# Survival of *Pseudomonas Putida* for Biodiesel Blend (B20) in Soil Bioremediation

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**Abstract.** The commercialization of biodiesel and their diesel blends in many countries can cause environmental pollution due to their spillage. This study aims to investigate the survival of *Pseudomonas putida* in soil contaminated with biodiesel/diesel blends (B20). The spillage simulation of B20 was conducted at laboratory scale for 24 days of incubation time. The experimental results show that contamination of biodiesel/diesel blends into the soil induced a reduction in growth of *Pseudomonas putida*. The growth of *Pseudomonas putida* was measured high in control compared to B20 sample. Soil microorganisms as well as *Pseudomonas putida* are very sensitive to any ecosystem perturbation therefore this study could notably serves as necessary information in soil bioremediation.

## Introduction

The fossil fuel resources are decreasing by day as global demand for the resources increased. The shortage of petroleum reserves will make renewable energy sources more attractive. Currently, the best option for a petroleum diesel fuel substitution is biodiesel. In order to meet the requirements of the Kyoto protocol (1997) and Madrid declaration (1994), several alternative sources of energy especially biodiesel are widely used as an alternative to diesel fuel due to its environmentally friendliness and other advantages [1]. Recently, biodiesel fuels have attracted increasing attention worldwide as blending components or direct replacements for diesel fuel in diesel engines. The most common biodiesel blend is B20 which qualifies for fleet compliance under the Energy Policy Act (EPAct) of 1992.

However, the commercialization of pure biodiesel and their diesel blends in many countries can cause environmental pollution due to their spillage. There is serious concern due to polyaromatic hydrocarbons in biodiesel and diesel have recalcitrant and mutagenic or carcinogenic properties [2]. The exposure of many living biological entity including human being to polyaromatic hydrocarbons may cause deoxyribonucleic acid mutation, reproductive defects, and increased risk of cancer and other adverse health effects through various pathways, such as ingestion of plants that uptake soil pollutants and also leaching of polyaromatic hydrocarbons from contaminated soil to ground and surface water used as drinking water [3].

The contaminated areas can be cleaned-up by the bioremediation technology process in which the hazardous contaminants are turned into carbon dioxide, water, and biomass [4] by action of microorganisms. In fact, this technology is cost-effective and environmentally friendly compared to physico-chemical treatments of pollutant for contaminated soil. Bacteria belonging to the family of *Pseudomonas*, *Acinetobact*or, *Coryneform, Flavobacterium*, and *Burkholderia* were often used [5] for biological treatment in soil contamination .Increasing attention has been paid to *P.putida* pure strains, which act as efficient oil-bioderadable agent in the soil bioremediation[6,7,8].

*P. putida* is endured to xenobiotics and play a essential role in the remediation of soil contaminated with petroleum hydrocarbons [9]. This pure strains occur in various environmental niches because of its metabolic versatility and low nutritional requirement [10]. 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 has some catabolic pathways capable of acting on recalcitrant substances [11].

Microbial growth is one of the consequences of the biodegradability of biodiesel/diesel blends. The aims of this study therefore, focusing on growth rate of *P. putida* to treat soil that has been contaminated with biodiesel/diesel blends.

## Materials and Methods

### Soil

The soil samples were collected from inside campus area. The samples were collected in the range of 2-3 kg from depth 5-10 cm beneath the surface by using spatula and kept in glass flasks and subsequently transported to laboratory. Soil was oven-dried at 105 C° for 24 hours, powdered and then passed through 2mm sieve to remove plants and debris. After that, the soil was put into a 2000 mL Erlenmeyer flasks and covered the flasks with aluminium foil paper so the soil would not be decomposed by light.

### Biodiesel and Diesel

The palm based-biodiesel samples which was produced by transesterification process were obtained from Biodiesel Pilot Plant, FTK, UTHM, Johor. The biodiesel was kept in a 1000 mL glass bottle prior to biodiesel/diesel soil mixture preparation. Refined fossil fuel used throughout this study is commercial petroleum diesel oil (EN 590: 2004) purchased from commercial diesel pump station at Parit Raja. The diesel was also kept in another glass bottle with a volume of 1000 mL . The bottles were covered by aluminium foil paper so the biodiesel and diesel would not be decomposed by light.

### *Pseudomonas Putida* Broth Culture

*P. putida* pure strains were purchased from USA in dry culti-loop form and stored in a freezer at 4 °C prior to cultivation procedure at Microbiology Laboratory, FTK, UTHM. The *P.putida* was grown and cultured in steriled nutrient broth before inoculated into contaminated soil with biodiesel and diesel. Approximately 16 g of medium (Brand: Merk Milipore, Germany) was suspended in a 2000 mL Erlenmeyer flask containing 2000 mL distilled water and the solution was stirred gently to homogenize them. The solution was then sterilized in a Hiramaya Autoclave Sterilizer (Model HV-85) for 15 minutes at 121 °C. The solution was allowed to cool down for a few minutes until its temperature drop in between 35 °C to 37 °C. One culti loops shaft containing *P.putida* pure strains were broke off from the handle directly into the warm medium according to the manufacturer's instruction. Keep stirred for a few minutes and then pH of samples were adjusted to optima pH 7 by adding sodium hydroxide solution before being stored in a incubator shaker at 35 °C for 9 days prior to further innoculation process.

### Nutrient Media (CHROM Agar *Pseudomonas*)

Approximately 22 g of powder was dispersed in a 500 mL Erlenmeyer flask containing 500 mL distilled water. The solution was stirred and heated on hot plate at 100 °C for 2 hours. After

that, the solution was sterilized in the autoclave at 15 psi and 121 ºC for 15 minutes. The solution was then allowed to cool down for a few minutes until its temperature drop to 48 ºC before pouring them into the sterile petri dishes. Media agar was stored in freezer at 4 ºC before conducting bacteria count experimentation.

**Soil-Biodiesel/Diesel and *Pseudomonas Putida* Mixture**

Spill simulations with biodiesel/diesel blends in soil were carried out in accordance with [12], with some modification. A 20% of biodiesel (100 mL) and 80% of diesel (400 mL) was added into a 1000 mL Erlenmeyer flask containing 1000 g of soil for sample B20. The contaminated soil samples (B20) were then inoculated with approximately 200 mL *P. putida* broth culture. As a control sample, another soils were prepared and then inoculated with *P. putida* without addition of both biodiesel and diesel .All samples were placed in the incubator shaker at 35 ºC. The day when the soil was mixed and innoculated with biodiesel/diesel blends as well as *P. putida* was considered as Day 0. The growth of *P. putida* was enumerated at interval of three day for up to Day 24 of incubation time (Day 0, 3, 6, 9, 12, 15, 18, 21 and 24).

**Enumeration of *Pseudomonas Putida***

First of all, dilution bottles containing 9 mL of distill water each was steriled at 15 psi and 121 ºC for 15 minutes prior to further procedures. After that, approximately 25g of inoculated soil samples (B20) were dissolved in a 250 mL Erlenmeyer flask containing 225 mL of sterilized distilled water. The flask was shaken on orbital digital shaker at 200 rpm for about 30 minutes. After being left for sedimentation for 2 hours, 1 mL of supernatant from the flask was placed into the first dilution bottle ($10^{-1}$). Next, 1 mL of the suspension from the 10-1 dilution bottle was transffered to the second dilution bottle ($10^{-2}$). This step was continued until the original sample is diluted to six dilution bottle ($10^{-7}$). Next, by applying membrane filtration method 9215D [13] each sample volume from dilution bottles was passed through 0.45 μm sterile grid membrane filter (Brand: S-PAK, USA) under partial vacuum and subsequently placed onto CHROM agar plate. The agar plate was stored into another incubator at 35 ºC for 24 hours prior to enumeration of *P.putida*. After being incubated for one 24 hours, growths of *P.putida* in the petri dishes were measured by using plate count agar. They were taken out of the incubator and placed on the counting chamber for bacteria counting. In this study, the dilution six in triplicates are chosen in bacteria counting experimentation.

**Results and Discussion**

Fig. 1 shows that the survival of *P. Putida* in the sample B20 was lower than control. These results verified that the presence of biodiesel/diesel blends inhibited growth of *P. putida* throughout the incubation time. Other than biodiesel/diesel blends content, the incubation time period also significantly affected the survival of *P. Putida* in the soil samples.

The *P. putida* starts to grow with a similar trend with maximum growth on Day 9 during the biodegradation in each sample as shown in Fig. 1. The peak point of growth is because of the amount of nutrients in both samples was highest on that day. The optimum nutrients stimulated their growth rapidly and resulted in their colonies number increases. The initial growth of *P. putida* in each sample was increased rapidly from Day 0 to Day 9 (log, or exponential growth phase) after inoculation and then started to decrease until Day 24 (death, or logarithmic decline phase) reaching values by the end of the process that were lower than their initial growth. The *P. putida* starts to growth progressively in the presence of nutrients and used them as its food and source of energy to survive well in the soil samples during the exponential growth phase. In the meantime, as the *P. putida* grow, they begin to use up not only the food but also oxygen in the soil samples. The

nutrients stimulated their growth and activity and resulted in their colonies number increases. PAHs were indeed being degraded and used as carbon and energy sources too [14-22] by *P. putida* in order to perform its microbiological process. More enzymes could be synthesized on active sites with the increase in bacteria growth. The active sites are the region at which the enzymes form a loose association with PAHs as its substrate [23]. Therefore, the more enzymes are secreted the more active sites will be provided for PAHs. Enzymes remain unchanged while they speed up degradation of PAHs.

Then, the growth was further decreased during the death phase because of *P. putida* continues to degrade biodiesel and diesel in which the nutrients and PAHs that found in the samples had been used by *P. putida* became fewer. This indicated that the deficiency of carbon and source energy in the samples had influenced their growth. Microbes  increased from the beginning but decreased throughout bioremediation period; however it also depends on fuel composition too [24-25]. It can be concluded that the increase in number of days in bioremediation period reduced the bacterial viability. Nevertheless, the amount of *P. putida* viability is still high which is at $10^6$ CFU/g even until up to Day 24.
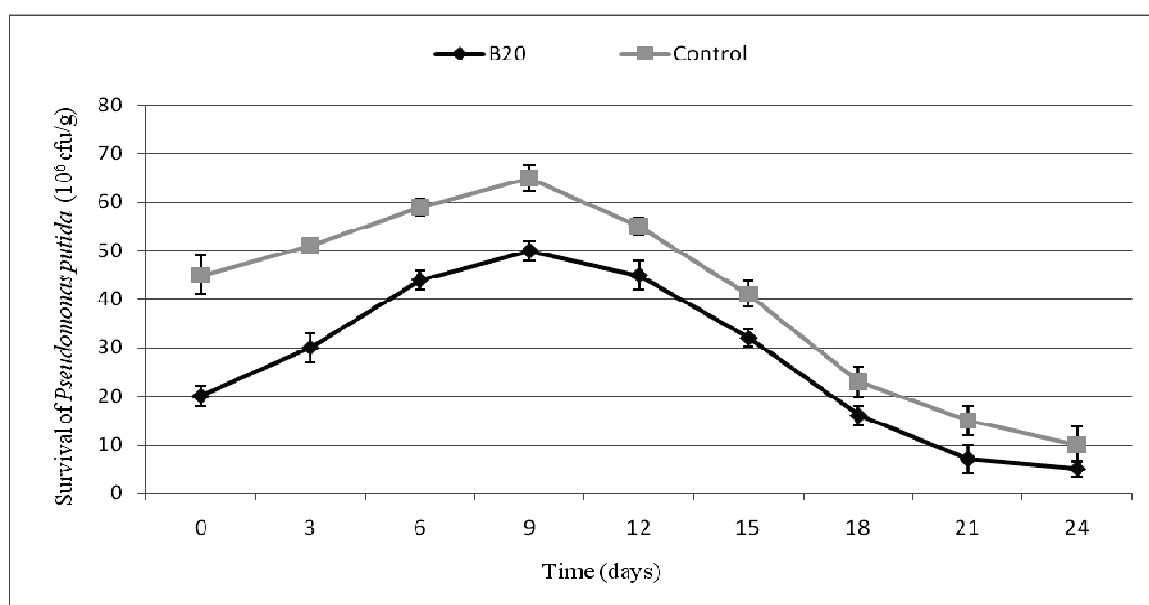


Fig. 1 The survival of *P.putida* ($10^6$ CFU/g) in the sample B20 and control.

## Conclusion

In conclusion, the contamination of soil with biodiesel/diesel blends demonstrated the effect on survival of *P. putida*. Even though there is inhibitory effect on growth of *P. putida* in sample B20, however the *P. putida* colonies counts was persisted high until the end of treatment period. The growth of *P. putida* in the samples gives good indication the bioremediation process occurred in soil biodiesel/diesel contamination. Future study was recommended by prolonged time length of bioremediation for necessary optimum survival rate of *P. putida* in biodiesel-soil contamination.

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