisture content is one of other factors that influenced the biodegradation activity by microorganisms and their cell growth. The optimum moisture content in soil samples will make the biodegradation of contaminants occurred effectively.

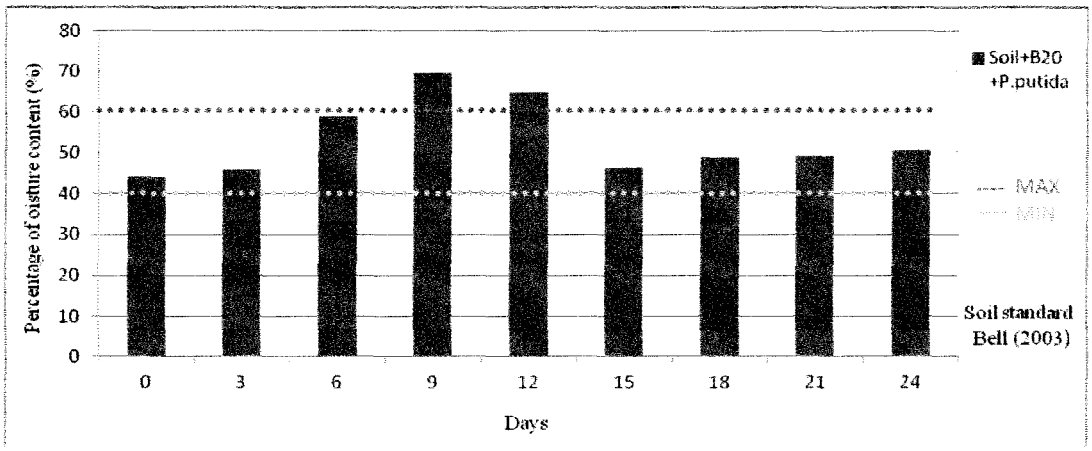

Fig. 1 Result of percentage moisture content in 24 days

The Fig. 2 shows the pH of sample B20 for 24 days of bioremediation treatment. The pH of soil sample was at pH 7.45 on Day 0. The pH of sample B20 was slightly fluctuated during the treatment. All pH of sample B20 were in the range of soil standard guidelines (pH 6-7.5) over time of treatment. According to Demirbas [24], commercial diesel fuel contains free fatty acid of approximately 45.62-46.48 mg KOH/g oil. The addition of pure diesel as well as *P. putida* into soil sample might be contributed to the decrement of pH for sample B20 during the treatment. Besides that, palm-based biodiesel is composed of methyl or ethyl esters of long chain fatty acids [25] and addition of acid catalyst in transesterification process to achieve better reaction and yield [26] also makes biodiesel in acidic conditions. Besides, pH of soil is one of other factors that influenced the microbial biodegradation activity and bacteria growth [12].

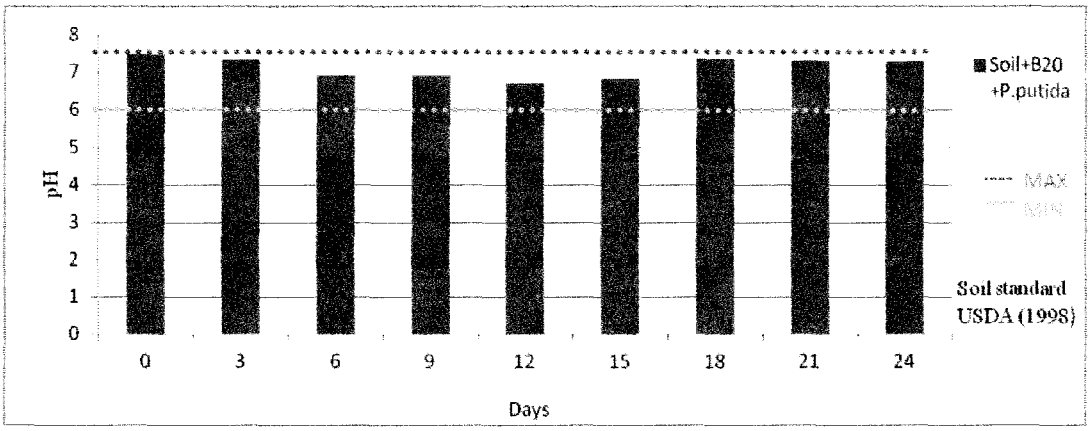

Fig. 2 Result of pH in 24 days

The Fig. 3 shows TOC results for 24 days of bioremediation. The reduction of TOC might be due to the *P. putida* has used carbon as an energy source using their metabolic process throughout the microbiological process in order to degrade, break down or transform hazardous contaminants [12] in samples to less toxic or nontoxic forms. It is thereby remedying or removing and eliminating pollutants from biodiesel/diesel contaminated soil. Overall, sample of B20 has TOC in the range of soil standard guidelines (0-0.05%) after treated with *P. putida.*

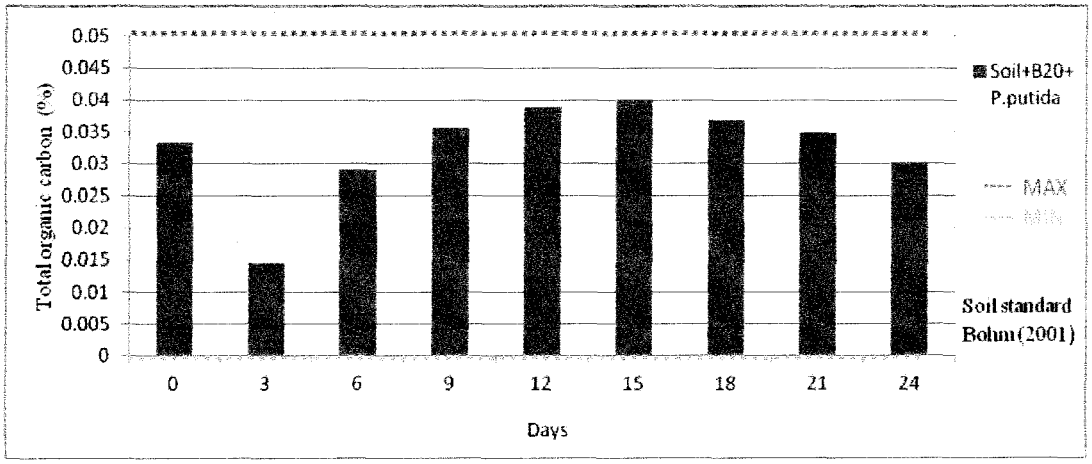

Fig. 3 Result of TOC in 24 days

The Fig. 4 shows TN results of sample B20 for bioremediation treatment. The TN shows decreasing pattern over time. The removal was observed up to 82% from Day 0 to Day 24. The nutrient like nitrogen is vital in soil bioremediation as it can enhance bacteria activities and their potential co-metabolism [14]. However, excessive amount of nutrients in soil cause microbial inhibition. Throughout the experiment, the TN was possibly used by *P. putida* to perform its microbiological process. This indicated by the reduction of nitrogen in the sample of B20. Overall, TN of sample B20 was in the range of soil standard guidelines (5-15 ppm) after 4 weeks of treatment with *P. putida.*

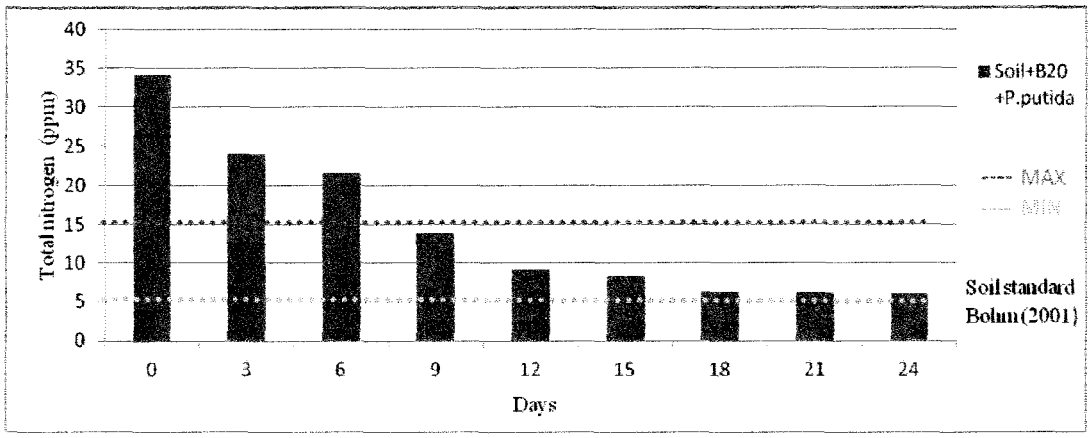

Fig. 4 Result of TN in 24 days

The Fig. 5 shows the results of sulfate amount during the bioremediation treatment. The results show the removal of sulfate up to 16% from Day 0 to Day 24. Most sulfate of sample was slightly over the range of soil standard guidelines (5-20 ppm). However, the amount of sulphate was in the safe limit start from Day 21 until Day 24. Sulfate is also vital in soil bioremediation process as it can enhance bacteria activities and their potential co-metabolism [27]. Throughout the experiment, the sulfate was used by *P.putida* to perform its microbiological process and result in reduction of sulfate in the sample of B20.

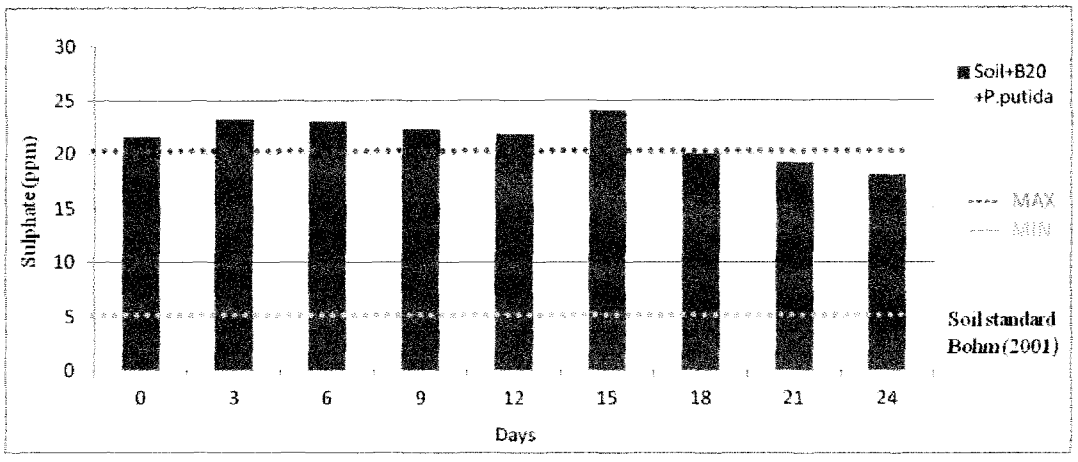

Fig. 5 Result of sulfate in 24 days

The Fig. 6 shows the amount of phosphate for during bioremediation treatment. The phosphate amount was decreased from Day 0 to Day 24. The highest removal of phosphate after treatment was up to 79%. During the bioremediation at a few days, the phosphate amount was observed not in the suggested suitable range of soil (0.5-0.9 ppm). The phosphate is also vital in soil bioremediation as it can enhance bacteria activities and their potential co metabolism [27]. However, the excessive amounts of phosphate in soil cause microbial inhibition. In this study, the phosphate was possibly used by *P. putida* to perform its microbiological process and result in reduction of phosphate in the sample

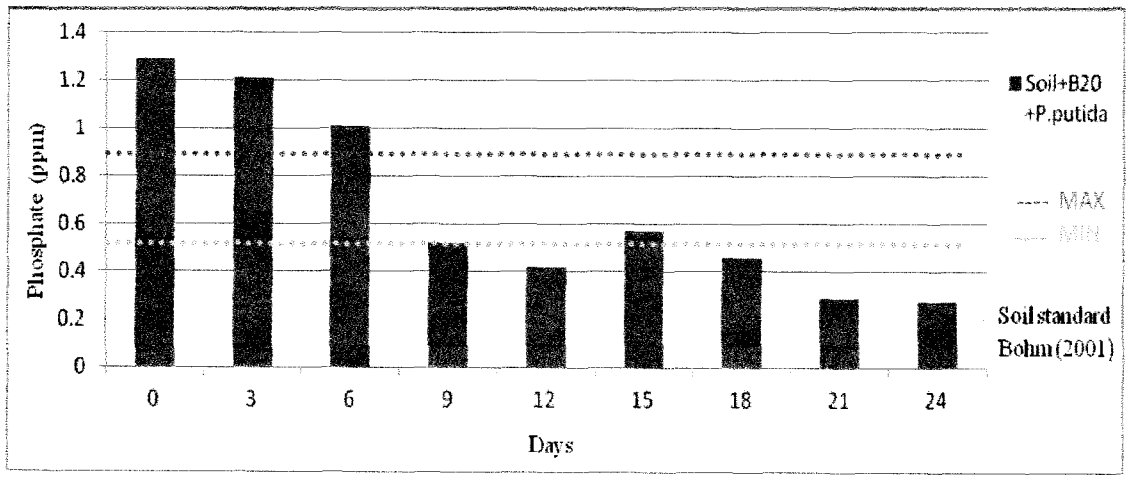

Fig. 6 Result of phosphate in 24 days

Table 1 Summary of the physico-chemical results

Parameter	Sample		Standard	Reference
	Soil + B20 + *P. putida* (Day 0)	Soil + B20 + *P. putida* (Day 24)		
pH	7.45	7.25	6-7.5	[14]
Total organic carbon (%)	0.0332	0.030	0-0.05%	[15]
Total nitrogen (ppm)	34.17	6.05	5-15 ppm	
Sulfate (ppm)	21.52	18.00	5-20 ppm	
Phosphate (ppm)	1.29	0.275	0.5-0.9 ppm	
Moisture content (%)	44.11%	50.35%	40-60%	[16]

Conclusion

Based on the obtained data it can be concluded that *P. putida* is an effective microorganism and suitable as oil-soil biodegradable agent. Physico-chemical tests proved that there is indication of nutrient consumed in soil-biodiesel/diesel mixture after 24 days of bioremediation. The *P. putida* was suitable to treat biodiesel/diesel-soil contamination based on these bioremediation results.

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