The feasibility of phytoremediation combined with bioethanol feedstock production on diesel-contaminated soil

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Abstract
The purpose of this study was to identify plant species capable of cleaning up diesel-contaminated soil and then to convert their biomass to bioethanol. Five selected plant species (*Catalpa ovata, Lolium perenne, Pinus densiflora, Populus tomentiglandulosa,* and *Thuja orientalis*) were cultured on an 8,000 mg/kg area of diesel-contaminated soil to assess their remediation properties. Lignocellulosic composition, concentration of reducing sugars, and saccharification yields were analyzed. In 120 days, diesel concentration in the planted soil, with fertilizer, was significantly decreased. However, no phytoremediation activity of plant species on diesel degradation was observed over the fertilization effect. Diesel contaminated soil resulted in reduced plant biomass of most tested plants. However, biomass of *P. densiflora* was not significantly decreased in the diesel contamination plot. The reducing sugar concentration ranged from 60.5 to 83.6 mg/g, depending on the tested plant species. The highest saccharification yield was obtained with *P. densiflora*.

Key Words
Bioethanol feedstock, diesel-contaminated soil, lignocellulose, phytoremediation, TPH.

Introduction

As a result of human economic and industrial activity, massive amounts of soil and water have been contaminated with oil and petrochemical products. Total petroleum hydrocarbon (TPH) contamination is recognized as a serious threat to environmental ecosystems. TPHs are a complex mixture of chemical substances such as alkanes, aromatics and asphaltene fractions (Admon *et al.* 2001) and are very toxic to living organisms. Phytoremediation has been proposed as a cost effective, non-intrusive, and environmentally friendly technology for the restoration of soils contaminated with TPH. Furthermore, biomass generated during phytoremediation can be used for production of bioenergy such as bioethanol. Bioethanol is a non-polluting alternative fuel derived from renewable sources of plant biomass. In this study, five plants were assessed in a greenhouse experiment in terms of their effectiveness in phytoremediation and to optimize the possibility of bioethanol production the resulting plant biomass.

Materials and methods

Preparation of the experimental soil

Sandy loam soil was collected from 5 to 10 cm depth. Collected soil had the following characteristics: pH = 5.65, EC = 0.03 dS/m, NO_3^--N = 8.66 mg/kg, NH_4^+-N = 1.9 mg/kg, P = 8.51 mg/kg, organic matter = 0.8%, CEC = 1.9 $cmol_c$/kg. The initial concentration of diesel in the experimental soil was set at 8,000 mg/kg. To ensure soil/diesel mixture homogeneity, multiple soil TPH analyses were carried out.

Plant materials and growing conditions

Five selected plants (*Catalpa ovata, Lolium perenne, Pinus densiflora, Poplar tomentiglandulosa,* and *Thuja orientalis*) were tested in a greenhouse for 120 days. These plant species were selected based on a previous study. The pots had an inside diameter of 23cm and a height of 25cm and were filled with 6 kg of diesel contaminated soil. Each pot also contained 5g of commercial compound fertilizer (NPK 21-17-17). Seedlings of the selected plant species were transplanted into the pots at one plant per pot. In the case of *L. perenne*, 50 seeds were sowed per pot. Control pots contained no plants, only contaminated soil with a) fertilizer application (5 g NPK 21-17-17) or b) no fertilizer application. Soil moisture was maintained at field capacity during the experiment. Each experiment was replicated three times.

Sampling and analysis
Three soil samples were collected with a soil auger from each pot at 0, 30, 75, and 120 days and deposited into amber glass jars at for analysis. Diesel levels in the experimental soils were determined by measuring TPH by GC-MS. Plant materials were harvested at 120 days after the start of the experiments, and dried at 75°C for 3 days. Shoot and root biomass was determined on a dry weight basis.

Lignocellulosic content of plant raw materials
The cellulose, xylan and lignin content of the biomass was determined by a two step H_2SO_4 hydrolysis method. Each sample (300 mg) of dried biomass was hydrolyzed in 3 mL of 72% (w/w) H_2SO_4 at 30°C for 1 hour. The mixture was diluted by adding 84 mL of distilled water, and further hydrolyzed at 121°C for 1 hour. The hydrolysis solution was filtered through preweighed filtering crucibles. The crucibles and insoluble lignin residue were dried at 105°C for 4 hours, and then burnt into ash in a muffle furnace at 575°C for 24 hours. Recorded weights of the residue and crucible before and after burning were used to calculate the concentration of insoluble lignin, according to the method from Sluiter *et al* (2004). The filtrate was captured to analyze the concentration of soluble lignin, glucose, and xylose. The concentration of soluble lignin in the hydrolysis liquor was calculated from the absorbance value of the sample at 320 nm (Sluiter *et al.* 2004). Glucose and xylose were analyzed using an HPLC instrument equipped with an ELSD detector. Sugars were separated on a Shodex Sugar SP0810 column at 30°C with 70% acetonitrile as an eluent, at a flow rate of 0.5 mL/min.

Saccharification experiment using cellulase
A hydrolysis mixture consisting of 0.2% biomass, 40 FPU cellulase (Celluclast 1.5L, Novozyme) per gram substrate, and 10 mL of sodium acetate buffer (pH 5.0) was incubated at 37°C in a rotary shaker at 150 rpm. Samples were taken from the reaction mixture at different time intervals and heated to 100°C immediately to denature the enzyme. Samples were cooled and then centrifuged for 10 min at 8000 rpm. Reducing sugars in the supernatant were determined using the 3, 5-dinitrosalicylic acid (DNS) method. Saccharification yield was calculated from the following equation: % Saccharification = reducing sugars × 0.9 × 100/carbohydrates in substrate. Cellulase activity was determined by the filter paper unit (FPU) method (Wood and Bhat 1988). (One FPU is defined as the amount of enzyme that releases 1 µmol of glucose equivalent from Whatman No.1 filter paper per minute.)

Results
At 120 days after the start of the experiment, the initial 8,000 mg·/kg diesel concentration had decreased to a range of 659.3 to 1,240.5 mg·/kg in the planted pots with the fertilization treatment, whereas a diesel concentration of 2,946.7 mg·/kg remained in the unplanted control pot without fertilizer application (Figure 1). In the control pots, decomposition of diesel was most marked with NPK fertilizer. When the NPK fertilization was used in the diesel contaminated pots, diesel levels were dramatically decreased regardless of the presence of plants. Much research has also reported that fertilizer application can positively influence diesel degradation by enhancing plant growth and microbial degradation (Merkl *et al.* 2005; Pichtel and Liskanen 2001).

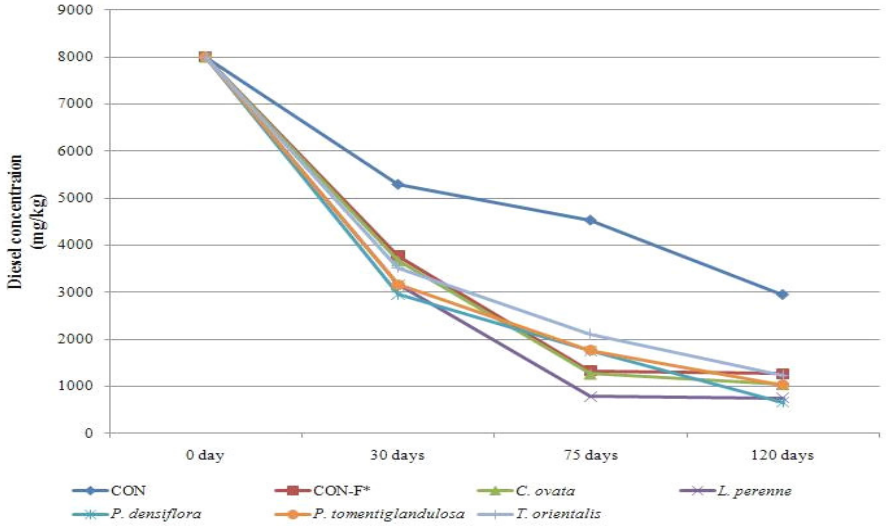

Figure 1. Effectiveness of TPH removal by two unplanted control pots and planted pots. Data were generated from TPH analysis of the soil samples collected after 120 days of phytoremediation.
*** Means unvegetated plot with fertilizer application.**

Plants do not normally grow in the harsh physical and chemical conditions, such as severely contaminated area (White *et al.* 2003). In this study, the changes in plant biomass depended on the plant species growing in the diesel-contaminated soil (Table 1). Most of tested plants were damaged by growth in 8,000 mg/kg diesel contaminated pots. In 120days, *L. perenne* had significantly lower shoot and root biomass in the diesel contaminated soil. In the case of *C. ovata* and *P. tomentiglandulosa*, shoot biomass in the diesel contaminated plot was not significantly decreased, but root biomass was severely decreased compared with uncontaminated plot. *P. densiflora* appeared to have a good tolerance of diesel contaminants, because the biomass of this plant was not significantly affected compared with the uncontaminated plot.

Table 1. Influence of diesel concentration on shoot and root biomass following a 120-day greenhouse study.

Plant species	Diesel concentration (mg/kg)	Parameters (g/pot)	
		Shoot biomass	Root biomass
C. ovata	0	32.29 ± 8.22	27.55 ± 13.4
	8,000	29.10 ± 7.54	7.05 ± 3.12
L. perenne	0	23.46 ± 2.69	19.55 ± 5.73
	8,000	8.21 ± 1.26	7.78 ± 2.24
P. densiflora	0	17.09 ± 5.23	5.23 ± 0.47
	8,000	16.06 ± 6.58	8.12 ± 0.12
P. tomentiglandulosa	0	30.61 ± 2.60	8.84 ± 0.07
	8,000	25.00 ± 1.98	4.78 ± 0.51
T. orientalis	0	7.84 ± 2.10	3.57 ± 1.55
	8,000	4.72 ± 1.53	2.98 ± 0.71

The biological process for converting the lignocellulose to bioethanol is an attractive technique to utilize its energy. The lignocellulosic composition varied among the plant species chosen for this study (Table 2). In all of the woody species, lignin was the major component, followed by cellulose and hemicellulose. However, in *L. perenne*, a kind of grass, there was a higher fraction of hemicellulose than in the woody species. *P. tomentiglandulosa* and *T. orientalis* had the highest content of cellulose.

Table 2. Lignocellulosic composition of the plant materials.

Plant species	Lignocellulose (g/100g)	Cellulose (g/100g)	Hemicellulose (g/100g)	Lignin (g/100g)
C. ovata	90.5	26.6	6.2	57.7
L. perenne	84.9	16.6	29.3	39.0
P. densiflora	88.4	33.0	7.2	48.2
P. tomentiglandulosa	83.8	37.2	3.0	43.6
T. orientalis	88.3	37.2	8.3	42.8

The reducing sugar concentrations and saccharification yields following hydrolysis of plant materials are given in Table 3. The lowest saccharification yield and reducing sugar concentration were obtained from *L. perenne* (7.56%). This may be due to its low content of cellulose. The highest saccharification yield was achieved for *P. densiflora* (10.4%). Although the cellulose contents of *P. tomentiglandulosa* and *T. orientalis* were higher than that of *P. densiflora*, the saccharification yield obtained for these were lower than that obtained for *P. densiflora*. This was probably due to the rigid structure of the lignin, which protects cellulose and hemicellulose against enzymatic hydrolysis (Béguin and Aubert 1994; Krisztina *et al.* 2009).

Table 3. Reducing sugar concentration and saccharification yield following the hydrolysis of plant materials.

Plant species	Reducing sugar (mg/g-substrate)	Saccharification yield (%)
C. ovata	78.1	9.76
L. perenne	60.5	7.56
P. densiflora	83.6	10.4
P. tomentiglandulosa	62.1	7.76
T. orientalis	71.5	8.94

Conclusion

The present study demonstrated that the combination of an adequate nutrient amendment, such as NPK fertilization, and the selection of highly tolerant plant species are key factors in successful phytoremediation. The efficiency of removing diesel in vegetated treatments with fertilization was as high as 85% while that in the corresponding unplanted control without fertilization was only 63%. The efficiency of remediation in

diesel contaminated soil was not significantly different between planted pots and unplanted pots when fertilizer was applied to diesel contaminated soil. This effect was considerable given the small pot volume and nutrient competition with microbials. The plants tested in this study were able to grow in 8,000 mg/kg diesel contaminated soil, but experienced seriously reduced shoot and/or root biomass, with the exception of *P. densiflora.* The reducing sugar concentrations and saccharification yields to produce bioethanol were varied among the plant materials. The highest saccharification yield was obtained on *P. densiflora*. This study is currently being expanded to field scale studies in order to assess phytoremediation of diesel contaminated areas and to optimize saccharification processes using plant biomass generated under these conditions.

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