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Physiological and genetic screening methods for the isolation of methyl *tert*-butyl ether-degrading bacteria for bioremediation purposes

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ABSTRACT

Bioremediation of groundwater contaminated with methyl *tert*-butyl ether (MTBE) has been widely described since their cost/efficient ratios are lower than other physic-chemical methodologies. The present study focused on the isolation and selection of MTBE degrading microorganisms from contaminated soil and groundwater samples based on results from growth on mineral media amended with MTBE and BTEX, presence or absence of the monooxygenase genes and specific ability to degrade MTBE. Three bacterial strains were selected and identified as *Rhodococcus ruber*, strains EE1 (CECT 8555), EE6 (CECT 8612) and A5 (CECT 8556), showing the ability to degrade 60.0, 36.0 and 10.0 mg l^{-1} MTBE, respectively. Moreover, all the *R. ruber* strains showed the presence of genes encoding MTBE-degrading enzymes. One isolated strain was identified as *Paenibacillus* sp. SH7 (CECT 8558) and demonstrated the greatest MTBE degradation value (100 mg l^{-1}), but together with the last strain selected and identified as *Agrobacterium* sp. MS2 (CECT 8557) did not result in positive amplification of any of the monooxygenase primers tested. The lowest toxicity (as EC_{50}) was observed after 4-days growth of *R. ruber* EE6 on MTBE-supplemented mineral medium. The potential application of these strains in bioremediation processes is discussed.

Introduction

Unleaded gasoline is a mixture of low-molecular-weight hydrocarbons (n-alkanes, isoalkanes, cycloalkanes, aromatic compounds) and various chemical additives such as ether oxygenates. Since the beginning of its consumption, the most widely used gasoline oxygenate, methyl *tert*-butyl ether (MTBE), has contaminated many water supplies worldwide largely due to its physico-chemical properties, making this substance a severe groundwater pollutant, which is mainly found together with other well-established gasoline compounds such as benzene, toluene, ethylbenzene and xylene (BTEX) mixtures within the same contaminated plumes (Schmidt et al., 2004).

Field studies agree that natural MTBE attenuation is slow and in some cases not detectable (Schirmer et al., 1998; Johnson et al., 2000). Therefore, the development of technologies to treat contaminated groundwater is of great importance. Among these, biological treatments are recognised as cost-effective and environmentally friendly options (Kharoune et al., 2001).

Numerous studies have focused on the improvement of isolation procedures and bacterial enrichment methods from different environmental samples (Mo et al., 1997; Hanson et al., 1999; Herman and Frankenberger, 1999; Hyman, 2000; Rohwerder et al., 2006; Lyew et al., 2007) using different growth media. To perform the selection of bacteria with degradation ability, several methods have been used: testing bacterial ability to grow on solid mineral media with fuel oxygenate as a sole carbon source (Fayolle et al., 1998), MTBE and *tert*-butyl alcohol (TBA) degradation testing (Hatzinger et al., 2001) and performing measurements of MTBE mineralization and utilisation by bacterial strains (Hanson et al., 1999).

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The biodegradation of ether oxygenates and *tertiary* alcohol metabolites occurs preferentially under aerobic conditions (Deeb and Alvarez-Cohen, 2000; Chen et al., 2011). The oxidation is carried out by monooxygenases as alkane hydroxylases and cytochrome P450s. Bacterial cytochrome P450s (CYPs) catalyse the O-dealkylation reactions of alkyl ethers and aralkyl ethers (Steffan et al., 1997; Hyman, 2013). Some bacterial strains use MTBE and TBA as a sole carbon and energy source for growth (Hanson et al., 1999; Hatzinger et al., 2001; Müller et al., 2008), whereas others, such as *Pseudomonas*, *Rhodococcus*, *Mycobacterium*, *Enterobacter* and *Achromobacter* are capable of degrading MTBE cometabolically but not TBA (Eixarch and Constantí, 2010; Smith and Hyman, 2010). In these cases, this metabolite often accumulates and increases the toxicity of the media. Therefore, before a certain MTBE bioremediation strategy can be used, an assessment of the risks associated with the accumulation of its breakdown products is essential.

The present study describes a precise isolation and identification methodology to obtain bacterial strains capable of using MTBE as a sole carbon and energy source based on physiological and genetic screening. Although at present the selection processes of bacteria tends to be quite short, the selection process described in this paper is much more specific and rigorous, and provides much more information about strains before more complex and expensive assays are carried out. The toxicity due to secondary metabolite accumulation in culture media tested by the Microtox® assay was a useful tool, in addition to the other assays, for determining microorganisms as potential bacterial inocula (alone or as a consortium) in several biological technologies for the treatment of fuel oxygenate-contaminated groundwater.

Materials and methods

Chemicals

All chemicals purchased were of reagent grade or of the highest purity available. The fuel ether MTBE (99.9% purity) was purchased from Sigma–Aldrich (Milwaukee, WI, USA). Benzene (99.5% purity) was purchased from Gruppo Montedison (Farmitalia Carlo Erba S.p.a, Div Analitica Milano, Italy), toluene (99.8% purity) from Lab-Scan (Analytical Sciences, Dublin, Ireland), ethyl-benzene (99% purity) from Merck Schuchard OHG (Hohenbrunn, Germany) and xylene (98.5% purity) from Panreac Quimica S.A.U. (Castellar del Vallés, Barcelona, Spain).

Growth medium

The growth medium used in the experiments was a modified mineral salts medium (FTW medium, Herman and Frankenberger, 1999) with the following composition: KH_2PO_4, 0.225 g l^{-1}; K_2HPO_4, 0.225 g l^{-1}; $(NH_4)_2SO_4$, 0.225 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.050 g l^{-1}; $CaCO_3$, 0.005 g l^{-1}; $FeCl_2 \cdot 4H_2O$, 0.005 g l^{-1}; and 1 ml of trace elements solution. Trace elements solution had the following composition: $ZnSO_2 \cdot 7H_2O$, 0.1 g l^{-1}; $MnCl_2 \cdot 4H_2O$, 0.03 g l^{-1}; H_3BO_3, 0.3 g l^{-1}; $CoCl_2 \cdot 6H_2O$, 0.2 g l^{-1}; $CuCl_2 \cdot 2H_2O$, 0.01 g l^{-1}; $NiCl_2 \cdot 6H_2O$, 0.02 g l^{-1}; and $NaMoO_4 \cdot 2H_2O$, 0.03 g l^{-1}. Moreover the medium was supplemented with 1 ml vitamin solution whose composition was: biotin 20 μg l^{-1}; folic acid 20 μg l^{-1}; pyridoxine HCL 100 μg l^{-1}; thiamine HCL 50 μg l^{-1}; riboflavin 50 μg l^{-1}; nicotinic acid 50 μg l^{-1}; pantothenate calcium 50 μg l^{-1}; p-aminobenzoic calcium 50 μg l^{-1}; lipoic acid 50 μg l^{-1} and cobalamin 50 μg l^{-1}.

For preparing solid modified FTW medium, agar–agar (16 g l^{-1}) was added.

Enrichment cultures and isolation of potential oxygenate-degrading microorganisms

To isolate microorganisms with oxygenate-degrading ability, two different methodologies were applied: direct isolation from environmental samples and isolation after subsequent enrichment steps.

Two different environmental sites were used to isolate potential MTBE-degrading microorganisms: an artificial hydrocarbon-contaminated soil within a pilot plant (Granada, Spain) (Silva-Castro et al., 2013), and a hydrocarbon-contaminated groundwater sample from a gas station at Catalonia (Spain) supplied by Repsol, S.A. For the direct isolation of oxygenate-degrading microorganisms from the contaminated soil and groundwater samples, the serial dilution technique and plating on solid-modified FTW medium supplemented with 150 mg l^{-1} MTBE were used. The inoculated plates were incubated in an MTBE-saturated atmosphere at 28 °C for seven days. The plates were observed daily and the isolated colonies were streaked onto solid-modified FTW plates for further studies. The isolation of oxygenate-degrading microorganisms after enrichment steps from the contaminated soil samples was performed using the methodology described by Purswani et al. (2008). For the isolation of microorganisms from the contaminated groundwater, 500 ml of the sample was filtered through a 0.45 μm sterile nitrocellulose filter (Millipore®). The filter was placed in a 50 ml sterile tube, to which 30 ml FTW medium was added and was then sonicated for 10 min. The supernatant was transferred to a sterile glass flask with 80 ml modified FTW medium supplemented with 500 mg l^{-1} MTBE, closed with polytetrafluoroethylene (PTFE) stoppers and incubated under controlled agitation and temperature (150 rpm and 28 °C) for three months in a rotatory shaker. Every 30 days, 10 ml of these cultures was transferred to fresh FTW medium (90 ml) supplemented with the corresponding concentration of MTBE, and was incubated under the same conditions. After three months, 1 ml aliquots of the enrichment cultures were serially diluted and spread onto solid-modified FTW plates supplemented with 200 mg l^{-1} MTBE. Plates were incubated in an MTBE-saturated atmosphere at 28 °C for 30 days. Different colony morphologies were selected and streaked onto solid-modified FTW plates with washed agar.

Pre-selection of MTBE-degrading bacterial strains

The bacterial strains isolated from soil and groundwater samples, as well as those previously isolated by Purswani et al. (2014) from the biofilm established on Bioflow 9® units within a lab-scale aerated submerged biofilter designed for the bioremediation of MTBE-contaminated groundwater samples (adapted to the fuel oxygenate and named FF2, FF5, DD1, DD6, EE1, EE5 and EE6), were tested for growth ($OD_{600\ nm}$) on modified FTW medium supplemented with MTBE and/or BTEX (benzene, toluene, ethylbenzene and xylene) as a sole carbon and energy source, using sterile 96-well plates.

Bacterial strains were pre-grown on tripticase soya broth (TSB, Difco, USA) under controlled temperature (30 °C) and agitation (150 rpm) for 4 days, followed by centrifugation of 1 ml of culture (for 1 min at 14,000 rpm). Subsequently, the pellet was resuspended in 1 ml phosphate-buffered saline solution (1 × PBS). Each well contained 90 μl modified FTW medium supplemented with MTBE and/or BTEX to reach a final oxygenate/aromatic compound concentration of 200 mg l^{-1} and was inoculated with 10 μl of bacterial suspension. The plates were kept at 28 °C in an MTBE-saturated chamber. Bacterial growth (as $OD_{600\ nm}$) was measured every 7 days in a plate reader (Fluostar Optima, BMG-Labtech) for 4 weeks. Before reading, each plate was shaken at 300 rpm for 1 min at 30 °C to prevent erroneous readings. The growth of the bacterial