International Biodeterioration & Biodegradation 87 (2014) 99–105

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

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

Diesel degradation potential of endophytic bacteria isolated from *Scirpus triqueter*

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ARTICLE INFO

Article history:
Received 3 July 2013
Received in revised form
11 November 2013
Accepted 12 November 2013
Available online 6 December 2013

Keywords:
Scirpus triqueter
Diesel
Degradation
Endophyte
Biosurfactant

ABSTRACT

Scirpus triqueter, a dominant species in wetland of Huangpu-Yangtze estuary can be used for phytoremediation. Endophytic bacteria from *S. triqueter* were investigated to evaluate the ability to degrade diesel. Bacterial incubation experiments were established to screen diesel degradation of endophytic microorganisms from *S. triqueter*. The oil-degrading bacterial strain J4AJ was discovered in vivo of *S. triqueter* and identified as *Pseudomonas* sp. U-3 bacterial strain also isolated from the root and stem of *S. triqueter* was identified to be the potent producer of biosurfactant as *Bacillus subtilis*. The minimum surface tension of U-3 was 30.9 mN/m. The bacterium manifested outstanding adaptation ability at extremes of temperature, pH and salinity. The removal ratio of J4AJ could be greatly improved by adding U-3 strain. However, the degradation ratio of J4AJ was obviously decreased by the addition of Alkyl Polysaccharide Glycoside (APG). The results suggested that J4AJ and U-3 inoculated were conducive to the degradation of diesel, which can be used for the remediation of oil-contaminated ecological environment.

1. Introduction

Diesel is a toxic mixture which consisted of paraffin, cyclic alkenes and aromatic compounds (Chandran and Das, 2011). Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of soil, surface water and ground water pollution (Moliterni et al., 2012). Many techniques of remediation of diesel contaminated soils have been developed, such as mechanical, chemical and biological degradation (Chandran and Das, 2011). Biodegradation is an efficient, environment-friendly, and cost-effective technology for both ex situ and in situ remediation of environments contaminated by hydrocarbons (Eapen et al., 2008; Eullaffroy et al., 2009; Liu et al., 2011). Proliferation of petroleum-degrading microorganisms can reduce available contaminants (Radwan et al., 2005; Davies et al., 2008). Current efforts are now focused on the microorganisms to restoration of oil-contaminated soil. Li et al. (2009) screened *Rhodococcus* sp. Moj-3449 to degrade alkanes with fast rate and high concentration.

Plant-endophyte is a type of non-pathogenic bacteria living inside healthy or symptomless plant tissues (Ho et al., 2012). Their associations rely on very close beneficial interactions where plants provide nutrients and residency for bacteria, which in exchange can directly or indirectly improve plant growth and health (Weyens et al., 2010). Germaine et al. (2006) found that the inoculation of a model plant, the pea (*Pisum sativum*), with a genetically tagged bacterial endophyte that naturally possesses the ability to degrade 2,4-dichlorophenoxyacetic acid. Endophytic inoculants have also been shown to transfer their degradative plasmids to other endophytes, thus increasing the overall degradation (Phillips et al., 2008). Endophytic bacteria such as *Pseudomonas*, *Arthrobacter Enterobacter* and *Bacillus strains* were isolated from the root, stem and leaf of two cultivars of poplar tree growing on a site contaminated with BTEX compounds (Moore et al., 2006).

Surfactant amendment may enhance the oil mobility and increase its availability, improving the biodegradation rates (Bordoloi and Konwar, 2009). Biosurfactants have gained increasing popularity in soil bioremediation. They are more environmentally friendly and efficient alternatives than synthetic surfactants, due to their excellent physicochemical properties and considerably lower toxicity. They can be produced from renewable sources by numerous microorganisms, which contributed to a large variety of biosurfactant, such as rhamnolipid, lipopeptide, phospholipid and so on. Desorption and solubility of hydrocarbons can be improved by biosurfactants, but it is not easy to comprehend their processing on bioavailability and biodegradation. Allen et al. (1999) found triton X-100 increased the rate of oxidation of the polycyclic

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http://dx.doi.org/10.1016/j.ibiod.2013.11.007

aromatic hydrocarbons (PAHs) with strain 9816/11 with the effect being most noticeable when phenanthrene was used as a substrate. However, the surfactant inhibited the biotransformation of both naphthalene and phenanthrene with strain B8/36 under the same conditions. Stelmack et al. (1999) indicated that the absorption and biodegradation of creosote were inhibited by surfactants because surfactants may simultaneously reduce the adhesion of bacteria to hydrophobic compounds.

Scirpus triqueter could survive in oil-contaminated wetland of Huangpu-Yangtze estuary. Liu et al. (2011) found that the removal ratio of diesel in *S. triqueter* planted soil was 16.51% higher than that in control soil. Moreover, the presence of S. *triqueter* could enhance the dissipation ratios of pyrene (Zhang et al., 2011). However, there was no study about the endophytic bacteria of *S. triqueter*. The objectives of this study are: 1) to isolate and identify endophytic diesel-degrading and biosurfactant producing bacteria from S. *triqueter*; 2) to analyze the stability of biosurfactant at various extreme conditions; 3) to ascertain the effect of diesel-degraded bacterium combined with biosurfactant producer bacterium or surfactants on diesel degradation; 4) to investigate the degradation characteristics of diesel under different treatments.

2. Materials and methods

2.1. Plant and materials

Scirpus triqueter, as the experiment plant was taken from the wetlands of Huangpu-Yangtze River Estuary, China. The rhizomes of *S. triqueter* were dug up carefully by using a garden spade. They were planted in diesel (10,000 mg/kg) contaminated soils and cultured in phytotron.

Diesel sample, No. 0, was purchased from Minghe petrol station of Sinopec in Baoshan district, Shanghai. All reagents were analytical grade and were purchased from sinopharm chemical reagent company, China.

2.2. Isolation of endophytic diesel-degraded and biosurfactant producer bacteria from S. triqueter

Healthy roots and stems of *S. triqueter* were collected from diesel (10,000 mg/kg) contaminated phytoremediation site located in the phytotron of Shanghai University, China. The bacteria were isolated from healthy roots and stems of *S. triqueter*. Endophytic bacteria isolating was based on the method of Phillips et al. (2008). The roots and stems of *S. triqueter* were cleaned with tap water. For isolated endophytic bacteria, rinsed roots and stems were surface disinfected by sequential washes with 75% ethanol (30 s) and 1.0% sodium hypochlorite (1 min), followed by a minimum of 5 rinses with sterile water. To assess surface sterility, 100 μL aliquots of the final rinse water were spread on nutrient agar (NA) plates. The sterile roots and stems were cut into small pieces and endophytic extracts were produced in 9 mL monopotassium phosphate (MPP) buffer (0.65 g K_2HPO_4, 0.35 g KH_2PO_4, 0.10 g $MgSO_4$ L^{-1} water) using a sterile mortar and pestle. The roots and stems extract were serially diluted in MPP and these 10-fold dilutions were used for culturable endophytic bacteria. The endophytic bacteria growing out from the extracts were cut by a sterile transferring loop and transferred to a new NA plate. The colonies were purified and stored at 4 °C.

Isolation of diesel-degraded bacteria were examined using the mineral salt medium (MSM) (K_2HPO_4 2.0 g, KH_2PO_4 0.5 g, NaCl 0.5 g, NH_4Cl 0.5 g, $MgSO_4$ 0.2 g, $CaCl_2$ 10 mg, $CaCl_2$ 15 mg, $FeSO_4 \cdot 7H_2O$ 2 mg, $Cu_2SO_4 \cdot 5H_2O$ 0.4 mg, KI 1 mg, $MnSO_4 \cdot H_2O$ 4 mg, H_3BO_3 5 mg, $CoCl_2 \cdot 6H_2O$ 1 mg, $Na_2MoO_4 \cdot 2H_2O$ 2 mg, $NiCl_2 \cdot 6H_2O$ 2 mg, H_2O 1 L, pH 7.0 ± 0.2) with diesel as the sole carbon and energy source (Zhao et al., 2009). The cultivation was carried out on rotary shaker (120 rpm) at 28 °C. After 7 days of incubation, 10 mL of bacterial suspension was transferred to a fresh MSM containing a higher concentration of diesel and incubated under the same conditions. The process was repeated several times with diesel concentration increasing. When no apparent growth of bacteria was observed in the MSM, the diesel concentration was considered as the highest diesel concentration tolerated by the tested bacteria. The size and color of the isolated colonies were recorded. Pure bacterial strains obtained were kept on LB (10.0 g NaCl, 10.0 g peptone, 5.0 g yeast extract, with or without 20 g agar in 1 L distilled water, and the solution pH was adjusted to 7.0.) slant culture at 4 °C. All the media were sterilized in autoclave at 121 °C for 20 min before use. J4AJ was chosen as diesel-degraded bacterium according to the highest removal ratio of diesel.

Biosurfactant producer bacteria were detected by oil spreading method (Anandaraj and Thivakaran, 2010) and surface tension determination of inoculum. The screened strains above were cultured in beef extract peptone medium on rotary shaker (50 rpm) at 28 °C for 3 days. Two hundred μl of bacterial suspension was transferred to 5 ml MSM, which was incubated on rotary shaker (80 rpm) at 28 °C for 4 days. The supernatant was used to determine oil spreading and surface tension. Maximum biosurfacant producing bacterium U-3 was selected as biosurfactant producer bacteria. The diesel-degraded bacterium (J4AJ) and the biosurfactant producer bacterium U-3 were identified by the genomic DNA (Zhang et al., 2009; Liu et al., 2011) (Shanghai Genecore Biotechnologies Company).

For the 16s rDNA sequencing the PCR analysis was performed as follows: universal 16S rRNA eubacterial primer (3′-AGAGTTTG ACCTGGCTCAG-5′, 5′-GGTTACCTTGTTA CGACTT-3′) was used for the amplification of DNA. The polymerase chain reaction was carried out on a thermal cycler (ABI 9700) in a 50 μl reaction mix. The reaction mix contained 10 × amplification buffer (5 μl), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 1 μl dNTP, and 0.25 μl Taq polymerase. After initial denaturation at 98 °C for 5 min, amplification was performed with 35 cycles of 35 s at 95 °C, 35 s at 55 °C, 1.5 min at 72 °C followed by a final extension at 72 °C for 8 min. PCR products were cloned using the pUCm-T vector and sequenced by ABI 3730 DNA sequencer. The 16s rDNA gene sequence was obtained by comparing with other bacterial sequences in NCBI database.

2.3. Stability analysis of biosurfactant

The stability of the biosurfactant at extremes of physical parameters like temperature (0, 40, 60, 80, 100 and 121 °C), pH (2, 4, 6, 8, 10 and 12), salinity (0%, 10%, 20%, 30%, 40% and 50%) were evaluated utilizing a digital tensiometer (Xia et al., 2011). LB medium without U-3 at the same proceedings was used as control sample (CK). All of the experiments were repeated thrice and the average values were taken as results in order to ensure its reproducibility.

2.4. Experimental design of diesel biodegradation

All experiments were carried out in a 100 ml Erlenmeyer flask containing 40 ml MSM supplemented with diesel (6000 mg/L) as the sole carbon and energy source (Zhao et al., 2009). J4AJ was inoculated in MSM containing 6000 mg/L of diesel on rotary shaker (120 rpm) at 28 °C. When the bacterial growth entered the late exponential phase, they were collected by centrifugation and resuspended in 0.9% sterile saline. 1% (v/v) of bacterial suspension was used as inoculums in all experiments.

The treatments in this trial were as follows: contaminated MSM without any bacteria (C, this treatment was set as blank test),