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# Enhanced remediation of diesel contaminated soil by the combined use of *Lolium perenne* and bacterial consortium

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**ABSTRACT:** *Rhizoremediation is a cost effective and aesthetically pleasing technology for the remediation of diesel polluted environment. The influence of diesel fuel contamination on Lolium perenne with bacterial consortium (Microbacterium schleiferi and Bacillus subtilis) on plant growth and hydrocarbon degradation was investigated. In this study, three different bacterial strains were grown at varying diesel oil concentration i.e. 0.5%, 1%, 1.5%, 2% and 2.5%. Bacteria were also screened for biosurfactant, indole 3-acetic acid production, phosphate solubilization, and antifungal bioassay. Microbacterium schleiferi and Bacillus subtilis were able to grow at 2% diesel oil concentration and exhibited plant growth promoting activities. Green house analysis revealed that augmentation with biosurfactant producing and plant growth promoting bacteria enhanced the plant growth and diesel oil degradation (80%) as compared to the treatments in which bacteria and plants were separately used. Therefore, the study concludes that application of selected strains with the Lolium perenne is a better approach for successful remediation of diesel oil contaminated soil.*

***Keywords:*** Rhizoremediation, Degradation, Diesel oil, *Lolium perenne*, Bacterial consortium

## INTRODUCTION

Soil contamination with petroleum products due to anthropogenic activities typically include accidental spills of diesel oil. The entry of these products in the environment cause impairment of soil structure, flora, and fauna which negatively affect crop productivity (Khalid et al., 2021; Kumar et al., 2021). To alleviate this environmental issue, the use of plants with symbiotic microorganisms has gain much attention in recent years as a proficient and aesthetically pleasing technology to restore diesel-contaminated soil (Devatha, 2019). Plants in conjunction with bacteria transform, stabilize or degrade the hazardous compounds to lesser toxic form which help in retaining the soil physiology resulting in a stable biome (Verma et al., 2019).

Among plants, grasses are potential candidates for diesel biodegradation owing to the highest biomass production, fast growth, and adaptation to environmental stress. Several studies have revealed that *Lolium perenne*, *Sorghum bicolor*, *Zea mays*, *Festuca arundinacea*, *Medicago sativa*, *Pennisetum purpureum*, *Cynodon dactylon*, and *Pteridium aquilinum* can be used for the remediation of on-site diesel contamination (Umeh et al., 2018; Jia et al., 2020; Lin, 2020; Lee et al., 2021; Lednev, 2021). The plants uptake, translocate, and accumulate diesel compounds in roots and shoots depending on the concentration, lipophilicity, solubility, and volatility (Ali et al., 2020). The root system of the grasses also facilitate the breakdown of pollutants by stimulating the rhizospheric bacteria (Herridge et al., 2021). Numerous studies have reported that microorganisms possess the ability to metabolize petroleum hydrocarbons but their hydrophobicity and low bioavailability hinder the process of degradation in the soil. To increase the microbial degradation, bacteria produce various types of biosurfactants which act as emulsifiers resulting in enhanced bioavailability and microbial growth (Gielnik, 2021). Moreover, plant growth promoting (PGP) capabilities such as phosphate solubilization, indole 3-acetic acid (IAA) production, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase reduce the environmental stress and allow to plant to grow in harsh conditions along with the microbes (Saeed et al., 2022).

While several reports indicate the rhizoremediation of diesel contaminated soil using plants and associated microorganisms, but the combined use of biosurfactant producing and plant growth promoting rhizobacteria (PGPR) is limited. Therefore, present study aimed to evaluate the application of bacterial consortium with *L. perenne* and *M. sativa* for diesel biodegradation.

## MATERIALS AND METHODS

### Bacterial strains

Three different bacterial strains *Kocuria rosea* 11, *Mycobacterium schleiferi* N24, and *Bacillus subtilis* M3 isolated and identified by Shehzadi et al. (2016) were used in this study.

**Seed collection**

The seeds of *Lolium perenne* (ryegrass) and *Medicago sativa* (Alfalfa) used in green house were obtained from local plant market of Sialkot Punjab, Pakistan.

**Selection of diesel tolerant plants**

Twenty seeds of each plant were sown in plastic container containing 80 g of contaminated soil. The experimental soil was contaminated with varying diesel oil concentrations i.e. 0.5%, 1%, 1.5% and 2%. Plants were also grown in un-contaminated (absence of diesel) agricultural soil. The experiment was performed in triplicates and plants were grown for 60 days. After two months, the biomass of each plant was recorded and compared.

**Selection of diesel degrading bacteria**

Tested strains were grown on minimal medium (M9) with varying diesel oil concentrations i.e. 0.5%, 1%, 1.5% and 2% for 7 days at 37$^{o}$C and 200 rpm. The percentage of diesel oil degradation was estimated gravimetrically as mentioned by Tahseen et al. (2020).

**Screening of biosurfactant producing bacteria**

Two methods were used to assess the biosurfactant production by the selected bacteria i.e. oil spreading and drop collapse assay followed by the procedure of Ismail et al. (2018).

To prepare the bacterial culture and supernatant, 100 mL minimal salt medium (MSM) along with diesel oil (1.0% w/v) was inoculated with 1% pure bacterial culture and placed at 37$^{o}$C for 7 days on shaking at 200 rpm. After 7 days, liquid culture was centrifuged followed by the filtration of supernatant through a filter paper of 0.45u pore size (Millipore). Cell-free broth was stored for further analysis.

**Biosurfactant extraction**

1M $H_2SO_4$ solution was used to adjust the pH of supernatant to 2. Later, chloroform: methanol solution (2:1) was added in the reaction mixture. The solution was carefully mixed and kept at room temperature for 24 h (Almansoory et al., 2019). The mixture of chloroform: methanol was evaporated. Dry weights were measured at the start and end of the experiment. Dry weight of residual material was estimated using the following formula:

Dry weight of bio-surfactants = (Final weight – Initial weight).

***In vitro* plant growth promotion (PGP) activities**

The selected bacterial strains were assessed for their ability to exhibit PGP activities. Antifungal activity was checked against *Fusarium oxysporum* on potato dextrose agar (PDA) plates for 7 days at 37ºC. After 7 days of incubation, bacteria/fungus inhibition zones were detected and compared to control (fungus absent) (Gizaw et al., 2022**)**.

Pikovskaya's agar plates were used to detect the phosphate-solubilization ability. The plates were spot inoculated followed by incubation for 5-7 days at 37ºC for 5-7 days. The plates were observed for halo zones (Rfaki et al., 2020).

Luria Bertani (LB) broth, augmented with tryptophan (0.1%), was inoculated by bacterial culture and kept in shaking incubator at 37ºC for 7 days. Non-inoculated broth culture was kept as control. After incubation, the indole acetic acid (IAA) production was determined by Salkowski method (Duca et al., 2020).

**Compatibility test for the formulation of bacterial consortium**

After selection, bacterial strains were checked for their compatibility to grow in combination following the protocol by (Fatima et al., 2015). One of the strains were spread on the LB agar plate and remaining strains were spot inoculated on it. The plates were incubated at 37ºC for 24h and checked for the zone of inhibition.

**Phytoremediation experiment**

Green house experiment was carried out with diesel contaminated soil (2.5g/Kg$^{-1}$). The plastic pots (20 cm*1000 cm) were filled with the 100 g of experimental soil with uncontaminated agricultural soil as control. Seeds of *L. perenne* were surface sterilized with 10% $H_2O_2$ for 10 min and washed thoroughly with sterile water and placed in each pot. The pots were given the following treatments.

T1= Uncontaminated agricultural soil+ *L. perenne*

T2= Diesel contaminated soil+ *B. subtilis*

T3= Diesel contaminated soil + *M. schleiferi*

T4= Diesel contaminated soil + *L. perenne*

T5= Diesel contaminated soil+ *L. perenne* + *B. subtilis*

T6= Diesel contaminated soil+ *L. perenne* + *M. schleiferi*

T7= Diesel contaminated soil+ *L. perenne* + Bacterial consortium

**Plant biomass**

The experiment was conducted for three months. After three months, plants were harvested and root/shoot length and fresh/dry biomass was determined as reported by Hostyn et al. (2022).

### Estimation of residual diesel oil concentration

The residual oil concentration by solvent extraction method was calculated gravimetrically using petroleum ether as extraction solvent at the end of the experiment by following Tahseen et al. (2020).

## RESULTS

### Selection of diesel oil tolerant plants

Both plants (*L. perenne* and *M. Sativa*) showed reduction in growth and biomass compared to the plants grown in un-contaminated (diesel absent) agricultural soil. However, biomass production of *L. perenne* was least effected by diesel oil concentration as compared to *M. Sativa* (Fig 1).

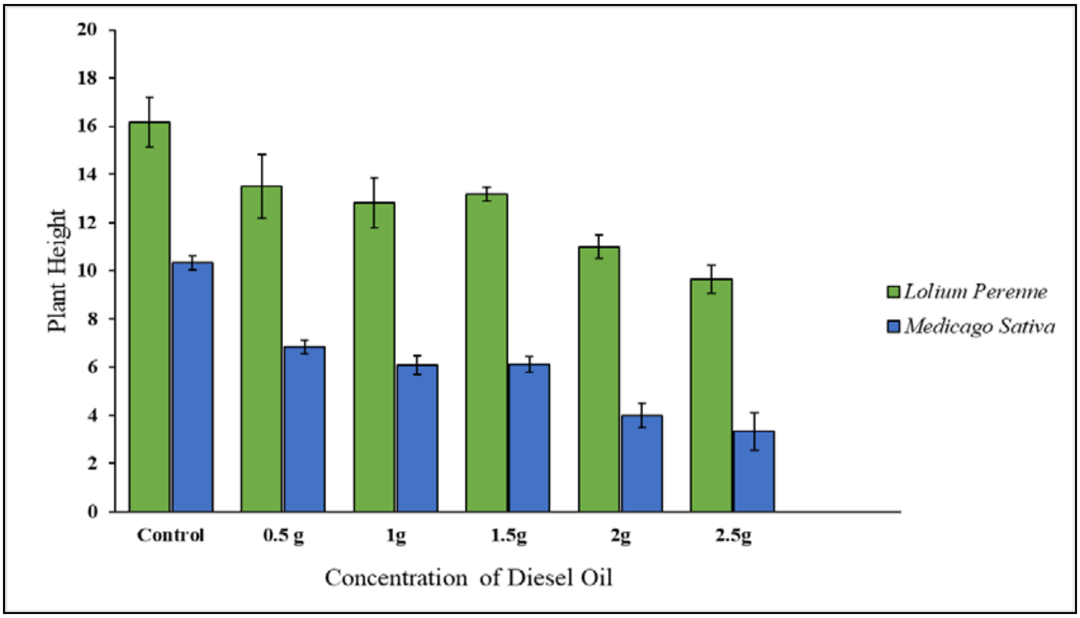


**Fig. 1.** Screening of diesel tolerant plant species on different concentrations of diesel

### Screening of diesel degrading bacteria

The highest percentage of crude oil degraded by *M. schleiferi* (11) was 70% and for *B. subtilis* (N24) was 65%. The least biodegradation (20%) was detected in *K. roses* (M3) (Fig 2).

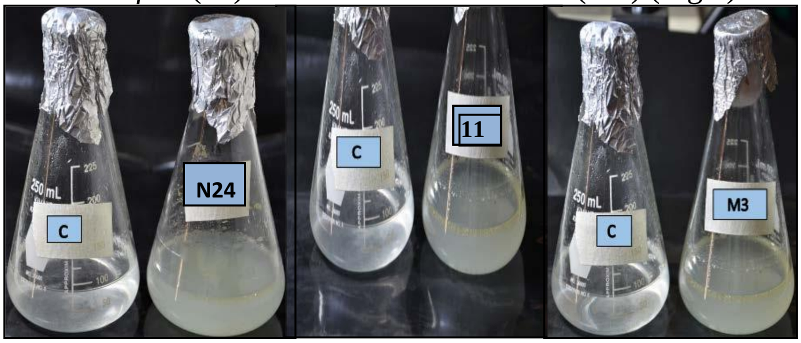


**Fig. 2.** Growth of bacterial strains at 2% diesel oil concentration

**Screening of biosurfactant-producing bacteria**

In oil displacement test, bacterial strains *K. roses* (M3), *B. subtilis* (N24), and *M. schleiferi* (11) were considered as positive strains as clear zones of 4 mm, 7 mm, and 2mm respectively were observed.

In drop collapse assay, *K. roses* (M3) produced a round-shaped droplet, while *B. subtilis* (N24) and *M. schleiferi* (11) developed a flat-shaped droplet.

**Extraction and measurement of biosurfactant**

*B. subtilis* produced the highest biosurfactant quantity i.e., 0.317g/100 mL while *K. roses* (M3) and *M. schleiferi* (11) displayed 0.17g /100 mL and 0.26g /100 mL of biosurfactant production respectively (Table 1).

**Table 1: Screening of bacterial strains for biosurfactant production**

| **Bacterial Strains** | **Oil spreading (zone)** | **Drop collapse** | **Biosurfactant** (g/100 mL) |
|---|---|---|---|
| **11** | + (2 mm) | +++ | 0.26 |
| **N24** | + (7mm) | ++ | 0.31 |
| **M3** | + (4mm) | + | 0.17 |

"+++" means drops collapse within one minute, "++" means drop collapse after one minute, "+" means drop collapse after 3 minutes.

**Plant growth promoting capability of bacteria**

In *in vitro* antifungal activity, bacterial strains *K. roses* (M3), *B. subtilis* (N24), and *M. schleiferi* (11) were able to inhibit the growth of fungi.

In Indole 3-acetic acid assay, bacterial strains *K. roses* (M3), *B. subtilis* (N24), and *M. schleiferi* (11) showed light pink color which is indicative of IAA production.

Phosphate solubilization assay showed that bacterial strains *B. subtilis* (N24) and *M. schleiferi* (11) formed clear halo zones on Pikovskaya's agar plate which indicates the solubilization of inorganic phosphate (Table 2).

**Table 2: Screening of *in vitro* PGP activities**

| Code | IAA | P-sol | Anti-fungal |
|---|---|---|---|
| **M3** | + | - | - |
| **M11** | + | + | + |
| **N24** | + | + | + |

**Compatibility test to formulate bacterial consortium**

Bacterial strains *B. subtilis* (N24) and *M. schleiferi* (11) showed growth on LB agar plate as no halo zone was observed around the growth so both strains were selected to formulate bacterial consortium.

**Effect of bacterial strains on diesel oil degradation and plant growth**

Highest diesel oil degradation (80%) was observed in T7 as compared to T5 and T6. T2 and T3 exhibited a lesser degradation ability while the lowest degradation of diesel oil was observed in the case of T4 (Fig. 3 and 4).

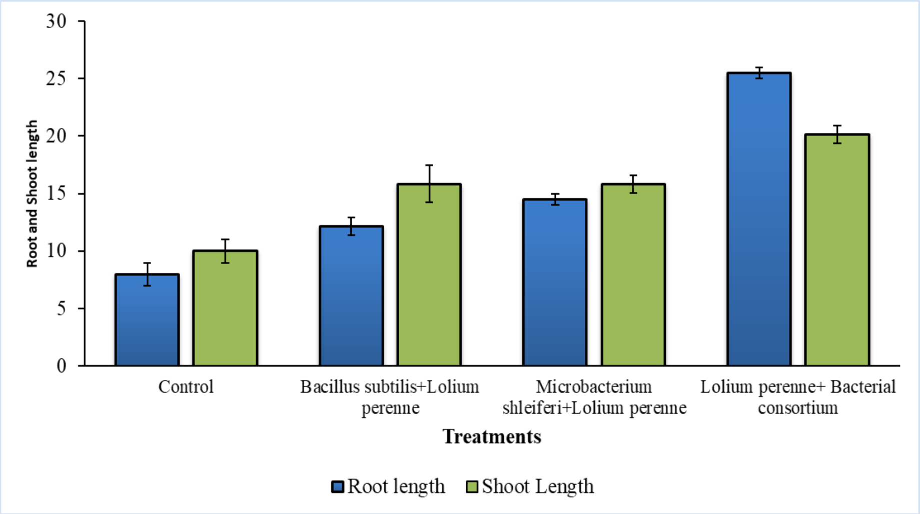


**Fig. 3.** Effect of bacterial inoculation on plant root and shoot length

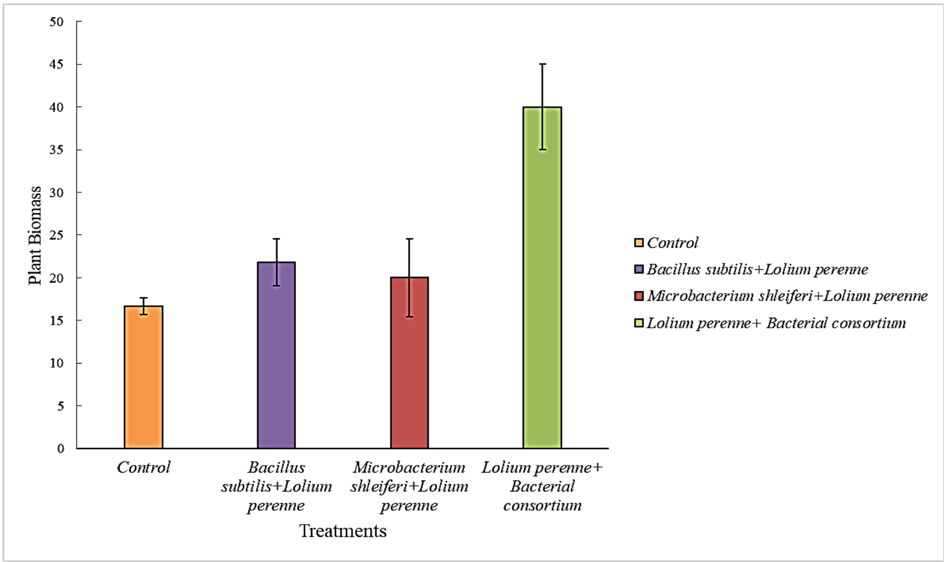


**Fig. 4.** Effect of bacterial inoculation on plant biomass at the end of experiment

## DISCUSSION

For rhizoremediation of diesel-contaminated sites, plants ought to overcome the contaminant(s) stress to initiate microbial degradation of petroleum products. In present study, *M. sativa* was more sensitive to diesel contamination than *L. perenne*. Consequently, under the applied experimental conditions, *L. perenne*, showed maximum biomass production owing to its diesel tolerance and fibrous root system. A fibrous root system offers a widespread area than taproots for colonization of microorganisms. Several studies (Castro-Mancilla et al., 2019; Hoang et al., 2021; Noni-Morales et al., 2019; Wu et al., 2019) have described the plant tolerance to petroleum-derived compounds.

As microorganisms play an integral role in rhizoremediation, biosurfactant producers can aid in overcoming the problem of reduced bioavailability of diesel (Goswami et al., 2019) (Jimoh and Lin, 2020). Oil spreading assay and drop collapse test revealed the biosurfactant producers in the order N24>11>M3 with highest to lowest production; hence, enhancing the diesel bioavailability, resulting in plant and bacterial uptake (Eze et al., 2022).

The success of rhizoremediation is interrelated with selection of particular plants and inoculated bacterial strains in diesel contaminated soil (Imron et al., 2020). The toxicants are transported to the plants shoots via roots upon interaction which ultimately affect the growth and biomass of plants (Aboelkhair et al., 2022). In this study, to reduce the harmful effects of diesel, inoculated bacterial strains having PGP activity were used which may assist in stimulating plant biomass and health (Iqbal *et al*., 2019). Based on the results, bacterial consortium improved the overall plant biomass, root and shoot length (T7) as compared to the treatments (T5 and T6) where individual strains were used. It is mainly due to the cooperative action of mixed microbial strains in the biodegradation of diesel oil and ultimately reducing the stress (Senan and Abraham, 2004).

## CONCLUSION

In conclusion, three different bacterial strains having biosurfactant producing and PGP activity were inoculated to *L. perenne.* The strains when used in consortium improved the plant growth and reduced the diesel oil concentration in the soil as compared to the strains when used individually.

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**Conflict of Interest**

The authors declare no conflict of interest.

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