International Biodeterioration & Biodegradation 97 (2015) 135–142

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

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

Rhizosphere as a tool to introduce a soil-isolated hydrocarbon-degrading bacterial consortium into a wetland environment

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ARTICLE INFO

Article history:
Received 16 April 2014
Received in revised form
7 November 2014
Accepted 10 November 2014
Available online 27 November 2014

Keywords:
Acorus calamus L.
Bacterial consortium
Diesel oil
Rhizosphere
Sweet flag

ABSTRACT

An attempt to incorporate a soil-isolated bacterial consortium capable of degrading hydrocarbons into the sweet flag rhizosphere was made in order to introduce the plant-bacteria combination into a wetland environment. The colonization of the rhizosphere occurred after a diesel oil degradation trial, where the plant was inoculated with the bacterial consortium. Both plant and bacterial growth were monitored. The 16S rRNA gene sequences method was used to verify the presence of the soil-isolates in the plants internal tissues and TTC-dehydrogenase measurements were employed to assess the microbial activity. The incorporation of bacterial cells into the sweet flag rhizosphere resulted in an increased diesel oil removal efficiency (by 50%) and enhanced bacterial and plant growth. Afterwards, the colonized sweet flag rhizomes were planted within a lake shoreline to evaluate the long-term prevalence of microorganisms in the natural environment. The bacterial DNA of the oil-degrading consortium species was detected in the harvested plants even after 8 months.

Introduction

An increasing interest in the field of bioaugumentation has been observed over the past years. Introduction of highly specialized microbes into contaminated sites has been recognized as a potentially promising strategy to improve the efficiency of bioremediation processes (Gentry et al., 2004a; Fantroussi and Agathos, 2005; Hosokawa et al., 2009). This approach is especially useful when the abundance of relevant genes among the members of indigenous microbial community is unsatisfactory. However, despite the growing number of reports describing potentially efficient degrading species, there are still many issues concerning this subject (Thompson et al., 2005). The field studies attempting to intensify the biodegradation efficiency through the introduction of highly-specialized strains into the environment have often ended in failure (Bouchez et al., 2000; Wagner-Döbler, 2003). Usually, the carefully selected microorganisms, very efficient in laboratory studies, presented little usefulness during field studies, if any. Several factors, both biotic and abiotic, are regarded as crucial for the application of bioaugumentation techniques. This includes competition with indigenous microflora, microbial predation and various environmental parameters (Gentry et al., 2004b). In order to successfully enhance the catabolic capabilities of degrading species it is important to minimize the influence of these limiting factors.

Numerous studies have shown that the presence of plants may contribute to increased growth of hydrocarbon degraders (Kirk et al., 2005; Kim et al., 2006; Zhuang et al., 2007; Gaskin et al., 2008). Simultaneous use of specific microorganisms and plants in an attempt to increase the bioremediation efficiency is known as 'rhizoremediation' (Kuiper et al., 2004). The occurrence of specific plant-bacteria interactions are fundamental for this phenomenon. The plants stimulate the growth of selected bacteria, either by the release of root exudates or by direct recruitment of endophytic species (Segura et al., 2009), while the microorganisms protect the plant from the toxic pollutant or contribute to increased plant growth (Hayat et al., 2010). Rhizoremediation has been extensively reviewed and this approach seems potentially promising for

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http://dx.doi.org/10.1016/j.ibiod.2014.11.006
0964-8305/

removal of organic contaminants (Chaudry et al., 2005; Gerhardt et al., 2009) as well as heavy metals (Lebeau et al., 2008; Ma et al., 2011). Several studies, such as those carried out by Siciliano et al. (2001, 2003) and Palmroth et al. (2005, 2007), demonstrated that some plants are capable of increasing the prevalence of catabolic genes involved in hydrocarbon degradation in the contaminated area. Moreover, numerous recent reports also show that some plants may exhibit a high potential for phytoremediation of petroleum hydrocarbons (>90%) (Agamuthu et al., 2010; Abioye et al., 2012; Dadrasnia and Agamuthu, 2013a,b). Taking this into account, it can be hypothesized that the use of specific plant-bacteria set-ups may potentially allow the microorganisms to overcome the environmental obstacles, result in their successful introduction into otherwise unfavorable niches and improve the overall removal efficiency of petroleum contaminants.

Most of the before-mentioned studies concerning the rhizoremediation of petroleum compounds were performed with the use of terrestrial plant species such as *Medicago sativa* L., *Lolium perenne, Brachiaria decumbens, Cymbopogon ambiguus, Microlaena stipoides* and trees such as *Populus nigra*). This approach is strictly dedicated to remediation of contaminated soil. However hydrocarbons are also common contaminants found in aquatic and semi-aquatic environments, such as marine shorelines and wetlands (Venosa and Zhu, 2003). Fundamentally different niches require other potential plant-bacteria pairings. Therefore this study is focused on assessing the possibility of incorporating an effective diesel degrading microbial consortium, isolated from a terrestrial area, into the rhizosphere of sweet flag (*Acorus calamus* L.), a common wetland plant. Both microbial and plant growth as well as the catabolic ability were assessed to ensure the occurrence of desirable plant-bacteria interactions. The long-term survival of such strains during field studies in a wetland environment was investigated. This type of study will give an insight on the usefulness of plants as mediators for introducing highly specialized microorganisms into the environment.

Materials and methods

Chemicals

Petroleum diesel fuel, produced according to EN 590:2004, was purchased from a petrol station (PKN Orlen, Poland). Before use in experiments diesel oil was sterilized by filtration (Millex, pore size of 0.2 μm; Millipore). Rhamnolipids, a mixture of anionic surfactants, were obtained from the Jeneil Biosurfactant Company (Saukvulle, WI, USA) as commercially available product – JBR-425 (25% rhamnolipid content), which contains mainly rhamnolipid RL1 (rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) and RL2 (L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β- hydroxydecanoate). Detailed information was previously described by Chrzanowski et al. (2012).

Plants

The rhizomes of sweet flag (*Acorus calamus* L.) were received from the commercial market, removed from the pots and rinsed with distilled water several times. Afterwards, the plants were transferred to a hydroponic culture in Hoaglands medium (medium A), which consisted of (g/l): NH_3PO_4, 0.15; KNO_3, 0.61; $Ca(NO_3)_2 \cdot 4H_2O$, 0.94; $MgSO_4$, 0.24. The microelements (mg/l): H_3BO_3, 2.85; $CuSO_4 \cdot 5H_2O$, 0.08; $ZnSO_4$, 0.22; $MgCl_2 \cdot 4H_2O$, 2.31; $Na_2MoO_4 \cdot 2H_2O$, 0.01; $FeSO_4$, 2.78; Na_2EDTA, 3.72.

Sweet flag was grown at 30 °C, 20 000 lux light intensity and a 16:8 photoperiod (lighting/darkness) until the leaves were about 20 cm long. The roots were subjected to bacterial DNA studies.

Microorganisms and preparation of the microbial inoculum

The microbial consortium used throughout this study was isolated from a crude oil contaminated site in Czarna, Poland in 2006, and exhibited an excellent biodegrading potential towards diesel oil (Owsianiak et al., 2009). The consortium was identified by restriction length polymorphism of 16S rDNA gene amplicons and sequenced. The main members of the diesel-degrading consortium belonging to the following bacteria: Pseudomonas alcaligenes, Ochrobactrum intermedium, Pseudomonas stutzeri, Achromobacter xylosoxidans, Citrobacter freundi, *Sphingobacterium multivorum, Pseudomonas aeruginosa, Pseudomonas fluorescens, Rhodoccocus rube, Pseudomonas putida, Bacillus subtilis, Staphylococcus aureus.* The relation between microorganisms of consortium shows the phylogenetic analysis (Fig. 1). No surfactants exertion was reported when the microbes were cultivated on diesel oil under conditions described in this study.

The stock cultures contained 1 loop full of cells, 50 ml of medium B and hydrocarbons (1 g/l). Medium B consisted of (g/l): $Na_2HPO_4 \times 2H_2O$, 7.0; KH_2PO_4, 2.8; NaCl, 0.5; NH_4Cl, 1.0; $MgSO_4 \times 7H_2O$, 0.01; $FeSO_4 \times 7H_2O$, 0.001; $MnSO_4 \times 4H_2O$, 5×10^{-4}; $ZnCl_2$, 6.4×10^{-4}; $CaCl_2 \times 6H_2O$, 1×10^{-4}; $BaCl_2$, 6×10^{-5}; $CoSO_4 \times 7H_2O$, 3.6×10^{-5}; $CuSO_4 \times 5H_2O$, 3.6×10^{-5}; H_3BO_3, 6.5×10^{-4}; EDTA, 0.001; HCl 37% (v/v), 0.0146 ml/l. The initial pH of the medium was 6.5. After 48 h, appropriate amounts of stock cultures were used for the inoculation of the final culture, with a base optical density of the final culture set to fit 0.1, measured spectrophotometrically at 620 nm.

Cultivation of plants with the bacteria

In the next step the plants were exposed to the final culture of the selected bacterial consortium. The medium composed for this purpose consisted of the two basic media (medium A/medium B) mixed at a 1:1 ratio and was designated as medium C. The roots were subjected to bacterial DNA studies.

Phytobiodegradation assays

The laboratory phytobiodegradation experiments were performed in 500 ml Erlenmeyer flasks containing 250 ml of the medium supplemented with 1% (v/v) of hydrocarbons. A biosurfactant, rhamnolipid, was added at a concentration of 150 mg/l in order to enhance the permeability of the plant tissues to the hydrocarbons. Four sets of experiments were carried out simultaneously, with ten replicate samples per experiment:

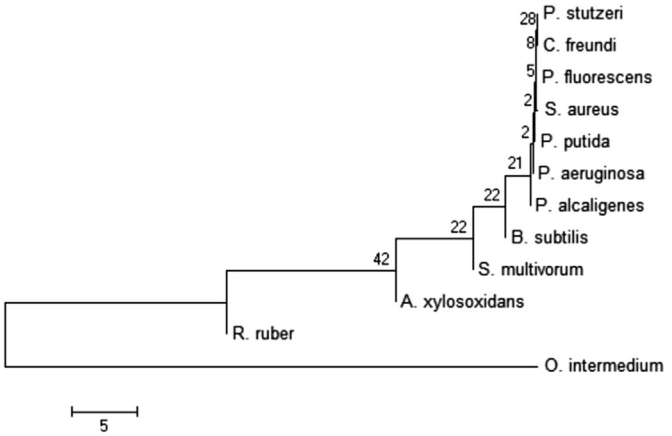

Fig. 1. Phylogenetic analysis based on 16S rRNA obtained from the microorganisms consortium used in the diesel oil biodegradation process.