

Rhizoremediation of fuel oil by *Vetiveria zizanioides* in association with *Kocuria* sp. no. MU1 and *Micrococcus luteus* WN01

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ABSTRACT: Pot experiments were conducted to determine the rhizoremediation potential of *Vetiveria zizanioides* in association with bacteria isolated from the oil-polluted area, in fuel oil degradation. The analysis of fuel oil-contaminated soil from Udonthani Province, Thailand, revealed 23 isolates based on their partial 16S rDNA sequences. Among these, only 6 isolates could grow on fuel oil MSM medium. Fuel oil biodegradability assay was performed with the six isolates and the established oil degrader strain *Micrococcus luteus* WN01. The results showed that *M. luteus* WN01 and *Kocuria* sp. MU01 utilized the fuel oil at maximal percentages (86.06% and 63.12%, respectively). Hence, they were chosen as inoculated bacteria in fuel oil-contaminated soil planted with vetiver grass, in 5 treatments: control, natural attenuation, rhizoremediation, bioaugmentation, and phytoremediation. The results revealed that plants in association with *M. luteus* WN01 or *Kocuria* sp. MU01 showed significant increases in total biomass, shoot height and root length, with higher relative growth rates. A significant decrease (> 50%) in total petroleum hydrocarbon (TPHs) concentration in soil, with the highest percentage of removal (50.25%), was observed in rhizoremediation with *M. luteus* WN01 after 45 days of treatment. Moderate percentages of removal (37–47%) were shown in rhizoremediation with *Kocuria* sp. MU01, bioaugmentation and phytoremediation. Natural attenuation exhibited the lowest percentage of removal (20.8%). These results suggested that mutual benefits between vetiver grass and bacteria *M. luteus* WN01 or *Kocuria* sp. MU01 can enhance the rhizoremediation of petroleum hydrocarbon contaminated soil.

KEYWORDS: fuel oil-contaminated soil, oil-degrading bacteria, *Vetiveria zizanioides*, TPH contaminated soil, *Micrococcus luteus* WN01, *Kocuria* sp. MU01

INTRODUCTION

Petroleum hydrocarbons (PHCs) are one of the natural resources that play very important roles in our daily life. PHCs in the form of stove oil, lubricants and fuel are used in heating and energy for engines and as fuel for industrial processes. Because of this, fuel oil is very popular due to the fact that it is extremely low priced than other petroleum products. The hydrocarbon residues are quite durable and can stay in the environment for a prolonged period [1]. The discharge of PHC to the environment is often associated with its consumption. Also, the discharge from accidents, such as leaks from pipes or containers, as well as transportation and oil spills from abandoned gas stations can result in its contamination in the environment. Oil spills are considered a serious environmental problem and

often have significant, long-term impacts on wildlife and human activities in affected areas, where environmental recovery may take several years [1].

Microbial bioremediation and phytoremediation have been widely investigated for the treatment of PHC contaminated sites. Recent research on the use of biosurfactant-producing *Bacillus subtilis* SE1 isolated from enriched petroleum-polluted soil in bioremediation of hydrocarbon contaminated environment in Thailand has been reported [2]. For phytoremediation, plants are used to degrade, stabilize and/or remove soil contaminants including organic compounds [3]. In the expansion of the two techniques, a convergence of phytoremediation and microbial bioremediation strategies has led to a more successful approach, namely microbe-assisted phytoremediation or rhizoremediation [3].

Rhizoremediation is the use of plants and microbiota associated with roots to remove, contain or render harmless environmental contaminants. It is a cost-effective, low maintenance on-site method for remediating petroleum-contaminated soils [3]. The degradation of PHCs in contaminated soil is the result of complex interactions involving roots, root exudates, rhizosphere soil, and microbes [3]. Sugar and organic acids in plant root exudates are commonly used as carbon and energy sources by soil microbes [4]. The microbial consortia can provide various benefits to plants, including the synthesis of compounds that protect the plants, chelators for plant nutrients and the degradation of contaminants [5].

The successful application of rhizoremediation is largely dependent on several factors, such as the tolerance of plants to PHCs, the high ability of bacteria or microbes to degrade PHCs and the capacity of microbes to efficiently colonize growing roots [6]. Therefore, rhizoremediation can be optimized by using suitable plant-microbe pairs, for example, a combination of plants and oil-degrading bacteria. Several species of bacteria are oil-degrading, such as *Bacillus*, *Micrococcus*, *Pseudomonas*, *Kocuria* [7, 8]. These bacteria are capable of degrading light crude oil, diesel oil and polycyclic aromatic hydrocarbons (PAHs). Microbial degradation can be accomplished by different bacterial species that are both native to the soil and added as effective degrading strains [9].

Plants suitable for rhizoremediation have to be tolerant to petroleum contaminants. Many plant species are sensitive to petroleum contaminants and showed inhibition of plant growth parameters, such as germination, shoot length and biomass [10]. Several plant species, including vetiver grass, tall fescue, sorghum, corn, and alfalfa, are effective in degrading total petroleum hydrocarbons (TPHs) and PAHs [11]. Grasses are generally tolerant to petroleum contaminants and have a fast growth rate with high biomass production. Besides, they have an extensive root system, which provides a large surface area for the colonization of oil-degrading bacteria.

Selection of tolerant plant species local to the area and native species of bacteria isolated from petroleum-contaminated soil should be preferred for effective rhizoremediation. Vetiver grass (*Vetiveria zizanioides*) has been proved to be an effective and tolerant plant for phytoremediation [11, 12]. Hence, vetiver grass was chosen in this study to investigate the potential for TPHs remediation from fuel oil-contaminated soil. Vetiver grass has been

grown in Thailand for a long time, mainly to prevent soil erosion due to its deep root system. It is promising for the amelioration of the soil and has shown tolerance towards petroleum contaminants [12]. It also has the potential for the removal of PAHs from diesel contaminated soil [11]. Thus, this study aimed to investigate the rhizoremediation of oil-polluted soil using *V. zizanioides* inoculated with oil-degrading bacteria isolated from oily sludge. The ability of vetiver grass to grow in oil-contaminated soil was evaluated. Total soil bacteria were also quantified to evaluate the effects of inoculated bacterial stimulation on oil biodegradation.

MATERIALS AND METHODS

Soil sample collection

A fuel oil-contaminated soil sample was collected from a PTT (Petroleum Authority of Thailand) petrol storage station (under the supervision of PTT Research and Technology Institute), Udonthani Province, Thailand. The physicochemical analyses were performed by the Department of Soil Science, Kasetsart University, Bangkok, Thailand. Soil texture was analyzed using hydrometer method; soil pH was measured using a pH meter after mixing soil and deionized water in a ratio of 1:1 w/v at room temperature; organic matter (OM) by Walkley-Black titration [13]; and cation exchange capacity (CEC) by ammonium saturation method with 1 N NH_4OAc , pH 7.0 [14]. The total nitrogen was measured by Kjeldahl digestion; available P by spectrophotometric measurement after extracting with Bray II method [15]; and available K by atomic absorption spectrophotometry after extracting with 1 N NH_4OAc , pH 7.0 [16]. Electroconductivity (EC) was measured by electrical conductivity meter after mixing soil and deionized water in a ratio of 1:5 w/v at 25 °C. Table 1 summarizes some of the physicochemical characteristics of the soil. The TPH concentration of soil from the petrol storage station was 20 000 ppm.

Isolation and identification of oil-degrading bacteria

Five grams of the oil-contaminated soil sample was added to 50 ml of Luria-Bertani medium (LB) medium and incubated at 30 °C, at 150 rpm for 24 h to isolate oil-degrading bacteria. Serially diluted soil samples were spread on LB agar plates and incubated at 30 °C for 48 h. Colonies with different morphological appearances were selected and purified in nutrient agar medium [17]. Chromosomal DNA of each strain was prepared from a single colony

Table 1 Some physicochemical characteristics of the oil-contaminated soil sample (from petrol storage station) and the spiked soil.

Characteristic	Contaminated soil		Analytical method
	Sample	Spiked soil	
pH	7.1	7.2	1:1 soil/deionized water
Organic matter (g/kg)	2.40	2.32	Walkley-Black titration
Texture	Silk clay	Clay	Hydrometer
Cation exchange capacity (cmol/kg)	31.40	28.20	Ammonium saturation
Total nitrogen (mg/kg)	1100	980	Kjeldahl
Available phosphorus (mg/kg)	40	28	Bray II
Available potassium (mg/kg)	149	162	AAS
Electrical conductivity (dS/m)	0.77	0.23	1:5 soil/deionized water
Total petroleum hydrocarbon (ppm)	20,000	10,000	Soxhlet

using the QIAamp DNA minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The 16S rDNA of bacterial isolates was amplified using the universal primers UFUL (GCCTAACACATGCAAGTCGA) corresponding to nucleotide position 39–58 (forward primer) and URUL (CGTATTACCGCGGCTGCTGG) corresponding to nucleotide position 530–511 (reverse primer). These partial sequences were matched in GenBank using the BLAST tool (blastn) on the NCBI web page (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with at least 97% similarity.

Screening of potential bacteria for fuel oil utilization

The 23 identified bacterial isolates were screened for their ability in oil degradation and to determine the most efficient degrader. Each of the bacterial cultures was serially ten-fold diluted with sterile 0.85% NaCl. After that, 100 µl of appropriate dilution, were spread on mineral salt medium (MSM) agar plates supplemented with 1% fuel oil [17]. The plates were incubated at 30 °C for 48 h to observe the colony appearance. Colonies were then counted and total microbial count/ml was calculated. The extent of bacterial growth was assessed by visual observation of the medium [18].

Fuel oil biodegradability assay

Fuel oil biodegradation assays were performed with the bacterial isolates having the ability to degrade oil and the oil degrader *Micrococcus luteus* WN01 deposited at the Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand. *M. luteus* WN01 was previously isolated from the wastewater treatment system at a PTT gas station plant; it could degrade and utilize hydrocarbon as

the sole carbon source [19]. The initial inoculation was 1 ml bacterial suspension (OD₆₀₀ about 0.5) in 50 ml of MSM medium prepared with 1% fuel oil as sole carbon source. The medium was sterilized at 121 °C for 15 min. The culture was incubated at 30 °C, 150 rpm for 7 days. Three replicates were performed for each bacterial isolate. Control without bacterial inoculum was also maintained. After the incubation, the extent of oil utilization was assessed by visual observation of the turbidity of the medium [18]. The level of microbial degradation of the oil was determined by gas chromatography (GC, Agilent Technology 6890 N (USA) equipped with a flame ionization detector (FID) and fused silica capillary column (30 m length; 0.25 mm i.d.; 0.25 mm film thickness), according to the US EPA 8015 method [20]. The bacterial strains that displayed high ability in biodegrading fuel oil were identified, and their percentages of removal efficiency were calculated to assess their potential use in the treatments of fuel oil-contaminated soils.

Rhizoremediation experiment

Soil and plant preparation

Uncontaminated soil with no history of TPH contamination, was purchased from Chatuchak Market, Bangkok, Thailand was used for spiked soil. Fuel oil (No. 5) was obtained from the PTT Research and Technology Institute. Fuel oil (1% w/w; 10 000 mg/kg soil) was mixed thoroughly with acetone (200 ml/kg of soil) before adding to the soil. The initial TPHs concentration achieved in spiked soil was 10 000 mg/kg soil. The soil was characterized for physicochemical characteristics (Table 1). Vetiver grass (*V. zizanioides*) Songkla 3 ecotype was obtained from the vetiver station in Pathum Thani Province, Thailand. Plants were acclimatized under

Table 2 Treatments and conditions.

Code	Treatment	Condition
T1	Control	Uncontaminated soil + vetiver grass
T2	Natural attenuation	Contaminated soil
T3A	Bioremediation	Contaminated soil + vetiver grass + <i>M. luteus</i> WN01
T3B	Bioremediation	Contaminated soil + vetiver grass + <i>Kocuria</i> sp. no. MU1
T4A	Bioaugmentation	Contaminated soil + <i>M. luteus</i> WN01
T4B	Bioaugmentation	Contaminated soil + <i>Kocuria</i> sp. no. MU1
T5	Phytoremediation	Contaminated soil + vetiver grass

prevailing natural environmental conditions for one week prior to the experiment.

Bacterial preparation

One ml of bacterial culture (the identified *Kocuria* sp. MU01 isolated from oil-contaminated soil or *M. luteus* WN01) was added to 50 ml of LB media and incubated at 30 °C, 150 rpm for 72 h. The cells were harvested at stationary phase and re-suspended in the MSM media before being inoculated into the soil by adding the bacterial suspension at 10 ml per kg of dry soil. The experimental soil was packed into plastic pots (19 cm in diameter and 22 cm in height) at 3 kg/pot. Each pot contained one plant.

Experimental design

The experiment was conducted in a greenhouse under prevailing natural environmental conditions with natural sunlight and ambient temperature ranged from 27 to 36 °C. There were 7 treatments (Table 2) and the experimental period was 45 days. Each treatment was performed in triplicate. The water supply was 200 ml of tap water every alternate day. Pots were arranged in a randomized complete experimental block design.

Determination of TPHs

TPHs were determined by the GC-FID system [20]. Two grams of soil sample were extracted with 10 ml of acetone:hexane (1:1, v/v) for 15 min using an ultra-sonicator (BANDELIN SONOREX SUPER RK 156 BH, Germany). The supernatant was dried by passing through a layer of anhydrous Na₂SO₄. Then the second cycle of extraction started using 10 ml hexane for 15 min. The extract was put under a stream of nitrogen gas until dry; then, 1 ml of n-hexane was added and 1 µl was injected into a GC system. The weight of residual TPHs was deter-

mined from subtracting the weight of empty vial from the weight of vial containing extracted TPHs. The percentage of degradation was calculated from

$$\% \text{ Degradation} = \frac{(\text{TPH}_{\text{initial}} - \text{TPH}_t)}{\text{TPH}_{\text{initial}}} \times 100,$$

where TPH_{initial} is initial soil TPH concentration, TPH_t is soil TPH concentration at time *t*, and *t* is 7, 15, 30 and 45 (day).

Bacterial plate count

At each sampling day of the experiment (0, 7, 15, 30, 45), samples were taken out from the pots and analyzed for viable cell count; and the extent of biodegradation was determined. The bacterial counts were determined with standard dilution plating technique using sterile nutrient agar; and the colony forming units were counted after incubation [21]. One gram of soil samples was mixed with 9 ml of 0.85% sterile NaCl solution and shaken vigorously until the mixture was thoroughly mixed. After appropriate serial dilutions, 1 ml each of three dilutions was inoculated on sterile LB agar plates and incubated at 30 °C for 3 days. The plates containing between 30 and 300 colonies were counted and expressed as a colony-forming unit (cfu)/g dry soil.

Data analysis

Plant growth was estimated by measuring the stem height, root length and dry biomass. The relative growth rate (RGR) was calculated by dividing the difference in plant weight between the initial and the final fresh weight. The percentage of oil biodegradation was determined by calculating the average of peak height area of each compound on the chromatogram of a biodegraded sample after different experimental periods to the initial sample [21].

Statistical analysis

Data were processed using analysis of variance (ANOVA) in the SPSS-18 statistical software package (SPSS, Inc.). Multiple comparisons of means of experimental parameters of each treatment, including the percentage of TPHs degradation (%) and counts of bacteria, were made using Tukey's HSD test at $p < 0.05$. The interpreted results represented the average of three replicates and were presented as the mean \pm standard error.

RESULTS AND DISCUSSION

Soil characteristics

The physical and chemical characteristics of oil-contaminated soil (spiked with TPHs) used in the rhizoremediation experiment are shown in Table 1. The soil pH was neutral (pH 7.2). The soil texture was clay soil with moderate levels of organic matter (2.32 g/kg) and the CEC value was 28.20 cmol/kg. In the soil background, the amounts of essential nutrients were relatively high and suitable for plant growth, especially the available phosphorus (28 mg/kg) and potassium (162 mg/kg). The TPHs content in contaminated soil was 10 000 ppm soil. From the results, all plants grown in both contaminated soil and uncontaminated soil appeared healthy and there were no abnormalities or discoloration of plant organs. They maintained 100% survival rate for the whole duration of the experiment (45 days).

Identification of isolates and their fuel oil utilizing potential

The analysis of fuel oil-contaminated soil from PTT petrol storage station revealed 23 bacterial isolates that were separated into three phyla: Proteobacteria with dominant species (15 isolates); Actinobacteria (2 isolates) including *Kocuria* sp. MU01 and *Kocuria* sp. MU02; and Firmicutes (6 isolates). Testing the isolates and *M. luteus* WN01 for their potential to utilize fuel oil as a sole carbon and energy source revealed that six isolates, *Exiguobacterium* sp., *Lysinibacillus* sp., *Kocuria* sp. MU01, *Ochrobactrum* sp., *Alcaligenes* sp., and *Achromobacter* sp. and *M. luteus* WN01 were able to utilize fuel oil.

Fuel oil biodegradability assay

The 6 bacterial isolates and *M. luteus* WN01 were assayed for fuel oil biodegradation. *M. luteus* and *Kocuria* sp. MU01 showed the highest percentage of TPHs degradation (86.08% and 63.12% degradation, respectively), followed by *Achromobacter*

sp. (56.02%). The moderate degradation percentage was observed in *Exiguobacterium* sp. (45.55%) and *Alcaligenes* sp. (40.02%). The lowest degradation percentage was found in *Ochrobactrum* sp. (23.46%), *Lysinibacillus* sp. (22.33%) and control (natural attenuation, 18.66%). Hence, *M. luteus* and *Kocuria* sp. MU01 were chosen for the rhizoremediation experiment. Similar bacteria were also isolated from PHC contaminated soil and water, for example: *Achromobacter*, *Micrococcus*, *Kocuria*, *Bacillus* [22]. The dominant species in the present study belonged to the genus *Bacillus* and *Proteobacteria*. The different capacities of the isolates in degrading fuel oil may be due to their different hydrocarbon degradation enzyme systems, i.e., some bacteria can degrade hydrocarbon more rapidly than others [7].

Rhizoremediation

The results of plant growth performance in the rhizoremediation study are shown in Table 3. Different growth parameters, including dry biomass, shoot height, root length, and relative growth rate (RGR), were measured at day zero and 45 days after exposure. In general, the dry biomass, shoot height and root length of *V. zizanioides* in all vegetated treatments (T1, T3A, T3B, T5) significantly increased with the exposure time ($p \leq 0.05$). In addition, all vegetated treatments did not show any significant difference in dry biomass production ($p > 0.05$). However, the dry biomass of plants grown in contaminated soil with bacterial inoculation exhibited an increase of 52.2% (*M. luteus* WN01, T3A) and 38% (*Kocuria* sp. MU01, T3B) when compared with control (T1) (Table 4). Vetiver grass grown in oil-contaminated soil alone showed a 29.4% decrease in dry biomass when compared with control (Table 4). The maximum total dry weight was found in T3A (*M. luteus* WN01, 5.13 g/plant) followed by T3B (*Kocuria* sp. MU01, 4.65 g/plant), T1 (control, 3.37 g/plant), and T5 (no bacteria, 2.38 g/plant) (Table 3).

The difference between the shoot height of vetiver grass grown in oil-contaminated soil (T5, 60 cm) and uncontaminated soil (T1, 74.5 cm) was significant ($p \leq 0.05$) (Table 3). The maximal shoot height was found in T3A (94.5 cm), followed by T3B (77.8 cm) and T1 (74.5 cm), while the minimal shoot height was shown in T5 (60 cm). Bacterial inoculation into contaminated soil positively affects the shoot height of vetiver grass. No significant differences in shoot height were observed among T1 (control), T3A (*M. luteus* WN01) and T3B (*Kocuria*

Table 3 Dry biomass production (DW, g/plant), shoot height (cm), root length (cm) and relative growth rate (RGR) of *V. zizanioides* grown in various soil treatments for 45 days.

Treatment code	Day	Dry biomass	Shoot height	Root length	RGR
T1	0	1.53 ± 0.05 ^{a,A}	20.00 ± 0.00 ^{a,A}	10.00 ± 0.00 ^{a,A}	0.000 ± 0.000
	45	3.37 ± 0.61 ^{a,B}	74.50 ± 0.70 ^{b,B}	19.00 ± 1.41 ^{a,B}	0.023 ± 0.003
T3A	0	1.46 ± 0.06 ^{a,A}	20.00 ± 0.00 ^{a,A}	10.00 ± 0.00 ^{a,A}	0.000 ± 0.000
	45	5.13 ± 1.92 ^{a,B}	94.50 ± 14.14 ^{b,B}	23.50 ± 7.77 ^{a,B}	0.035 ± 0.009
T3B	0	1.47 ± 0.11 ^{a,A}	20.00 ± 0.00 ^{a,A}	10.00 ± 0.00 ^{a,A}	0.000 ± 0.000
	45	4.65 ± 1.11 ^{a,B}	77.75 ± 3.88 ^{b,B}	18.00 ± 7.07 ^{a,B}	0.033 ± 0.005
T5	0	1.52 ± 0.15 ^{a,A}	20.00 ± 0.00 ^{a,A}	10.00 ± 0.00 ^{a,A}	0.000 ± 0.000
	45	2.38 ± 1.31 ^{a,A}	60.00 ± 4.24 ^{a,B}	19.50 ± 0.70 ^{a,B}	0.017 ± 0.011

Values are mean ± SE ($n = 3$). Those followed by the same letter did not differ; small letters showed differences between treatments; capital letters showed differences between exposure time at $p < 0.05$ according to the Tukey's HSD test.

Table 4 Range of influence of bacterial inoculation on changes in plant growth parameters in various soil treatments.

Treatment code	Range of variation (% increase)		
	Dry biomass	Shoot height	Root length
T1	100.0	100.0	100.0
T3A	52.2	26.9	23.7
T3B	38.0	4.4	-5.3
T5	-29.4	-19.5	2.6

sp. MU01). In addition, the bacteria did not show any significant influence in root length among all treatments ($p > 0.05$). The increases of 26.9% in shoot height and 23.7% in root length were observed with *M. luteus* WN01 when compared with control (Table 4).

When the RGR values of all treatments were compared, the highest RGR values were shown in treatments with bacterial inoculation (0.033–0.035), followed by those of control (0.023) and treatment without bacteria (0.017) (Table 3). Vetiver grass is a high-biomass plant with a long massive, finely complex structured root system. It is capable of withstanding extreme harsh environmental conditions such as temperature, salinity, heavy metal, and hydrocarbon contamination [12]. Moreover, vetiver grass showed their potential in removing various environmental contaminants from both soil and water [23, 24]. In the present study, *V. zizanioides* showed a promising behavior in degrading fuel oil-contaminated soil (10 000 mg/kg). The successful growth of vetiver grass showed the over time increases in dry biomass, shoot height and root length in all treatments (with or without

bacterial inoculation). However, Nisa et al [11] reported the adverse effect of diesel contamination on the growth of *V. zizanioides*, which was slightly suppressed with a decrease in biomass. Considerable decrease of plant biomass, as well as shoot height and root length by the presence of PHCs, was found in several cases, including wheat, ryegrass, corn, fescue, sorghum, and common flax [25]. Plant growth inhibition can be caused by toxic compounds in PHCs, especially low molecular weight hydrocarbons. Hydrocarbons can also alter soil properties because of their hydrophobicity, which may result in a reduction in water and nutrient availability and plant growth [10, 26].

Bacterial inoculated treatments showed significant increases in growth parameters when compared with treatments without inoculation. These results were supported by Omokeyeke et al [27], who reported that the interactions between plants and rhizosphere bacteria in oil-contaminated soil caused an increase in plant biomass. In general, soil conditions at contaminated sites are very low in nutrients. The presence of rhizosphere bacteria can improve plant growth due to their plant growth-promoting activities, such as the production of phytohormones, ACC (1-amino-cyclo-propane-1-carboxylic acid) deaminase, siderophore production, and phosphorus solubilization [28].

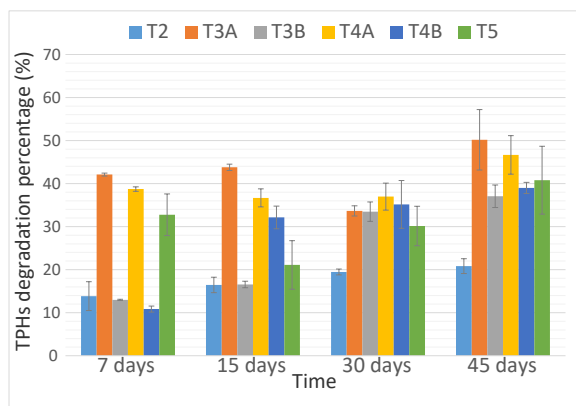
Biodegradation of TPHs

The residual concentrations of TPHs remaining in soil and percentages of TPHs removal in different treatments are shown in Table 5 and Fig. 1, respectively. TPHs concentrations decreased in all treatments after 45 days of the experiment. The statistical analysis revealed that the TPHs removal

Table 5 Residual TPHs concentrations in contaminated soils of different treatments.

Treatment code	TPHs concentration in soil (mg/kg) [†]				
	0 days [*]	7 days [*]	15 days [*]	30 days [*]	45 days [*]
T2	9241.05 ± 97.68 ^{a,A}	8314.00 ± 730.79 ^{a,AB}	8398.33 ± 307.70 ^{a,AB}	7483.75 ± 135.26 ^{a,B}	7356.68 ± 271.75 ^{a,B}
T3A	9801.19 ± 79.20 ^{a,A}	5725.33 ± 71.97 ^{b,B}	5508.98 ± 56.37 ^{b,C}	6806.79 ± 219.64 ^{ab,B}	4878.26 ± 935.92 ^{c,C}
T3B	9060.15 ± 275.65 ^{a,A}	7883.58 ± 259.14 ^{a,B}	7557.89 ± 134.64 ^{ab,B}	6164.52 ± 476.79 ^{bc,C}	6145.60 ± 172.24 ^{ab,C}
T4A	9602.26 ± 147.35 ^{a,A}	7056.04 ± 38.77 ^{ab,BC}	6077.68 ± 192.22 ^{bc,B}	7766.30 ± 307.53 ^{a,C}	6411.15 ± 708.03 ^{ab,C}
T4B	9744.11 ± 753.55 ^{a,A}	8999.78 ± 572.61 ^{a,A}	6838.41 ± 51.97 ^{ab,A}	6519.87 ± 382.11 ^{b,A}	6161.94 ± 569.87 ^{ab,A}
T5	10043.85 ± 981.22 ^{a,A}	6721.23 ± 735.94 ^{b,C}	7924.45 ± 24.67 ^{a,B}	6985.66 ± 33.32 ^{ab,BC}	5892.32 ± 539.55 ^{ab,C}

^{*} Days after treatment. [†] Values are mean ± SE ($n = 3$). Those followed by the same letter did not differ; small letters showed differences between treatments; capital letters showed differences between exposure time at $p < 0.05$ according to the Tukey's HSD test.

**Fig. 1** TPHs degradation efficiency in contaminated soil with different treatments.

could be divided into 3 levels, i.e. highest, moderate and lowest removal percentages. The highest percentage of TPHs removal (50.2%), with the lowest residual TPHs concentration (4878 mg/kg), was shown in rhizoremediation treatment with vetiver and *M. luteus* WN01 (T3A). The moderate removal percentages (37.1–46.7%) were shown in phytoremediation (T5, 40.8%), bioaugmentation (T4A, 46.4%; T4B, 39%) and rhizoremediation with *Kocuria* sp. MU01 (T3B, 37.1%). These treatments exhibited medium concentrations of residual TPHs (5892–6411 mg/kg). The lowest removal TPHs percentage (20.8%), with the highest TPHs residual concentration (7356.7 mg/kg), was found in natural attenuation (T2).

A rapid increase in the degradation (around 40%) was observed at the initial period of remediation after 7 days for T3A (vetiver grass + *M. luteus* WN01) and T4A (*M. luteus* WN01 alone) (Fig. 1). Treatments with the inoculation of *Kocuria* sp. MU01 showed a slight delay in degradation, i.e., 15 days for T4B (*Kocuria* sp. MU01 alone) and

30 days for T3B (vetiver grass + *Kocuria* sp. MU01). In T2 (natural attenuation) and T5 (phytoremediation) treatments, there was a gradual increase in TPHs degradation from day 7 to day 30 (30–32%) and became higher (41%) on day 45 (Fig. 1).

According to this study, TPH removal efficiency, as well as plant growth, was improved by the addition of oil-degrading bacteria. Several reports showed that the rhizosphere bacterial inoculation could enhance plant growth and remediate contaminants. Inoculation of maize (*Zea mays*) with *Bacillus subtilis* can successfully enhance phytoremediation of petroleum hydrocarbons [29]. Afzal et al [30] found that the maximum hydrocarbon degradation (83%) and toxicity reduction (84%) were observed in ryegrass inoculated with *Burkholderia phytofirmans* PsJN. Buffalo grass (*Brachiaria mutica*) and Kallar grass (*Leptochloa afusca*) showed the potential to remediate soil contaminated with crude oil (78%) when combined with a consortium of *Acinetobacter* sp. strain BRSI56 and *Pseudomonas aeruginosa* strain BRR154 [31]. *Enterobacter cloacae* VW4&CAL2 induced the growth of tall fescue (*Festuca arundinacea*) and enhanced TPH degradation [32]. *Gordonia* sp. S2RP-17 inoculation could enhance remediation efficiency as well as promote the growth of maize in diesel-contaminated soil [33].

As previously stated, the rhizoremediation treatments with inoculation of *M. luteus* WN01 or *Kocuria* sp. MU01 showed a high percentage of TPH removal (37–50%) after 45 days. Species of *Kocuria* and *Micrococcus* have been consistently isolated from oil-polluted soil and implicated in PHC degradation. *Micrococcus* was reported to degrade low molecular weight PAHs, diesel oil and TPHs [7]. Similar to our study, Jampasri et al [19] also reported that rhizoremediation of fuel oil and Pb co-contaminated soil using *Chromolaena odorata*

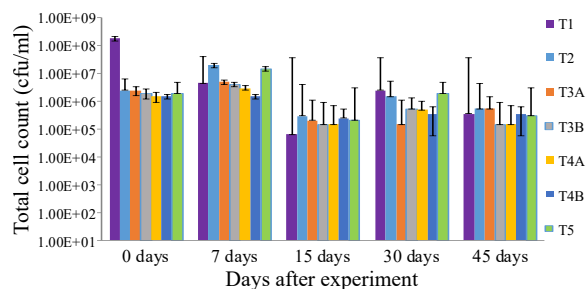


Fig. 2 Comparison of total bacterial population over a 45-day experiment. The result was expressed as colony forming unit (CFU).

inoculated with *M. luteus* WN01 showed a 52% reduction of TPHs. Several species of *Kocuria* were able to degrade PAHs, diesel oil and crude oil [8, 34]. El Mahdi et al [35] also confirmed that *Kocuria* sp. can utilize crude oil as the sole carbon source and has a high ability to degrade crude oil up to 70% in 28 days. *Kocuria sediminis* DDK6 had the ability to degrade C₁₄–C₁₉ compounds in diesel oil [8]. Ahmed et al [34] also demonstrated that *K. flava* and *K. rosea* showed the ability to degrade naphthalene, phenanthrene, fluoranthene, and crude oil. Several studies reported that the production of biosurfactants by *Kocuria* enhanced bacterial oil degradation [36]. Biosurfactant derived from *Kocuria* sp. emulsified more than 50% of the crude oil. Sarafin et al [37] also highlighted that *K. marina* produced biosurfactants reflecting its efficiency in oil degradation.

In this study, the addition of *M. luteus* WN01 and *Kocuria* sp. MU01 to the contaminated soil showed the rapid reduction of TPH during the first 2 weeks. This result confirms the report of Haritash [38], who also found the degradation of low molecular weight PAHs by two bacterial species, *M. luteus* and *Kocuria rosea*, after 15 days. The maximal degradation was observed for naphthalene (68.7%) followed by phenanthrene (62.9%), fluoranthene (61.4%) and pyrene (61.3%). Bento et al [39] also reported that the number of oil-degrading bacteria increased in the first two weeks. These suggested that the lower chain alkane (C₁₄–C₁₈) was degraded rapidly within 15 days due to their labile properties; so they are easy to degrade by bacteria to obtain carbon source for growth and activities. In theory, the rates of hydrocarbon degradation are in the following order: linear alkanes > branched alkanes > aromatic hydrocarbons > polycyclic hydrocarbons. In addition, the rapid TPH degradation during the

first two weeks might be due to a greater bacterial population and sufficient nutrient availability for the degrading microbes.

Total bacterial population

The results revealed that from the initial day to the end of the experiment, when compared between planted soils with or without bacteria (T1, T3A, T3B, and T5) and unplanted soils (T2, T4A, T4B), the planted soil treatments showed higher total bacterial population than the unplanted. The total bacterial population showed a rapid rise until day 7 in all treatments (Fig. 2). The highest number of bacteria (1.8×10^8 cfu/ml) was observed in T1 at the initial day, followed by T3A ($2.5\text{--}5 \times 10^6$ cfu/ml) and T3B ($2\text{--}4 \times 10^6$ cfu/ml). A rapid initial increase in the microbial population in all treatments indicated the abundance of carbon sources in oil-contaminated soil. Gradually, the microbial population tended to slightly decline and became stable until the end of the experiment with the reduction in residual alkane concentration. The lack of carbon sources due to degradation with time could be related to the decrease in the microbial population in the soil. The results of the overall microbial population showed that the plant and the fuel oil could promote the growth of microorganisms in contaminated soil. After 45 days, the number of the colonies was in the range of 10^{-5} – 10^{-8} cfu/ml for planted soil.

In this study, the total bacterial populations in the planted soils were higher than those in the unplanted, demonstrating the bacterial effects of rhizosphere-bacteria interactions [27, 28]. The growth of hydrocarbon-degrading bacteria can be stimulated because plant root exudates contain compounds structurally similar to various PHCs [28]. More nutrients in the soil can be accounted for more microbial population. It has been shown that plants may contribute to the dissipation of PAHs by an increase in microbial numbers [29–31].

Mezzari et al [40] reported that the increase in bacterial population correlated with the presence of grasses, indicating that rhizosphere stimulated bacterial growth which ultimately enhanced hydrocarbon removal. The microbial population present in the root zone or rhizosphere can degrade hydrocarbons or make them more bioavailable. The removal of these compounds becomes more feasible through the metabolic transformation processes of plant-microbe interaction [4].

CONCLUSION

The present study was designed to determine the rhizoremediation potential of *Vetiveria zizanioides* in association with bacteria isolated from the oil-polluted area, in fuel oil degradation. Based on the results of the present work, *V. zizanioides* possess the ability to grow well and withstand the fuel oil concentration upto 10 000 mg/kg soil. It is interesting to mention that the inoculation of bacteria increased plant growth as well as hydrocarbon degradation. The results showed that *M. luteus* WN01 and *Kocuria* sp. MU01 utilized the fuel oil at maximal percentage of removal (> 50%). Therefore, the utilization of vetiver grass and specially the interaction of vetiver grass and oil degrading bacteria suggests an alternative strategy for effective rhizoremediation of soils contaminated with petroleum hydrocarbon. Furthermore, a lab-scale experimental system for rhizoremediation is widely performed due to low cost. It does not only reduce the period of plant growth and length of exposure, but also reduces both the space needed for experiments and variability due to other environmental factors. However, lab scale testing results still need to be confirmed by field performance trials.

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