Fuel 116 (2014) 292–298

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

Phytoremediation of diesel-contaminated soil and saccharification of the resulting biomass

Sujit Sadashiv Jagtap [a,1], Seong Min Woo [b,1], Tae-Su Kim [a], Saurabh Sudha Dhiman [a], Dongwook Kim [b,*], Jung-Kul Lee [a,*]

[a] Department of Chemical Engineering, Konkuk University, Seoul 143-701, South Korea
[b] Phygen Research Institute, Daejeon, South Korea

HIGHLIGHTS

- The three plant species are grown in 6000 mg/kg diesel-contaminated soil.
- The addition of a microbial consortium and fertilizer increases remediation efficacy.
- It reports saccharification of woody biomasses using lignocellulases from *Armillaria gemina*.
- *A. gemina* can be a good option for reducing sugar production from woody biomasses used for phytoremediation.

ARTICLE INFO

Article history:
Received 16 January 2013
Received in revised form 10 June 2013
Accepted 7 August 2013
Available online 22 August 2013

Keywords:
Fungi
Diesel-contaminated soil
Phytoremediation
Plant
Saccharification

ABSTRACT

In this study, we aimed to identify plant species capable of remediating diesel-contaminated soil and to convert their biomass to bioethanol. Three plant species (*Pinus densiflora*, *Populus tomentiglandulosa*, and *Thuja orientalis*) were grown on an area of soil contaminated with 6000 mg/kg of diesel to assess the effects of addition of a microbial consortium and fertilizer on remediation efficacy. Diesel-contaminated soil resulted in reduced plant biomass for most of the tested plants. However, in diesel-contaminated *P. densiflora* pots containing the microbial consortium, shoot biomass was greater than that in pots treated with diesel alone. Additionally, fertilizer application was found to be the most important factor for efficient diesel degradation. Plant biomass in diesel-contaminated soil was pretreated and used as a substrate for hydrolysis using lignocellulases from *Armillaria gemina*, a newly isolated fungal strain. The strain showed the highest β-glucosidase (15 U/mL), cellobiohydrolase (34 U/mL), endoglucanase (146 U/mL), endoxylanase (1270 U/mL), laccase (0.16 U/mL), mannanase (57 U/mL), lignin peroxidase (0.31 U/mL) and filter paper (1.72 U/mL) activities. The highest saccharification yield was obtained with *P. densiflora* (52%). The *A. gemina* enzymes hydrolyzed the woody biomass used for phytoremediation and resulted in a high level of reducing sugar (375 mg/g-substrate).

1. Introduction

Massive amounts of soil and water have been contaminated with total petroleum hydrocarbons (TPHs), including diesel and petrochemical products, because of economic and industrial activities. TPHs are a complex mixture of chemical substances such as alkanes, aromatics, and asphaltene fractions [1]. Diesel fuel obtained by the distillation of crude oil has a carbon range between C_8 and C_{26} [2] with high content of polyaromatic hydrocarbons (PAHs) [3]. Diesel is more toxic to plant than is crude oil, because of diesel's high light-hydrocarbon content. Although diesel is a commonly used fuel for vehicles and machinery, it is recognized as a serious threat to ecosystems. Many soil remediation technologies, including soil washing and thermal treatment, are applied to petroleum contaminated soil. However, these physical and chemical technologies destroy soil ecology and are very expensive. Phytoremediation has been proposed as a cost effective, non-intrusive, and environmentally friendly technology for the restoration of soils contaminated with TPH. Moreover, since phytoremediation is an in situ method, it may cause only minimal damage to the soil environment during the remediation process.

Many field and greenhouse studies have assessed phytoremediation of TPH contaminants [4,5]. Plants enhance water infiltration and oxygen diffusion deep in the soil and have a positive impact on the diversity of the rhizosphere microflora [6,7]. A range of organic

* Corresponding authors. Tel.: +82 42 822 4864; fax: +82 42 822 4865 (D. Kim), tel.: +82 2 450 3505; fax: +82 2 458 3504 (J.-K. Lee).
E-mail addresses: dwsoil88@daum.net (D. Kim), jkrhee@konkuk.ac.kr (J.-K. Lee).
[1] These authors equally contributed to this manuscript.

0016-2361/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fuel.2013.08.017

compounds that exude from plant roots stimulate microbial activity [8]. Vigorous microbial activity in the rhizosphere may enhance the degradation of TPH contaminants. However, because of competition for nutrients between microorganisms and plants, sufficient nutrient support is required. Furthermore, fertilizer management is recommended in order to optimize plant growth and microbial activity in the contaminated area.

As a further benefit, biomass generated during phytoremediation can be used for production of bioenergy such as bioethanol. Bioethanol is a non-polluting alternative fuel derived from plant biomass, a renewable resource. The enzyme system for the conversion of cellulose to glucose involves at least three types of cellulases, including endoglucanase (EG, E.C.3.2.1.4), cellobiohydrolase (CBH, E.C.3.2.1.91), and β-glucosidase (BGL, 3.2.1.21) [9]. In lignocellulose, the linear cellulose polymers are highly crystalline and are usually surrounded by lignin and xylan; this reduces their accessibility to hydrolytic enzymes. Several pretreatment techniques to remove lignin and xylan have been used to increase the hydrolysis of lignocellulosic biomass: enzymes, dilute acid, ammonia recycle percolation, lime, steam explosion [10], and alkaline and acidic wet oxidation [11]. Biodegradation of lignin and xylan requires laccase (EC.1.10.3.2), lignin peroxidase (EC. 1.11.1.14), endo-1,4-β-D-xylan xylanohydrolase (EC.3.2.1.8), β-xylosidase (EC.3.2.1.37), and several other accessory enzymes [12]. Enzymatic saccharification of cellulosic biomass has been considered to be an eco-friendly method that replaces conventional chemical treatments.

In this study, a microbial consortium was used to enhance the degradation of organic pollutants and enhance plant growth in the phytoremediation system. The microbial consortium consisted of *Stenotrophomonas acidaminiphila* sp. nov. and *Pseudomonas putida* (Trevisan) Migula. These microorganisms are reported to degrade TPH effectively [13,14]. Plants were selected mainly on the basis of pollutant resistance, plant biomass, and capacity for pollutant degradation. In the present study, *Pinus densiflora*, *Thuja orientalis*, and *Populus tomentiglandulosa* were evaluated for their ability to degrade diesel. They were also evaluated as feedstock for the production of reducing sugar by using lignocellulases from *A. gemina*.

2. Materials and methods

2.1. Preparation of experimental soil

The soil materials used in the greenhouse study were air-dried for 7 days and then sieved through a 2 mm mesh sieve. The 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 cmolc/kg. In the previous reports, about 6900 mg/kg of a diesel-contaminated field at a transport company was used for a biodegradation test using a hot-air injection method [15]. Therefore, commercial diesel fuel (GS Caltex, South Korea) was mixed with the soil at an initial concentration of 6000 mg/kg for this study. To ensure the homogeneity of the soil/diesel mixture, multiple soil TPH analyses were performed. Prior to planting, the diesel-contaminated soil was stabilized for 2 weeks to ensure the evaporation of the unstable components in the diesel.

2.2. Plant materials and growing conditions

The pots had an inside diameter of 48 cm and a height of 65 cm and were filled with 70 kg of diesel-contaminated soil (D). Three plant species (*P. densiflora* (Pd), *T. orientalis* (To), and *P. tomentiglandulosa* (Pt)) were grown on the contaminated soil in a greenhouse for 150 days. Strains of the bacteria *S. acidaminiphila* (ATCC 21910) and *P. putida* (Trevisan) Migula (ATCC 39270) were purchased from the American Type Culture Collection (ATCC). The two bacterial strains were cultured as recommended by the ATCC and combined in a 1:1 ratio. The microbial consortium (M) was applied to the soil in the pots at a concentration of 10^{10} cells per strain. A commercial compound fertilizer (NPK 21-17-17) was also added to the experimental soil. The fertilizer was supplied to each pot at 0 g (F), 60 g (F1), and 120 g (F2). *P. tomentiglandulosa, T. orientalis* and *P. densiflora* pots with fertilizers (PtF1, PtF2 or ToF1, ToF2 or PdF1, PdF2), treated with diesel and fertilizers (PtDF1, PtDF2 or ToDF1, ToDF2 or PdDF1, PtDF2), and treated with diesel, fertilizers, and microbial consortium (PtDF1 M, PtDF2 M or ToDF1 M, ToDF2 M or PdDF1 M, PdDF2 M) were tested for shoot and root biomass production, respectively.

2.3. Sampling and analysis

Three replicate soil samples were collected from each treatment every 50 days. The soil samples were collected from the rhizosphere region and stored in a refrigerator at 4 °C, until they were measured. At the end of the experiment, shoot and root biomass and diesel concentration were recorded (TPH analysis, PLFA, bacterial biomass, etc.).

2.4. Lignocellulosic content and pretreatment of plant biomass

The cellulose, xylan, and lignin content of the biomass was determined using 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 h. The mixture was diluted by adding 84 mL of distilled water and further hydrolyzed at 121 °C for 1 h. The hydrolysis solution was filtered through preweighed filtering crucibles. The crucibles and insoluble lignin residue were dried at 105 °C for 4 h and then burnt to ash in a muffle furnace at 575 °C for 24 h. Recorded weights of the residue and crucible before and after burning were used to calculate the concentration of insoluble lignin, according to the method described by Sluiter et al. [16]. The filtrate was collected 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. Glucose and xylose were analyzed using high-performance liquid chromatography (Dionex model Ultimate 3000 Standard, DIONEX, Sunnyvale, CA) equipped with an evaporative light-scattering detector (ChromaChem ELSD detector, ESA Inc., Sunnyvale, CA). Sugars were separated using a Shodex sugar column (SP0810, 8 × 300 mm) at 30 °C with 70% acetonitrile as an eluent, at a flow rate of 0.5 mL/min.

Ten grams (dry weight) of solid material and 60 mL of aqueous sodium hydroxide (2.0%) were added to 200-mL Erlenmeyer flasks with a solid to liquid ratio of 1:6. Sodium hydroxide pretreatment was performed in an autoclave at 121 °C for 80 min. After the reaction ended, the solid residue was washed with distilled water several times. The sodium hydroxide pretreated samples were stored at 4 °C for enzymatic hydrolysis [17].

2.5. Isolation and identification of a new fungal strain

Soil samples were collected from the Osaek Hot Springs trail head located in Sorak Mountain (South Korea) by using capillary tube methods. Samples were diluted in sterile dilution solution (0.9% saline), aliquots were spread on potato dextrose agar plates, and plates were incubated for 3 days. Approximately 1560 isolates were screened for cellulase activity by using the Congo red agar plate technique [18]. Plates were prepared with medium containing 14 mL $(NH_4)_2SO_4$ (10%), 15 mL KH_2PO_4 (1 M), 6 mL urea