Toxicity and biofilm-based selection for methyl tert-butyl ether bioremediation technology

I. M. Guisado, J. Purswani, L. Catón-Alcubierre, J. González-López and C. Pozo

ABSTRACT

Extractive membrane biofilm reactor (EMBFR) technology offers productive solutions for volatile and semi-volatile compound removal from water bodies. In this study, the bacterial strains *Paenibacillus etheri* SH7 (CECT 8558), *Agrobacterium* sp. MS2 (CECT 8557) and *Rhodococcus ruber* strains A5 (CECT 8556), EE6 (CECT 8612) and EE1 (CECT 8555), previously isolated from fuel-contaminated sites, were tested for adherence on tubular semipermeable membranes in laboratory-scale systems designed for methyl tert-butyl ether (MTBE) bioremediation. Biofilm formation on the membrane surface was evaluated through observation by field-emission scanning electron microscope (FESEM) as well as the acute toxicity (as EC50) of the bacterial growth media. Moreover, extracellular polymeric substance (EPS) production for each strain under different MTBE concentrations was measured. Strains A5 and MS2 were biofilm producers and their adherence increased when the MTBE flowed through the inner tubular semipermeable membrane. No biofilm was formed by *Paenibacillus etheri* SH7, nevertheless, the latter and strain MS2 exhibited the lowest toxicity after growth on the EMBFR. The results obtained from FESEM and toxicity analysis demonstrate that bacterial strains *R. ruber* EE6, A5, *P. etheri* SH7 and *Agrobacterium* sp. MS2 could be excellent candidates to be used as selective inocula in EMBFR technology for MTBE bioremediation.

Key words | biofilm, EMBFR, EPS, microtox assay, MTBE

INTRODUCTION

Methyl tert-butyl ether (MTBE) has been the most widely used oxygenate compound in reformulated gasoline. Consequently, as a result of accidental spills and tank corrosion leakage from gasoline stations and refineries, this substance has been detected in many saline and fresh water aquifers worldwide (Johnson et al. 2000; Rosell et al. 2007). MTBE levels in background environmental samples are generally below 2 μg/L; when higher concentrations are found, it is an indication of an unknown point source and must be investigated. Several investigations (Schmidt et al. 2004; Shih et al. 2004) have focused on the maximum detected levels of MTBE in European and American groundwater (those varied from 120 μg/L in the Netherlands to 830 mg/L in the United Kingdom).

Bioremediation technologies to palliate these aquifers are sought for their relatively cheap and low-disturbance in the environment compared to other chemical remediating strategies. Biofilm-based technologies are highly advantageous over planktonic technologies since the protection conferred by the biofilm matrix include high resilience after disturbance, greater bacterial density and consequently a larger presence of enzymes involved in the degradation pathway of the xenobiotic compounds (Decho 2000; Singh et al. 2006; Mitchell et al. 2008).

The extractive membrane biofilm reactor (EMBFR) used in previous studies (Livingston 1995; Freitas dos Santos & Livingston 1994; Ferreira & Livingston 2000) could be successfully used for the treatment of MTBE contaminated groundwater. The basis of this technology was the physical separation of the air supply (preventing air stripping), the contaminated influent, and the biofilm, yet allowing for the contaminant to diffuse from the influent compartment to the biological one through a semipermeable membrane, while the microorganisms...
thrive in their medium without altering the effluent (Katsivel et al. 1999).

Effective biofilm formation on the inert support is crucial for the application of biofilm-based technologies and the extracellular polymeric substance (EPS) production is indicative of the possibility of adequate biofilm formation.

EPS are biopolymers located outside of the microbial cells (in a layer around the cell) consisting mainly of carbohydrate homo-/heteropolymers, which may additionally contain various organic and inorganic substituents. The main function of EPS in biofilms is to provide adequate adhesion to the substrate as well as strength and consistency to the biofilm, increasing microbial survival and growth of the microorganisms (Sutherland 2001; Poli et al. 2007). Environmental signals/factors alter the quantity and composition of EPS produced by a microorganism, such as nutritional stress, presence of xenobiotic compounds, different carbon sources or microbial growth inhibitors (Sponza 2002).

Microbial transformation of MTBE may lead to the accumulation of metabolites, such as tert-butyl alcohol (TBA), which are even more toxic than the contaminant itself. Thus, monitoring the toxicity effects of degrading strains should be performed, especially if metabolite accumulation occurs.

The technology utilized in this study is based on the use of semipermeable tubular membranes through which contaminants migrate to the biological compartment (‘biomedium’) in which microorganisms, capable of metabolizing MTBE, may grow and form active biofilm layers on the external surface of the membrane. The majority of the bacterial strains with MTBE degrading capacities exhibit low growth and poor biomass yield, nevertheless the application of membrane technology increases cell density. This technology has been used before with a dense compound (1,3-dichloropropene, ρ: 1.217 g/mL), however, we tested this system with a lighter compound such as the fuel oxygenate MTBE, may grow and form active biofilm layers on the external surface of the membrane. The majority of the bacterial strains with MTBE degrading capacities exhibit low growth and poor biomass yield, nevertheless the application of membrane technology increases cell density.

The bacterial strains used in this work were pre-grown on modified FTW medium amended with 150 mg/L MTBE as previously described by Purswani et al. (2008). The composition of this medium was (g/L): KH₂PO₄, 0.225; K₂HPO₄, 0.225; (NH₄)₂SO₄, 0.225; MgSO₄7H₂O, 0.050; CaCO₃, 0.005; FeCl₃4H₂O, 0.005 and 1 mL of trace elements solution. Trace elements solution had the following composition (g/L): ZnSO₄7H₂O, 0.1; MnCl₂4H₂O, 0.03; H₃BO₃, 0.3; CoCl₂6H₂O, 0.2; CuCl₂2H₂O, 0.01; NiCl₂6H₂O, 0.02 and NaMoO₄2H₂O, 0.03. Moreover, the medium was supplemented with 1 mL vitamin solution whose composition was (mg/L): biotin, 20; folic acid, 20; pyridoxine HCL, 100; thiamine HCL, 50; riboflavin, 50; nicotinic acid, 50; pantothenate calcium, 50; p-aminobenzoic calcium, 50; lipoic acid, 50 and cobalamin, 50. When solid plates were used, agar-agar (16 g/L) was added. In all cases, MTBE was added by filtration (Millipore, 0.22 μm) to growth media previously sterilized by autoclaving.

For EPS production experiments, both modified FTW medium and MY medium (Moraine & Rogovin 1966) were used. The composition of MY medium was the following (g/L): yeast extract, 3.0; malt extract, 3.0; protease peptone, 5.0; and glucose, 5.0.
EPS production, extraction and characterization

EPS production by the selected bacteria was evaluated according to the methodology proposed by Quesada et al. (1993). Briefly, precultures of each bacterial strain were grown in modified FTW medium containing 150 mg/L MTBE during 4 days (100 rpm, 28 °C). After this time, each inoculum (5 mL) was added to an Erlenmeyer flask with 500 mL of FTW medium supplemented with 50 or 150 mg/L MTBE as carbon source. Controls were included using the same conditions, without MTBE. EPS production on MY medium was carried out using the same conditions as those with the modified FTW medium. Following incubation, the cells were harvested at 5 °C by centrifugation at 10,000 rpm for 45 min. The supernatants were mixed with double volume ice cold ethanol (96% v/v) and kept at 4 °C for 24 h to precipitate the EPS. After this, the mixtures were centrifuged at 5 °C during 15 min at 7,500 rpm. The pellet was resuspended in deionized distilled water and dialyzed using Midicell dialysis membranes (12–14 kDa pore size) in distilled water for 24 h to remove the salts. The samples were frozen at −80 °C in Petri dishes until lyophilization. The samples were weighed and dry weight of EPS (g/L of culture) was determined for each sample. Carbohydrate and protein concentrations within the EPS produced were determined following the methodologies proposed by Dubois et al. (1956) and Bradford (1976), respectively.

Biofilm formation in tubular semipermeable membrane

Biofilm formation assessment by the bacterial strains on the external surface of the semipermeable tubes was achieved using the constructed laboratory-scale system shown in Figure 1. Erlenmeyer flasks (1 L of total volume), sealed with a silicon stopper and filled with 400 mL modified FTW medium were used. Semipermeable silicone tubes (63.9 cm length and 3 mm internal diameter, VWR) were immersed in the medium and connected to a peristaltic pump. To prevent MTBE loss outside the flask, two different materials were used as connecting tubes: Marprene® (Watson Marlow, Wilmington, MA, USA) and Teflon (PFTE). These tubes reduced (Marprene®) or inhibited (Teflon) MTBE loss through gas diffusion. The pump operated at a constant speed required to reach adequate influent flow (77.6 mL/day) for a hydraulic retention time (HRT) of 1.4 h. The HRT is the residence time of the contaminant inside the length of the semipermeable tubing which was submerged within the biomedium. A low Reynold’s number indicates a laminar flow within the tube. The medium was inoculated with the bacterial strains (5 mL) previously pre-grown on modified FTW medium amended with 1 g/L yeast extract during 4 days under agitation and controlled temperature (150 rpm, 30 °C).

The biological compartment, containing the inoculated strain and the diffused MTBE, was tested for MTBE concentrations in the abiotic controls.

In order to determine differences in the adhesion behavior of the bacteria according to the presence or absence of the fuel oxygenate as carbon source, abiotic and biotic controls were included. The biotic control did not contain MTBE, and the abiotic control did not contain the strain.

Determination of MTBE diffusion through the tubular semipermeable membrane

MTBE diffusion through the tubular semipermeable membranes to biological compartment was determined. Prior to
bacterial inoculation, the system was operated for 24 h, after which 1.5 mL of the culture within the biological compartment (biomedium) was collected and the MTBE amounts were determined following the methodology described by Purswani et al. (2014).

The samples (in triplicate) were placed into 2 mL vials and clamped. Then they were heated at 90 °C during 90 min, followed by the injection of 50 μL of the gas phase into the gas chromatography mass spectrometry (GC/MS) equipment (Hewlett-Packard 6890 GC coupled to a MS Hewlett-Packard 5973 mass selective detector, Palo Alto, CA, USA). The analysis was performed on a Quadrex capillary column (007-1, Dimethylpolysiloxane-PHAT Phase, 20 M × 0.18 mm × 6.0 μm). The temperature program was: 40 °C (3.5 min), 10 °C min⁻¹ up to 85 °C, and 7 °C min⁻¹ up to 235 °C. Helium was used as the carrier gas at a flow rate of 0.4 mL min⁻¹. Quantification of MTBE was performed using an external standard calibration (R > 0.99).

**Biofilm observation using field-emission scanning electron microscopy**

Observation of the biofilm established on the surface of semipermeable tubing immersed in the biomedium, was carried out as described by Purswani et al. (2011). After an incubation time of 10 days, the systems were unassembled and three 1 cm portions of the immersed tubing were cut out. The units were fixed in 2.5% glutaraldehyde in 1 x phosphate buffered saline (PBS) pH 7.4 during 24 h at 4 °C. The samples were then washed three times in 1 x PBS during 20 min. Fixation was followed by addition of 1% osmium tetroxide, kept in the dark during 1 h at room temperature and washed three times with distilled water. Samples were dehydrated in increasing ethanol concentrations, desiccated and covered by the evaporation of carbon with a HITACHI evaporator prior the observation using a LEO 1530 field-emission scanning electron microscope (FESEM) available at Centro de Instrumentación Científica (CIC) from the University of Granada.

**Microtox® test**

The Microtox® bioassay was used to measure acute toxicity (as EC₅₀) of each bacterial culture inside the flasks (biomedium) at the end of the experiment. The toxicity test is based on the bioluminescence reduction of *Vibrio fischeri* after 15 min exposure to the medium using the Microtox® Model 500 toxicity analyzer (Instrumentación Analítica S.A., Madrid, Spain). The effective concentration for 50% inhibition of luminescence (EC₅₀) after 5 min incubation was calculated with data reduction software using the methodology proposed by the manufacturer.

**Statistics**

Multi-factor analysis of variance using the software package STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) was performed to identify significant differences between the treatments. A significance level of 95% (P < 0.05) was selected.

**RESULTS AND DISCUSSION**

Poor microbial growth on MTBE is observed in a variety of studies (Fortin et al. 2001; Hatzinger et al. 2000); however, this does not mean that MTBE degradation is not possible. The cell density of MTBE degrading strains under unlimited MTBE concentrations are calculated to be low (Müller et al. 2007) and a minimum of 100 mg/L should be used to observe growth. Given the probable low cell density, EMBFR technology provides compartmentalization of the biological strains from the contaminated flow, thus allowing for optimal growth conditions in the biological compartment, while providing a membrane for biofilm formation where the concentration of the contamination is at its highest.

**Bacterial EPS production**

The amount of EPS (as g/L) produced by each bacterial strain after incubation under different MTBE concentrations were noted as observed in Figure 2.

Maximum EPS production was accomplished by *R. ruber* EE6 at 50 mg/L MTBE, with no further increase in EPS production at higher MTBE concentrations. Strangely, a significant increase in EPS production was accompanied with increasing MTBE concentrations for *R. ruber* EE1 and A5 being the values achieved when the media were amended with 150 mg/L MTBE significantly higher as the statistical analysis indicated. When the bacterial strains were grown on MY medium (Moraine & Rogovin 1966), EPS production was >4-fold higher in all strains with the exception of *Paenibacillus etheri* SH₇, for which the amount of EPS produced was very low and similar in both media, and A5 whose minimal EPS production was inhibited in MY medium (data not shown).
Table 1 shows the carbohydrate (A) and protein (B) percentages within the EPS produced by the bacteria in the modified FTW medium. Several studies have reported that the yield and composition of EPS produced by bacteria can be influenced both by changes in culture conditions and media composition (Cerning et al. 1986, 1990). Onbasli & Aslim (2009) observed how the monomer composition of the EPS produced by some strains of *Pseudomonas* spp. changed in the presence of different organic pollutants. So, depending on the organic compound used as carbon source, different monomer composition of the EPS could be detected. Thus culture conditions and the type of carbon source could affect the amount and composition of EPS produced. On the other hand, proteins play an important role in aggregation processes, on structural functions and other such enzymatic and biological functions (i.e. bacterial adhesion in the biofilm formation process) (Higgins & Novak 1997). Gao et al. (2013) showed that protein was the most important fraction in the EPS, but this trend could change according to the

Table 1 | (A) Carbohydrates (as %) in the EPS produced by the bacterial strains in each growth media. Values are media ± SD of three data. | (B) Proteins (as %) in the EPS produced by the bacterial strains in each growth media. Values are media ± SD of three data

<table>
<thead>
<tr>
<th>MTBE (mg/L)</th>
<th>A5</th>
<th>EE1</th>
<th>EE6</th>
<th>MS2</th>
<th>SH7</th>
</tr>
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<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>16.0 ± 1.59</td>
<td>16.2 ± 0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.3 ± 2.89</td>
<td>8.9 ± 0.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>16.5 ± 1.93&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.0 ± 0.51</td>
<td>15.9 ± 1.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.1 ± 1.20</td>
<td>–</td>
</tr>
<tr>
<td>150</td>
<td>9.6 ± 0.70</td>
<td>14.2 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.9 ± 1.30</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Bacterial strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>2.8 ± 2.44</td>
<td>2.8 ± 0.11</td>
<td>2.9 ± 0.13</td>
<td>4.2 ± 0.32&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.1 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>3.1 ± 0.17</td>
<td>3.1 ± 0.10</td>
<td>3.7 ± 0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.3 ± 0.03</td>
<td>–</td>
</tr>
<tr>
<td>150</td>
<td>3.2 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2 ± 0.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.2 ± 0.22</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Data followed by (*) significantly differ among experiments for the same bacterial strain. Data followed by (Ø) significantly differ among bacterial strains for the same experimental conditions.
environmental conditions, i.e. high temperature or carbon source.

In our study, the carbohydrate fraction was always higher than that of protein due to the C/N ratio in the media used for EPS production analysis. No significant increase in relative EPS carbohydrate or protein percentages was accompanied with increasing MTBE concentrations. In fact, only the protein percentage was significantly higher when the bacterial strains A5 and EE1 grown in media amended with 150 mg/L MTBE.

**Bacterial attachment observation by field-emission scanning electron microscopy**

FESEM analysis was carried out in order to determine the adhesion capacity of the bacterial strains on the surface of the immersed silicone tubing after a 10-day period. In Figure 3, two microphotographs are shown for each strain with (150 mg/L) and without MTBE contaminated influent. Microphotographs from the external surface of the non-inoculated system (abiotic control) were also included.

Both *R. ruber* A5 and *Agrobacterium* sp. MS2 showed great adherence on the external surface of the silicone tubing despite being the bacterial strains that exhibited the low production of EPS. Moreover, higher cell densities were observed in the presence of contaminant. Adhesion molecules and structures such as pili contribute to colonization processes, biofilm formation, and microbial community development in numerous bacterial genera (*Streptococcus, Vibrio, Agrobacterium* or *Pseudomonas*) (Nobbs et al. 2003; Zechner et al. 2012). Thus, for example, MS2 contains 11 genes related to pilus assembly and secretion proteins (data not shown). No differences in cell density were observed in the biofilms established by *R. ruber* strains EE1 and EE6 on tubing surface when the influents were amended with MTBE (data not shown). The only strain that did not form a biofilm under any condition was SH7.

An MTBE concentration of 124 mg/L in the biomedium of the abiotic assay confirmed adequate diffusion of MTBE across the tubing. Thus, the increase of cell density on the semipermeable tubing is a response to microbial growth using the carbon available.

*P. etheri* SH7 could still be considered as a ‘good candidate’ for use as a member of a bacterial consortia in a bioremediation strategy, due to its high MTBE degradation capacity (66.6% removal in 4 days; Guisado et al. 2015), despite the small EPS amounts produced and the incapacity to attach to our semipermeable tubing.

**Figure 3** | Scanning electron microscope images of biofilm developed on tubular semipermeable membranes with/without MTBE. Microphotographs from tubing surface of abiotic controls have been also included. Samples were taken from the bottom of immersed tubing after '10 days' recirculation. Magnification of 5,000× on all images.

In a study carried out by Katsivela et al. (1999), five bacterial strains with trans-1,3-dichloropropene (DCPE) degradation capacities were isolated from the metabolically active biofilm of an EMBFR and further investigated in batch experiments. Both our and their system intend to limit volatilization of the xenobiotic. The biofilm, attached to a silicone membrane, was also able to mineralize the contaminant after its diffusion through the membrane.

Biofilm formation of our strains A5, MS2, EE1 and EE6 were more abundant than that of the consortia *Acinetobacter calcoaceticus* M10/R. *ruber* E10 observed on the MTBE remediation biofilter (Purswani et al. 2011). Purswani et al. (2011) observed how the selective inoculation of an aerated submerged biofilter designed for treating contaminated MTBE groundwater with a strain with high capability of
biofilm formation (*A. calcoaceticus* M10), with another bacterial strain exempt of this capacity (*R. ruber* E10) but highly effective in the degradation of MTBE, yielded a biofilm even more abundant and was more efficient in the removal of the contaminant than that with *A. calcoaceticus* M10 on its own. A mixture between biofilm forming and non-forming strains with different MTBE capacities may yield a more effective inoculum than a single-microbial inoculum.

**Toxicity analysis**

Acute toxicity (as EC$_{50}$) of the biomedium at the end of each experiment was tested by Microtox® test. The Figure 4 illustrates the EC$_{50}$ values after 10-day incubation under controlled conditions. The samples from media inoculated with the strain *R. ruber* EE6 showed the lowest levels of toxicity (EC$_{50}$ = 85.8) followed by those from *Agrobacterium* sp. MS2 (EC$_{50}$ = 75.0) and *R. ruber* A5 (EC$_{50}$ = 43.0). However, *R. ruber* EE1 culture medium exhibited the highest toxicity data (EC$_{50}$ = 4.2) being thus similar to those obtained from abiotic control medium (EC$_{50}$ = 3.0). Abiotic controls were not inoculated with the bacterial strains but had the same concentration of MTBE as inoculated flasks.

The presence of genes encoding MTBE-degrading enzymes (alkane or cytochrome P450 monooxygenases) in bacterial strains *R. ruber* EE1, EE6 and A5 have been determined previously by Guisado *et al.* (2015). These bacterial strains were able to use MTBE in batch experiments as sole carbon source on modified FTW-medium amended with 150 mg/L MTBE. The acute toxicity analyses performed after an 8-day incubation indicated that growth media inoculated with strains *R. ruber* EE1 and EE6 exhibited (as in this study) the highest and the lowest toxicity values, respectively. The accumulation of some toxic metabolites from MTBE degradation (i.e. tert-butyl-alcohol or formaldehyde) by *R. ruber* EE1 could be the reason for acute toxicity values exhibited by this microorganism, however no detectable TBA was observed.

Toxicity values of our systems were much lower than those from a previous MTBE bioreactor study (Purswani *et al.* 2014; a biofilter simulating a recent MTBE spill with 100 mg/L MTBE). In the biofilters, the EC$_{50}$ maximum values were approximately 20%, i.e. between three and four times more toxic than our results with strains MS2 and EE6, and one to two times more toxic than A5 and SH7T within the same experimental period. Since EE1 toxicity mimics that of abiotic controls, this result clearly means that strain EE1 is not a good candidate as selective inoculum in a MTBE bioremediation.

In the current study, the acute toxicity results for *P. etheri* SH7T was low. This strain did not develop any biofilm, and in a previous study (Guisado *et al.* 2015) lacked amplification of either known MTBE monooxygenase (alkane or cytochrome P450 monooxygenase), though in the same study, important biotransforming/degrading MTBE capacity was observed in batch experiments. A draft genome of this strain is available (Purswani *et al.* 2016), however, we do not have clear gene cluster similarities with other known MTBE-degraders. Further research is needed to clarify which enzymes are involved in the degradation pathways of MTBE by these bacterial strains.

**CONCLUSIONS**

The selection of bacterial strains as selective inocula for bioremediation purposes must not only be determined by...
the ability to biodegrade a target pollutant but also by other factors, such as the facility to adhere to supports and form a biofilm, as well as obtain an adequately low acute toxicity level exhibited as a consequence of their biodegradation activities. EPS production is not exclusively indicative of biofilm formation, since also flagella, fimbriae and pili exhibit strong adhesive properties. Thus, all aspects should be contemplated when selecting an adequate selective inoculum.

The bacterial strains Agrobacterium sp. MS2, P. etheri SH7T and R. ruber strains EE6 and A5 showed appropriate characteristics for their use as selective inocula (sole or in consortium) in an EMBFR designed for MTBE contaminated groundwater bioremediation.

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CONFLICT OF INTEREST

All the authors of the present manuscript have no conflict of interest to declare.

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