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Evaluation of Bioremediation Effectiveness by Resolving Rate-Limiting Parameters in Diesel-Contaminated Soil

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Abstract The biodegradation rates of diesel oil by a selected diesel-degrading bacterium, *Pseudomonas stutzeri* strain Y2G1, and microbial consortia composed of combinations of 5 selected diesel-degrading bacteria were determined in liquid and soil systems. The diesel degradation rate by strain Y2G1 linearly increased (R^2=0.98) as the diesel concentration increased up to 12%, and a degradation rate as high as 5.64 g/l/day was obtained. The diesel degradation by strain Y2G1 was significantly affected by several environmental factors, and the optimal conditions for pH, temperature, and moisture content were at pH 8, 25°C, and 10%, respectively. In the batch soil microcosm tests, inoculation, especially in the form of a consortium, and the addition of nutrients both significantly enhanced the diesel degradation by a factor of 1.5 and 4, respectively. Aeration of the soil columns effectively accelerated the diesel degradation, and the initial degradation rate was obviously stimulated with the addition of inorganic nutrients. Based on these results, it was concluded that the major rate-limiting factors in the tested diesel-contaminated soil were the presence of inorganic nutrients, oxygen, and diesel-degrading microorganisms. To resolve these limiting parameters, bioremediation strategies were specifically designed for the tested soil, and the successful mitigation of the limiting parameters resulted in an enhancement of the bioremediation efficiency by a factor of 11.

Key words: Biodegradation, diesel, hydrocarbon, bioremediation, limiting factors

Environmental contamination due to mineral oil hydrocarbons from industrial wastes, transport, and storage accidents is currently widespread. Diesel oil is one of the major contaminants of soil and ground water near gas stations [2]. The demand for cleaning up oil-contaminated sites has also increased with increasing public concern over the preservation of the environment. One efficient and ecologically acceptable treatment method for the decontamination of many oil-polluted areas is bioremediation, which attempts to accelerate natural biodegradation rates by overcoming the limiting factors [1, 7]. The microbiological decontamination (bioremediation) of oil-polluted soils has been claimed to be an efficient, economic, and versatile alternative to physicochemical treatments [6, 9].

The ability of microorganisms to degrade organic compounds and the rates at which degradation occurs depend on the interaction of numerous factors. These factors include the chemical structure, concentration, and availability of the contaminant to the microorganisms, the nature of the microbial population, and the physicochemical environment. In addition, environmental factors, such as temperature, moisture, pH, physicochemical properties of the soil, and aeration, often influence the activity of a microbial population in the biodegradation of contaminant compounds [7, 11, 39, 42]. It is well established that petroleum hydrocarbon compounds are biologically degraded under optimal environmental conditions. However, in natural environments, such as soil ecosystems, environmental and other parameters frequently limit the biodegradation rate of the contaminants of interest such that bioremediation is not a feasible option for site remediation. In many cases, preliminary laboratory studies have yielded effective biodegradation rates because environmental conditions could be easily controlled in laboratory settings. However, when these applications were scaled up to the field level, substantially different contaminant removal rates were frequently observed relative to those observed in the laboratories. This is largely due to the failure to fully account for the various rate-limiting factors prevalent in soil systems.

One key factor that all effective soil bioremediation applications share is that the application design must effectively compensate for the rate-limiting effects of various parameters, microbiological and environmental. Bioremediation

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should not be used indiscriminately, in isolation from other procedures, or of without adequate knowledge of the existing environment. Successful bioremediation programs also require application methodologies specifically tailored to the environmental parameters at each contaminated site.

In this study, the feasibility and efficacy of the bioremediation of an oil-contaminated soil environment were evaluated using techniques resolving the rate-limiting factors. To enhance the oil degradation, techniques such as the addition of inorganic nutrients (biostimulation), the addition of acclimated bacteria (bioaugmentation), aeration by tilling or venting, increased bioavailability through the application of surfactants, and adjustment of the pH and moisture content were applied, and their treatment differences compared.

Materials and Methods

Microorganisms and Culture Conditions

Five diesel-degrading bacterial strains, *Pseudomonas stutzeri* Y2G1 (KCTC 18049P), *Aquaspirillum* sp. Y1K3, *Pseudomonas* sp. Y2K4 (KCTC 18050P), *Pseudomonas* sp. Y5K2, and *Sphingomonas* sp. D3K1 (KCTC 8935P), were isolated from oil-contaminated soils collected in the Yosu industrial area, Republic of Korea. The strains were cultured on sterile mineral salts medium (MSM, $NaNO_3$, 4 g; KH_2PO_4, 0.15 g; $Na_2HPO_4 \cdot 12H_2O$, 1.25 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeCl_3 \cdot 6H_2O$, 0.0005 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; distilled water, 1-l, final pH 7.0 ± 0.2) containing 4% (v/v) diesel (Sigma 6, LG Caltex, Korea) at 25°C for 6 days with shaking at 150 rpm.

Effect of Environmental Factors on Diesel Degradation by the Strain Y2G1

Preparation of bacterial suspension: The bacterial cells were grown on diesel, as described above, and harvested at 1,300 ×g for 15 min. After washing twice with sterilized MSM, the cells were suspended in fresh MSM to give a cell density of 2×10^5 CFU/ml. Fifty-milliliter aliquots of the prepared cell suspension were transferred to 250-ml Erlenmeyer flasks, and incubated under the required conditions for 6 days with shaking at 150 rpm. Flasks under the same conditions without inoculum served as abiotic controls, and all experiments were performed in duplicate.

Effect of diesel concentration: Diesel was added to Erlenmeyer flasks containing the cell suspension to make final concentrations of 2%, 4%, 8%, or 12% (v/v). The residual diesel was analyzed after 6 days of incubation at 25°C.

Effect of pH: The initial pH of the MSM was adjusted to 5, 6, 7, 8, or 9 using 1 N HCl or NaOH and cell suspensions were prepared with each pH. Diesel (4%) was added to the flasks, which were then incubated for 6 days at 25°C.

Effect of surfactants: Seven different nonionic surfactants, Triton X-100, Tween 20, Tween 40, Tween 60, Tween 80 (Sigma, St. Louis, MO, U.S.A.), Biosolve (Jeil Co., Korea), and Sophorolipid were individually added to the suspension at a concentration of 0.01% (w/v), which is low enough to prevent the inhibition of microbial activity by the surfactants. Biosolve is a synthetic mixture of biosurfactant and inorganic nutrients, which contains 316.4 g-carbon/l, 119.5 g-nitrogen/l, and 0.9 g-phosphorous/l. The Sophorolipid was produced using *Torulopsis bombicola* (ATCC 22214), as previously described by Cooper and Paddock [19].

Preparation of Diesel-Degrading Bacterial Consortia

To construct the consortia, each of the 5 bacterial strains was cultured on MSM containing 4% (v/v) diesel. The bacterial cells were washed twice with sterilized MSM after centrifugation at 1,300 ×g for 15 min and then resuspended in MSM to give a cell density of 1×10^7 CFU/ml. Identical volumes of each of the prepared cultures were mixed in various combinations and 1 ml of each mixed culture was placed in an Erlenmeyer flask containing 50 ml of MSM with 4% (v/v) diesel oil. The degradation of diesel was measured after 6 days of incubation at 25°C in a rotary shaker at 150 rpm.

Experimental Conditions of Soil Microcosms

Characterization of soil: The soil used in the experiments was collected from diesel-free ground around Uiwang, Kyunggi-do, Republic of Korea. The characteristics of the soil were analyzed using ASTM methods [4], D2216-90, D854-91, D3282-91, D2974-87, and G51-84, and the Hilgard cup method for the determination of the water-holding capacity (WHC) of the soil [30]. The soil had a pH of 8.6, organic content of 3.9%, moisture content of 6.9%, specific gravity of 2.69, and WHC of 51% (w/w). The soil was almost coarse sand (98%) and the content of silt and clay was negligible (2%).

Diesel degradation in soil batch microcosms: Two-liter glass beakers were used as the batch microcosms. Each batch microcosm consisted of 1 kg of diesel-contaminated soil, which was prepared by homogenous mixing after addition of diesel (0.4%, w/w) in the soil. The soil was then inoculated with strain Y2G1 or a mixture of strains Y2G1, Y2K4, and D3K1 to make a final cell density of 1×10^7 CFU/g. The water content of the soil was initially adjusted to 9.8, 19.6, 29.4, or 39.2% of its WHC, using sterilized distilled water. To the soil reactors with a water content of 19.6% of WHC, nonionic surfactants, Triton X-100, Tween 60, Sophorolipid, or Biosolve were individually added at a concentration of 0.01% (w/w). As inorganic nutrients, agricultural fertilizer (Chobi Co. Ltd. Korea) was added to the soil in a C/N/P ratio of 100:10:3. One gram of the fertilizer consisted of 257.0 mg urea-N, 100.4 mg ammonia-N, 0.8 mg nitrate-N,

0.1 mg nitrite-N, and 33.0 mg phosphate-P. Silica and latex were added to the fertilizer as support material. The microcosms were loosely covered with aluminum foil and the diesel degradation was monitored for 30 days at ambient temperature.

Diesel degradation in soil columns: Acryl-columns (4 cm× 20 cm) were packed with 750 g of soil contaminated with 0.4% (w/w) diesel. The soil was inoculated with the cells of strain Y2G1 or a mixture of strains Y2G1, Y2K4, and D3K1 to make a density of 1×10^7 CFU/g-soil, and the water content was adjusted to 10% with sterile distilled water. An abiotic soil column was prepared by poisoning the soil with formalin (0.2%, v/w) to measure any abiotic loss of diesel. Aeration was accomplished by injecting forced air upward from the bottom of the column at a flow rate of 50 ml/min.

Residual Oil Analysis

After incubation, the residual diesel in the culture medium or soil was extracted using the EPA 3510C method or EPA 3550 method, respectively [40, 41]. For the analysis of residual diesel, total GC detectable hydrocarbons in the extract was quantitatively measured using gas chromatography (Hewlett-Packard Model 6890 plus, U.S.A.) equipped with a flame ionization detector and 5% phenyl methyl siloxane capillary column (HP-5, Hewlett-Packard, 0.32 mm×1.0 μm× 60 m). The operating conditions were: carrier gas (helium) flow rate, 5 ml/min; injector (split) temperature, 200°C; split ratio, 20:1; oven temperature, 45°C for 3 min, increasing to 275°C at a rate of 12°C/min with holding for 12 min; detector temperature, 200°C.

RESULTS AND DISCUSSION

Effects of pH and Temperature on Diesel Degradation in Liquid by Strain Y2G1

The diesel degradation by strain Y2G1 was at a maximum at 25°C and pH 8 (data not shown). The growth of the strain on Nutrient broth medium (Difco, U.S.A.) is also optimal at 25°C, plus the alkaline optimal pH gives a great advantage to strain Y2G1 as an inoculum to remediate diesel-contaminated soil with an alkaline pH. Dibble and Bartha [21] also reported that maximum hydrocarbon metabolism typically occurs within a temperature range of 20°C to 30°C at a slightly alkaline pH. The diesel degradation by strain Y2G1 was compared at various diesel concentrations (Fig. 1). Hydrocarbon biodegradation has been known to slow down at concentrations above 5% [24], and to be definitely inhibited at concentrations above 10% [7, 21]. However, in this study, the degradation rate proportionally increased as the diesel concentration increased up to 12%, and a diesel degradation rate as high as 5.64 g/l/day was obtained. The results of this study

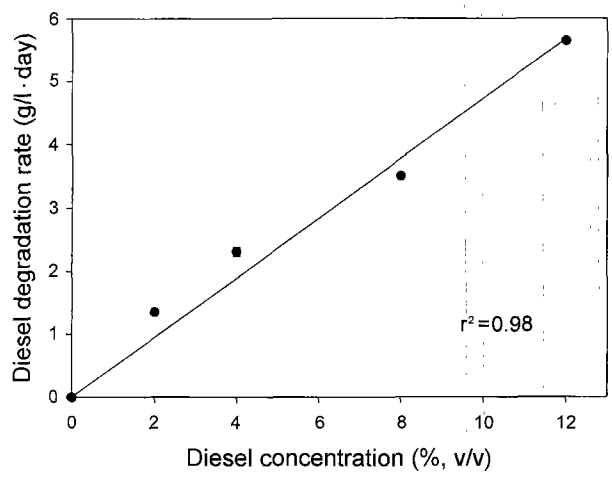

Fig. 1. Effect of diesel concentration on degradation by *P. stutzeri* Y2G1.
The strain was cultured on diesel for 6 days at 25°C with shaking at 150 rpm.

indicate that strain Y2G1 is a typical mesophilic hydrocarbon-degrading bacterium with an appreciable degradation rate, which has a high potential applicable to heavily oil-contaminated environments.

Effect of Various Surfactants on Degradation of Diesel Oil

The biodegradation of organic compounds with limited water solubility is often slow, because of the low availability of these compounds to microbial cells. The availability of slightly soluble organic compounds can be enhanced by surfactants, which can increase the aqueous dispersion by several orders of magnitude [45]. In order to enhance availability, nonionic surfactants were selected in this study because of their higher hydrocarbon solubilizing activity, weaker adsorption to charged sites, lower toxicity to bacteria, and reduced foaming properties. This class of surfactants is also known to be efficient in enhancing the metabolism of various polycyclic aromatics [3, 38].

Among the seven surfactants, Biosolve, Sophorolipid (hydrophilic/lipophilic balance, HLB, ≒ 12), Tween-60 (HLB, 14.9), Tween-80 (HLB, 15.0), and Triton X-100 (HLB, 13.5) enhanced the diesel degradation in liquid medium compared to the control (Fig. 2). Less degradation was observed with the addition of either Tween 20 (HLB, 16.7) or Tween 40 (HLB, 15.6). It would seem that the HLB of the surfactants was negatively correlated to the diesel degradation by strain Y2G1. Bruheim *et al.* [14] also reported that surfactants that are more hydrophobic (Tergitol 15-S-7 and Tergitol 15-S-3) increase the rate of alkane oxidation by *Rhodococcus* sp., whereas less hydrophobic surfactants (Tergitol 15-S-150 and Tergitol 15-S-30) do not significantly increase the rate of alkane oxidation. The authors explained that the effect of nonionic surfactants on

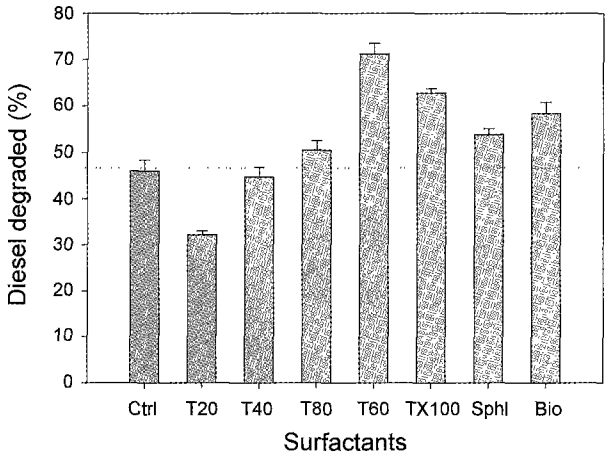

Fig. 2. Effect of various surfactants (0.01%, w/v) on diesel degradation by *P. stutzeri* Y2G1.
The strain was cultured on diesel (4%, v/v) for 6 days at 25°C with shaking at 150 rpm. Ctrl, Control; Bio, Biosolve; Sphl, Sophorolipid; T20, Tween-20; T40, Tween-40; T60, Tween-60; T80, Tween-80; TX100, Triton X-100.

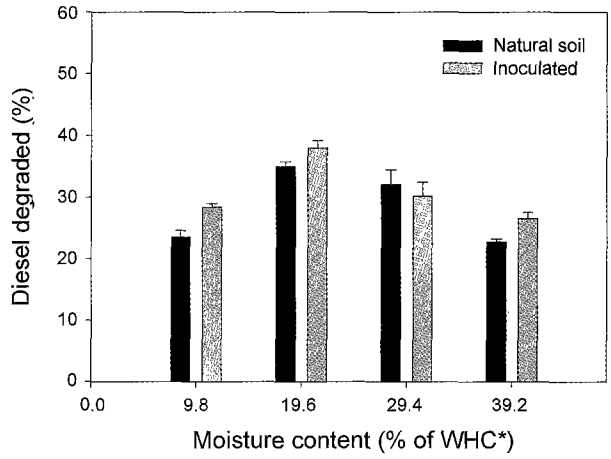

Fig. 3. Effect of water contents on diesel degradation by *P. stutzeri* Y2G1 in a soil batch microcosm.
The soil was contaminated with 0.4% (w/w) diesel, and the amount of degraded diesel was measured after 30 days of incubation at ambient temperature. Natural soil, soil without inoculum; Inoculated, soil received inoculum. *WHC: water holding capacity.

hydrocarbon degradation is not due to the general amphiphilic properties of the surfactants, but rather to specific interactions determined by the physicochemical properties of the surfactants and the bacterial cell surfaces. When combined with the current results, this implies that surfactant-solubilized hydrocarbons can be utilized when the repulsion between the charged bacterial cells and the surfactants is minimized, and the ability of bacterial cells to access surfactants is determined by their HLB values.

Diesel Oil Degradation by Consortia

Although each of the five bacterial strains degraded 40 to 50% of the supplied diesel (4%, v/v) during 6 days, the bacterial consortia composed of strain Y2G1 and other strains showed a significantly higher diesel-degrading activity than any of the single strains (Table 1). Among the constructed consortia, the combination of Y2G1/Y2K4/Y5K2 and Y2G1/Y2K4/D3K1 exhibited a diesel-degrading activity twice as high as the single strains. It would appear that assemblages of different bacterial species with limited substrate specificities offer a greater capacity to degrade multi-component hydrocarbon mixtures. The construction of microbial consortia to efficiently degrade hydrocarbon mixtures has already been successfully demonstrated in aerobic and anaerobic systems [8, 15, 44].

Table 1. Diesel degradation using different combinations of bacterial strains in liquid MSM after 7 days of incubation at 25°C.

Strains	Diesel degradation (%)	Strain	Diesel degradation (%)
1	46.0	1+2+4	72.1
1+2	76.5	1+3+4	94.4
1+4	75.3	1+3+5	93.5
1+5	82.1	1+3+4+5	70.2

1. *Pseudomonas stutzeri* Y2G1; 2. *Aquaspirillum* sp. Y1K3; 3. *Pseudomonas* sp. Y2K4; 4. *Pseudomonas* sp. Y5K2; 5. *Sphingomonas* sp. D3K1.

Degradation of Diesel in Soil Microcosms

Batch microcosms: The soil texture and organic matter content are both known to strongly influence the soil microenvironment, namely its WHC, permeability, cation exchange capacity, surface area, and adsorption capacity [31, 32, 35]. The diesel degradation in the contaminated soil was significantly affected by the moisture content of the soil (Fig. 3). The batch microcosm studies indicated that soil with a water content of 10% of its WHC showed the highest diesel-degrading activity regardless of the inoculation.

In many soil systems, contaminants are known to be unavailable due to contaminant hydrophobicity, sorption onto the soil particles, or complexation with soil organic matter [2]. The biological availability of contaminants to soil bacteria can frequently be a rate-limiting factor with regard to the biodegradation of these contaminants. In this study, surfactants were added to the contaminated soil to enhance the diesel degradation by increasing the bioavailability. As shown in Fig. 4, none of the surfactants, except for Biosolve, was as effective as in liquid tests. Several investigators have already studied the effects of surfactant addition on hydrocarbon degradation. While some research groups found that the presence of surfactants enhanced biodegradation [3, 28, 36, 43], others claim that the presence of surfactants inhibits biodegradation [17, 20, 26, 37]. Surfactants increase the solubility of hydrocarbons by forming micelles [22, 23, 34]. The surfactants begin to assemble

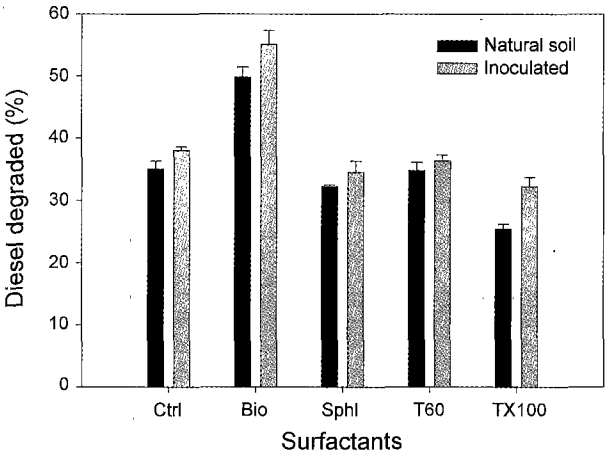

Fig. 4. Effect of various surfactants (0.01%, w/w) on diesel degradation by *P. stutzeri* Y2G1 in soil batch microcosm.
The soil was contaminated with 0.4% (w/w) diesel, and the amount of degraded diesel was measured after 30 days of incubation at ambient temperature. Ctrl, Control (soil without the surfactant); Bio, Biosolve; Sphl, Sophorolipid; T60, Tween-60; TX100, Triton X-100.

into micelles at the critical micelle concentration (CMC), and the interiors of the micelles then provide a hydrophobic environment for solubilizing nonpolar compounds, such as hydrocarbons. The marginal effect of surfactants in the current study was presumably because the concentration (0.01%) of the surfactants was too low to enhance the biodegradation, as it is already known that surfactants at a concentration below the CMC may not enhance solubility [34]. Laha and Luthy [25] also observed that the application of nonionic surfactants at a low dose does not enhance hydrocarbon degradation in a soil-water system due to the sorption of the surfactants onto the soil particles.

The enhancing effect of Biosolve did not appear to be related to the effect of the surfactant itself, but rather due to the effect of the inorganic nutrients present in Biosolve. This postulation is strongly supported by the results that the degradation of diesel by strain Y2G1 was significantly enhanced from 63.7 to 270 mg/kg/day with the addition of fertilizer in a C:N:P ratio of 100:10:3 (Fig. 5). When compared to MSM that does not have any limiting inorganic nutrients, the low level of nutrients in natural soils usually limits microbial activity. The acceleration of oil degradation by the addition of inorganic nutrients is a well-known phenomenon that has been observed in many previous studies [12, 13, 29].

While the addition of strain Y2G1 marginally enhanced the diesel degradation from 51.3 to 63.4 mg/kg/day, the addition of the consortium, Y2G1/Y2K4/D3K1, enhanced the diesel degradation rate by a factor of 1.5 (Fig. 5). This was probably due to the better ability of the consortium to maintain its metabolic activity and compete with indigenous microorganisms in the contaminated soil. Various investigators have suggested that mixed cultures of hydrocarbon degraders would be optimal as inocula for seeding because of the relatively diverse and complementary hydrocarbon-degrading capabilities [5, 18]. The diesel degradation rates due to the consortium were also highly enhanced in the presence of Biosolve or fertilizer by a factor of 1.5 and 4, respectively (Fig. 5). These observations imply that inorganic nutrients primarily limited the diesel degradation in the tested soil, whereas the presence of diesel-degrading microorganisms only limited diesel degradation to a minor extent.

Soil columns: From the column packed with soil poisoned with formalin (0.2%, v/w), about 40% of the contaminated diesel was lost (Fig. 6), probably via evaporation or chemical

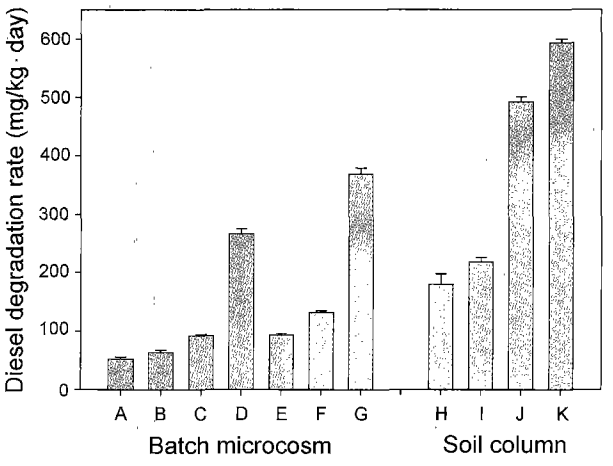

Fig. 5. Diesel degradation in soil reactors by *P. stutzeri* Y2G1 or consortium of strains, Y2G1, Y2K4, and D3K1.
The soil was contaminated with 0.4% (w/w) diesel, and the amount of degraded diesel was measured during 30 days of incubation at ambient temperature. A, Natural soil; B, Y2G1; C, Y2G1+Biosolve; D, Y2G1+Nutrient; E, Consortium; F, Consortium+Biosolve; G, Consortium+Nutrient; H, Natural soil; I, Consortium; J, Natural soil+Nutrient; K, Consortium+Nutrient.

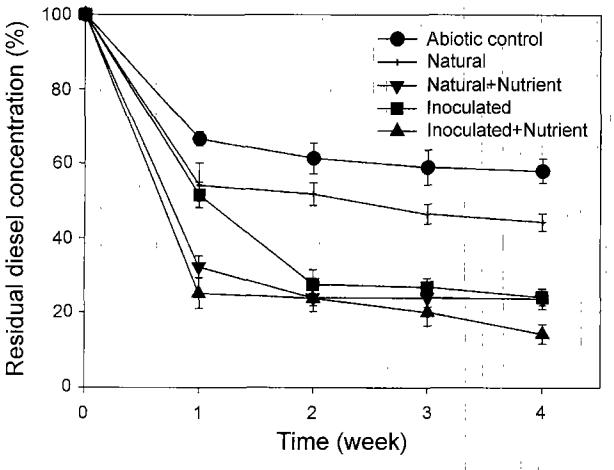

Fig. 6. Diesel degradation by *P. stutzeri* Y2G1 in soil columns.
The soil was contaminated with 0.4% (w/w) diesel, and the amount of degraded diesel was measured during 30 days of incubation at ambient temperature. The soil columns were aerated at a rate of 50 ml/min.

transformation [27]. However, in the biologically active soil columns, the diesel degradation mainly occurred during the first week at an appreciable rate and no significant degradation was observed thereafter. When compared to the degradation rates (51–369 mg/kg-soil/day) in the batch microcosms, the enhancement of the degradation rates in the soil columns (179–593 mg/kg-soil/day) was very obvious (Fig. 5). It would seem that the available oxygen limited the diesel degradation in the tested soil and bioaugmentation further increased the oxygen demand of the soil for diesel degradation. Song and Bartha [33] observed that jet fuel degradation was three to five orders of magnitude higher near the surface of soil columns than in deeper portions of the columns. The authors claimed a reduced oxygen availability as a plausible cause for the difference. The results in this study suggest that oxygen availability could have limited the diesel degradation in the batch reactors, while the aeration of the column reactors successfully mitigated any oxygen limitation. While bioaugmentation of the soil increased the total amount of diesel degraded during 4 weeks, the augmentation itself did not significantly accelerate the degradation rate (Fig. 6). However, the addition of fertilizer into both the augmented and nonaugmented soil columns significantly accelerated the initial diesel degradation, and a diesel degradation rate as high as 593 mg/kg/day (1,595 g/m^3/day) was obtained. Stimulatory effects of nutrient amendment on initial hydrocarbon degradation have also been observed in many other studies [16, 29]. The rates of biodegradation under optimal laboratory conditions and *in situ* conditions have previously been reported to be 2,500–100,000 [10] and 0.001–60 g/m^3/day [7, 9], respectively. In the current study, because the soil columns were operated at ambient temperature without any deliberate optimization of the operation conditions, the obtained removal rates could be further increased by manipulating several environmental factors.

The results of this study clearly showed that biodegradation efficiencies in diesel-contaminated soil greatly depend on the treatment strategy. As summarized in Fig. 6, the degradation of diesel was highly enhanced by introducing diesel-degrading microorganisms, especially in the form of a consortium, by the addition of inorganic nutrients, and by aeration. This study also suggests that feasibility and efficacy tests on oil-contaminated soil environments should be conducted in ways to determine the rate-limiting parameters and optimize treatment conditions for resolving these limiting parameters. Further laboratory experiments that closely model real environmental conditions or field demonstrations are necessary to establish specific bioremediation strategies.

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