UNIVERSIDAD DE GRANADA

DEPARTAMENTO DE MICROBIOLOGÍA. FACULTAD DE CIENCIAS
INSTITUTO UNIVERSITARIO DE INVESTIGACIÓN DEL AGUA

PROGRAMA DE DOCTORADO EN BIOLOGÍA FUNDAMENTAL Y DE SISTEMAS

Estudio Biológico de los Sistemas de Biorreactores de Membrana Extractiva con Desarrollo de Biopelícula para el Tratamiento de Agua Subterránea Contaminada con Oxigenantes de las Gasolinas

Biological Study of Extractive Membrane Biofilm Reactor Systems for the Treatment of Groundwater Contaminated by Fuel Oxygenates

Isabel María Guisado Requena

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Memoria para la obtención del grado de Doctor con mención internacional

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RESUMEN / ABSTRACT

RESUMEN

Hoy en día, la aplicación de nuevas tecnologías para el tratamiento biológico de aguas contaminadas con metil *tert*-butil éter (MTBE, compuesto oxigenante presente en la formulación de las gasolinas) es un hecho clave para conseguir una óptima calidad de las masas de agua tratada. Con ello se logra por un lado, proteger el medioambiente y por otro la adecuación de las mismas para consumo humano y fines agrícolas. En los últimos años una alternativa emergente y de gran importancia en el campo de la biorremediación, es la basada en el uso de biopelículas fijas establecidas sobre membranas semipermeables dentro de biorreactores. Desde un punto de vista biológico, la incorporación de los procesos de biopelícula fija confiere diversas ventajas a los sistemas de biorreactores de membrana extractiva (*EMBR*, Extractive Membrane BioReactor), tales como una mayor actividad y resistencia de la biomasa a sustancias xenobióticas. El biorreactor de membrana extractiva con desarrollo de biopelícula (*EMBFR*, Extractive Membrane BioFilm Reactor) se muestra como una novedosa configuración de la tecnología *EMBR* y viable alternativa para el tratamiento de aguas subterráneas contaminadas con oxigenantes de las gasolinas, ofreciendo la posibilidad de controlar las condiciones de crecimiento de los microorganismos para así asegurar una eficiente degradación de los compuestos contaminantes mediante la formación de una biopelícula activa sobre las membranas semipermeables, independientemente de las condiciones en el efluente a tratar.

En esta Tesis Doctoral se ha abordado el *Estudio biológico de los sistemas de biorreactores de membrana extractiva con desarrollo de biopelícula para el tratamiento de agua subterránea contaminada con oxigenantes de las gasolinas.*

Inicialmente se procedió al aislamiento y selección desde distintas muestras medioambientales de 20 cepas bacterianas con capacidad para biotransformar MTBE. La mayoría de las cepas bacterianas aisladas estaban afiliadas a los filo *Firmicutes* y

Actinobacteria, representando el 60% del total de los aislamientos. Las cepas bacterianas restantes se agruparon dentro del filo *Proteobacteria*.

Basándose en los resultados de crecimiento y degradación en medios adicionados de MTBE y compuestos aromáticos (BTEX), así como en los datos generados de la búsqueda de genes implicados en la degradación de éteres, se seleccionaron un total de 5 cepas bacterianas (*Rhodococcus ruber* A5, *Rhodococcus ruber* EE1, *Rhodococcus ruber* EE6, *Agrobacterium* sp. MS2 y *Paenibacillus* sp. SH7) para estudios posteriores.

La capacidad de estas cepas para establecerse en biopelícula sobre las membranas tubulares semipermeables, componentes de los futuros *EMBFR*, así como la producción de exopolisacárido (EPS) por parte de las mismas en medios de cultivo adicionados de MTBE, fue evaluada a escala de laboratorio. Para ello, se diseñaron sistemas *in batch* a escala de laboratorio con membranas tubulares semipermeables los cuales fueron inoculados con las cepas anteriormente citadas. La toxicidad aguda exhibida por los cultivos bacterianos fue evaluada mediante el bioensayo Microtox®.

Las cepas bacterianas *Agrobacterium* sp. MS2, *Paenibacillus* sp. SH7 y *Rhodococcus ruber* EE6 mostraron apropiadas características en cuanto a la degradación del contaminante, formación de biopelícula, y ecotoxicidad (EC_{50}) para su futuro uso como inóculos selectivos (solas o en consorcio).

Para evaluar la eficiencia de la tecnología *EMBFR* para la eliminación de MTBE de muestras de agua contaminada con este compuesto, se diseñaron y construyeron biorreactores a escala de laboratorio los cuales fueron inoculados selectivamente con las tres cepas anteriormente citadas. Los biorreactores inoculados se mantuvieron en funcionamiento durante 28 días (7 días en recirculación y 3 semanas en continuo) bajo tres distintos tiempos de retención hidráulica (1 h, 6 y 12 h). Diversos parámetros tales como pH, temperatura, oxígeno disuelto y crecimiento bacteriano en suspensión (como densidad óptica) en el biomedio de los sistemas fueron determinados diariamente, así como las

concentraciones de oxigenante presentes tanto en la entrada de los sistemas (influente sin tratar) como en el biomedio y en el efluente tratado mediante cromatografía de gases-espectrometría de masas (GC-MS). Al final de cada ensayo, se evaluó la formación de biopelícula sobre las membranas tubulares semipermeables mediante microscopia electrónica de barrido por emisión de campo (FESEM) así como la ecotoxicidad exhibida por el biomedio y el efluente mediante el bioensayo Microtox®.

En el desarrollo de esta Tesis Doctoral también se ha realizado el estudio taxonómico de una especie bacteriana perteneciente al género *Paenibacillus* (*Paenibacillus* sp. SH7), aislada y seleccionada en los anteriores ensayos por su capacidad para utilizar MTBE como única fuente de carbono y energía. Los resultados de los estudios morfológicos, fisiológicos, quimiotaxonómicos y filogenéticos, así como los procedentes de la hibridación ADN-ADN han confirmado que dicha cepa es una nueva especie del género *Paenibacillus,* la cual se ha propuesto como cepa tipo y designada como *Paenibacillus oxygenati nov*.

Esta cepa bacteriana junto *Agrobacterium tumefaciens* MS2 fueron los dos microorganismos seleccionados que aunque degradaron significativas cantidades de MTBE, no mostraron la presencia de ninguno de los genes conocidos que codifican para enzimas involucradas en la degradación de este compuesto xenobiótico (alcano monoxigenasa y/o citocromo P-450) tras la búsqueda de genes efectuada.

Los resultados obtenidos en esta Tesis Doctoral muestran en primer lugar la gran importancia del uso de nuevos métodos complementarios tanto fisiológicos, genéticos como ecotoxicológicos para el aislamiento y selección de bacterias con capacidad para degradar xenobióticos (caso de MTBE), para su posterior aplicación en procesos de biorremediación. También, han revelado que la selección de cepas bacterianas como inóculos para su aplicación en distintas tecnologías, no sólo debe estar determinada por la capacidad para degradar un contaminante, sino también por otros factores tales como la

facilidad de las cepas bacterianas para adherirse a soportes y establecerse como biopelícula o el nivel de toxicidad aguda exhibida debido a sus actividades de degradación. Por último, la tecnología de biorreactores de membrana extractiva con desarrollo de biopelícula (*EMBFR*), se muestra como una eficiente alternativa (bajo determinadas condiciones de operación) para la biorremediación de agua contaminada con compuestos semi-volátiles (caso del MTBE), resolviéndose los problemas presentados por otras tecnologías como la baja densidad bacteriana desarrollada y la volatilización del oxigenante desde la fase acuosa.

ABSTRACT

The application of new technologies for the biological treatment of groundwater contaminated with methyl *tert*-butyl ether (MTBE, an oxygenate compound present in the formulation of fuel) is an instrument of crucial importance in the search to optimise the quality of treated water. The aim of this approach is, on the one hand, to protect the environment and, on the other, to make the water thus treated fit for human consumption and agricultural purposes. In recent years, a new approach has been proposed, one that is highly important in the field of bioremediation, based on the use of fixed biofilms attached to semipermeable membranes within bioreactors. From a biological standpoint, incorporating fixed biofilm processes benefits extractive membrane bioreactor (EMBR) systems in various ways, such as the increased activity and greater resistance of the biomass to xenobiotics. An extractive membrane biofilm reactor (EMBFR) is presented, as a novel configuration of EMBR technology and a viable alternative for the treatment of groundwater contaminated with fuel oxygenates. EMBFR makes it possible to control the growth conditions of microorganisms and thus ensure the efficient degradation of pollutant compounds, by forming an active biofilm on the semipermeable membranes, regardless of the conditions in the effluent to be treated.

This Doctoral Thesis is concerned with the *Biological study of extractive membrane biofilm reactor systems for the treatment of groundwater contaminated by fuel oxygenates*.

In the initial phase, we examined various environmental samples and isolated and selected 20 bacterial strains capable of biotransforming MTBE. Most of the bacterial isolates (60%) belonged to the phyla *Firmicutes* and *Actinobacteria*. The other bacterial strains corresponded to the phylum *Proteobacteria*.

Taking into account the results obtained for the growth and degradation of MTBE and aromatic compounds (benzene, toluene, ethylbenzene and xylene – BTEX) in the

media examined, together with the data generated from the search for genes involved in the degradation of ethers, five bacterial strains were selected for further study: *Rhodococcus ruber* A5, *Rhodococcus ruber* EE1, *Rhodococcus ruber* EE6, *Agrobacterium* sp. MS2 and *Paenibacillus* sp. SH7.

The ability of these strains to become attached as a biofilm on semipermeable tubular membranes, the components of the future EMBFR, and the production of exopolysaccharide (EPS) by these strains in culture media to which MTBE had been added, was assessed in the laboratory. For this purpose, laboratory-scale batch systems were designed, with semipermeable tubular membranes that were inoculated with the above-mentioned strains. The acute toxicity exhibited by the bacterial cultures was then evaluated by the Microtox® bioassay.

The bacterial strains *Agrobacterium* sp. MS2, *Paenibacillus* sp. SH7 and *Rhodococcus ruber* EE6 were found to have appropriate characteristics regarding pollutant degradation, biofilm formation and ecotoxicity (EC_{50}) for use as selective inocula (either alone or in consortium).

To evaluate the efficiency of EMBFR technology for the elimination of MTBE from samples of water contaminated with this compound, laboratory scale bioreactors were designed and built, and then selectively inoculated with the three strains mentioned above. The inoculated bioreactors were operated for 28 days (7 days in recirculation and 3 weeks continuously), under three different hydraulic retention times (1, 6 and 12 h). Various parameters, such as pH, temperature, dissolved oxygen and bacterial growth in suspension (measured as the optical density) in the biomedium of each system, were measured daily, together with the concentrations of oxygenate present in the input to the systems (untreated influent), in the biomedium and in the treated effluent, using gas chromatography-mass spectrometry. After each assay, the formation of biofilm on the semipermeable tubular membranes was assessed by scanning electron microscopy field emission and by the

ecotoxicity present in the biomedium and in the effluent, according to the Microtox® bioassay.

In the development of this Thesis, we also performed a taxonomic study of a bacterial species belonging to the genus *Paenibacillus* (*Paenibacillus* sp. SH7), which was isolated and selected for the above assays on the basis of its ability to use MTBE as the sole source of carbon and energy. The results of the morphological, physiological, chemotaxonomic and phylogenetic studies conducted, together with those of DNA-DNA hybridisation, confirmed that this strain is a new species of the genus *Paenibacillus*, which is proposed as a type strain, to be designated *Paenibacillus oxygenati nov*.

This bacterial strain and *Agrobacterium tumefaciens* MS2 were the two microorganisms selected because, although they degraded significant quantities of MTBE, a gene search did not reflect the presence of any known genes encoding for enzymes involved in the degradation of this xenobiotic compound (monooxygenase alkane and/or cytochrome P-450).

The results obtained and presented in this Thesis highlight the major importance of new complementary methods – physiological, genetic and ecotoxicological – for the isolation and selection of bacteria capable of degrading xenobiotics (such as MTBE), for subsequent use in processes of bioremediation. The results also show that the selection of bacterial strains as inocula for application in different technologies should be determined not only by their ability to degrade a pollutant, but also by other factors, such as the capability of bacterial strains to adhere to supports and to become attached as a biofilm, and the level of acute toxicity achieved by their degradation activities. Finally, extractive membrane biofilm reactor technology is shown to be an efficient alternative (under certain operating conditions) for the bioremediation of water contaminated with semi-volatile compounds (such as MTBE), overcoming the problems encountered by other technologies such as low bacterial density, or the volatilisation of the oxygenate from the aqueous phase.

I. INTRODUCCIÓN GENERAL

En las últimas décadas el "Desarrollo Industrial" ha sido concebido como sinónimo de progreso y evolución, pero actualmente aspectos tales como “contaminación” y “residuos tóxicos", también podrían ser incluidos dentro de este concepto.

Cuando se habla de **tóxicos ambientales**, se suelen imaginar únicamente gases en la atmósfera, pero lo cierto es que **tóxicos** también son sustancias que están presentes en aguas tanto superficiales como subterráneas, y en suelos ya sean cultivables o no. La mayoría de estas sustancias se caracterizan por poseer una elevada persistencia o permanencia, provocando un efecto indeseado, ya que si no causan un efecto directo, tienen la capacidad potencial de causarlo tanto en el medio ambiente como en los seres que en él viven.

El agua es un elemento indispensable para la vida como lo es el aire que respiramos. La sociedad en que vivimos no tiene conocimiento o éste es muy limitado sobre la magnitud del problema ya que no hay consciencia de la contaminación que sufren nuestros mares, lagos, ríos y acuíferos, siendo un problema devastador sin otorgarle la importancia que tiene. Concretamente las aguas subterráneas son una de las principales fuentes de suministro para uso doméstico y para riego en muchas partes del mundo, y especialmente en España.

La contaminación de aguas subterráneas supone un gran problema ambiental, ya que mientras el periodo de permanencia medio del agua en los ríos es de días, en un acuífero es de cientos de años, lo que hace muy difícil su depuración y limpieza. Además, las aguas subterráneas suele ser más difíciles de contaminar que las superficiales, pero cuando esta contaminación se produce, es más difícil de eliminar.

Las dimensiones de la problemática de la contaminación de las masas de agua son actualmente desconocidas, ya que no existe sobre los contaminantes que se vierten en los cursos de agua, una información completa. Por ello, existen cada vez más leyes preventivas contra la posible contaminación de las aguas por parte de estas sustancias.

1. Historia del uso del metil *tert*-butil éter

Hasta la década de 1970 el uso de plomo en la formulación de las gasolinas había sido la forma más económica para aumentar su rendimiento. Sin embargo y debido a los problemas de contaminación generados por la combustión de las mismas, al inicio de los años 80 se redujo su uso y en algunos estados de EEUU se sustituyó, en la gasolina reformulada, por compuestos ricos en oxígeno ("compuestos oxigenantes") mejorando así su octanaje y disminuyendo las emisiones tóxicas a la atmósfera (Barceló, 2007). El octanaje es un número de referencia que indica la presión y temperatura a la que puede ser comprimido un combustible carburado (es decir, ínfimamente mezclado con aire) sin auto encenderse.

Entre los compuestos oxigenantes más utilizados en la formulación de las gasolinas están: etil *tert*-butil éter (ETBE), *tert*-amil metil éter (TAME), *tert*-butil alcohol (TBA), di-isopropil éter (DIPE), metanol, etanol y metil *tert*-butil éter (MTBE), siendo este último el compuesto más utilizado en la gasolina reformulada y objeto de estudio de esta Tesis Doctoral.

De conformidad con las enmiendas de 1990 a la Ley de Aire Limpio (CAA) en Estados Unidos, el MTBE fue añadido para mejorar la calidad del aire, ya que su uso reducía considerablemente los niveles de monóxido de carbono y ozono a la atmósfera. Posteriormente, en 1992, las zonas que aún no cumplían con esta ley, fueron obligadas a usar gasolina oxigenada para la reducción de dichos contaminantes. Aunque esta normativa fue aplicada, aún en 1995 existían áreas que seguían incumpliéndola y éstas fueron obligadas a usar gasolina reformulada (conteniendo compuestos oxigenados) durante todo un año.

Al comienzo de la década de los 90, el 61% de la gasolina que era consumida en Estados Unidos contenía MTBE en su formulación (Andrews, 1998); en 1999, en este

mismo país, se producían más de 200 mil barriles por día de MTBE (USEPA, 2006). En la Unión Europea, en el año 2001 fue aprobada la normativa para retirar totalmente las gasolinas con plomo del mercado (EEA, 2002) y se reguló la incorporación de oxigenantes en su formulación.

Varias fueron las razones para el uso masivo de MTBE en la formulación de las nuevas gasolinas. Así, podía ser transportado a través de los gasoductos existentes, era relativamente fácil de producir con compuestos disponibles en la mayoría de las refinerías con bajo costo y además, se mezclaba fácilmente con la gasolina (Squillace y colaboradores, 1997).

Sin embargo, la introducción en el mercado del MTBE supuso una serie de consecuencias ambientales que se desconocían hasta esa fecha; su solubilidad en agua llega a ser casi 100 veces más alta que la del tolueno y 30 veces mayor que la del benceno; además, su gran movilidad da lugar a que su incorporación en acuíferos sea más rápida ocasionado graves problemas de contaminación. Al final de la década de los noventa, en California, empezó a ser notable y alarmante la presencia de MTBE en el agua subterránea destinada al consumo humano, preocupación que se extendió rápidamente hacia todo el territorio de los Estados Unidos.

En el año 1995 se inició el consumo de gasolina sin plomo (adicionada de oxigenantes) en España, siendo en el año 1996, 34,7% el porcentaje de consumo de gasolina sin plomo del total de los carburantes utilizados (el más bajo a nivel Europeo). En el año 2002 este porcentaje se incrementó hasta 63,5%. Durante años se quiso sustituir al MTBE por un aditivo basado en potasio, el cual fue retirado en el año 2005.

La variación media en la concentración de MTBE que muestran las gasolinas europeas es de aproximadamente un 2% (v/v), aunque entre países el valor puede variar extraordinariamente. Así, en España por ejemplo, la variación entre gasolinas sin plomo de 98 octanos puede llegar a ser un 10% y un 4% en gasolinas sin plomo de 95 octanos.

El uso de MTBE en España ha ido disminuyendo en los últimos años, dejando paso al etil *tert*-butil éter (ETBE), a consecuencia del incremento en la producción de bioetanol. Este compuesto (ETBE) se puede producir a partir de etanol e isobutileno en una reacción catalítica y también a partir de bioetanol (Bio-ETBE).

A nivel europeo han sido muchos los expertos que han defendido los efectos de carácter positivo que presenta el uso de los oxigenantes, en contra de otros muchos que han sostenido los efectos negativos que generan. Sobre este tema, se han impartido diversas conferencias sobre MTBE y otros oxigenantes en Europa; la primera de ellas fue en el año 2003 en Dresden (Alemania), a la que siguieron otras más. Una en 2004 en Barcelona (España) y una tercera en Amberes (Bélgica) en el año 2007.

El MTBE es uno de los contaminantes con mayor presencia en los recursos hídricos de países como Alemania, Francia, Reino Unido, España y Bélgica (Schmidt y colaboradores, 2002; Klinger y colaboradores, 2002; Barceló, 2007).

La presencia de este oxigenante en masas de agua tanto superficiales como subterráneas ha dado lugar a una intensa evaluación de su transporte y destino en el medioambiente, siendo de especial interés su biodegradabilidad.

2. Propiedades fisicoquímicas del MTBE

El metil *tert*-butil éter (MTBE) puede presentarse con los siguientes sinónimos: 2-metoxi-2-metilpropano, éter-metil-*tert*-butílico, *tert*-butil-metil-éter; metil-1,1-dimetiletiléter y 1,1,1-tri metil-dimetil éter.

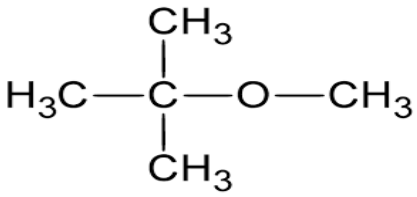

Figura 2.1. Fórmula química del metil *tert*-butil éter (MTBE).

Es un líquido volátil e incoloro, de olor similar a los terpenos y es producido mediante procesos químicos de síntesis a partir del metanol e isobutileno (**Figura 2.2**), a diferencia de las gasolinas que son producidas por la refinación del petróleo.

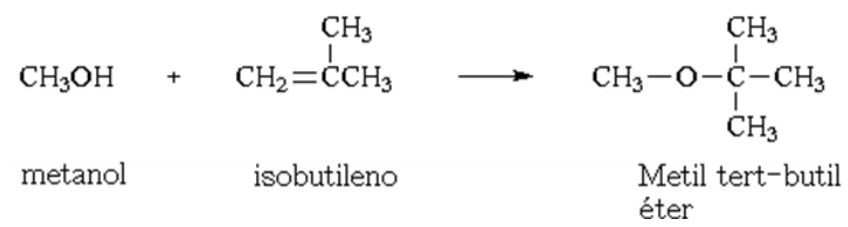

Figura 2.2. Reacción química de metanol e isobutileno para la producción de MTBE.

Este compuesto se adiciona (en concentraciones entre 11-15 % v/v) a las gasolinas en el momento en que éstas son formuladas para su distribución y posterior venta al público. Sólo una cantidad minoritaria se utiliza para otros fines, tales como disolvente en lugar de éter dietílico o éter diisopropílico en industrias químicas, farmacéuticas y laboratorios.

Es altamente soluble, siendo su solubilidad en agua en torno a 50.000 mg / L; presenta una baja constante de Henry ($5{,}3 \times 10^{-4}$ - 3×10^{-3} atm.m^3 / g.mol). Una sustancia química con una constante de Henry baja, prefiere la fase acuosa a la fase de gas, por lo que el MTBE se disuelve fácilmente en agua y en hidrocarburos, y se particiona en el agua en lugar de en el aire (Squillace y colaboradores, 1997).

Uno de los hallazgos más comunes en un sitio de derrame de gasolina, es que el MTBE migra por delante de otros componentes de la gasolina como el benceno, tolueno, etilbenceno y xilenos (BTEX). Esto sucede porque el MTBE tiene una mayor movilidad y está menos sujeto a la biodegradación durante su transporte. Además, el MTBE, en soluciones acuosas, es más volátil que otros oxigenantes y tiende a evaporarse sin dificultad a la atmósfera (Huttenen y colaboradores, 1997). También, puede entrar en masas de agua subterránea poco profundas a través de la precipitación. Según Squillace y colaboradores (1997), la alta presencia de este oxigenante en zonas como gasolineras y aparcamientos, donde la concentración atmosférica de MTBE es alta, puede provocar una contaminación medible en zonas próximas a las aguas pluviales y en aguas subterráneas.

Las principales alternativas potenciales para el uso del MTBE son otras formas de éteres tales como el ETBE, TAME y alcoholes, pero estas alternativas no están exentas de problemas. El etanol es más costoso de producir, plantea desafíos al sistema de distribución de gasolina y es poco probable que esté disponible en cantidad suficiente a corto plazo. Por otra parte, su metabolito acetaldehído es un posible carcinógeno, que se somete a una reacción fotoquímica en la atmósfera para producir el irritante respiratorio nitrato de peroxiacilo.

3. Presencia de MTBE en aguas superficiales y subterráneas

La presencia de este compuesto oxigenado en distintos compartimentos medioambientales ha provocado una intensa discusión sobre los efectos negativos y positivos que conlleva su utilización.

Aunque su uso efectivamente mejora la calidad del aire y aumenta la eficiencia de la combustión, la Agencia de Protección Medioambiental de Estados Unidos (EPA) a partir del año 2000, investigó la presencia de MTBE y éteres relacionados en el agua subterránea de todo el territorio de EEUU debido a las filtraciones reiteradas desde tanques subterráneos de almacenamiento (Halden y colaboradores, 2001).

Efectivamente este contaminante puede detectarse en aguas subterráneas a consecuencia de derrames accidentales desde los contenedores de almacenamiento, fugas en los sistemas de conducción ó fugas desde tanques de gasolineras, favorecidos por su tendencia a lixiviarse y su alta solubilidad en agua. También se puede detectar en aguas superficiales debido a los desagües pluviales, a una liberación directa, a la descarga de aguas subterráneas contaminadas, como consecuencia de la precipitación (Squillace y colaboradores., 1997), o por las emisiones desde embarcaciones motorizadas (Keller y colaboradores, 1998). La **Figura 3.1.** muestra la movilidad de este compuesto en el medioambiente.

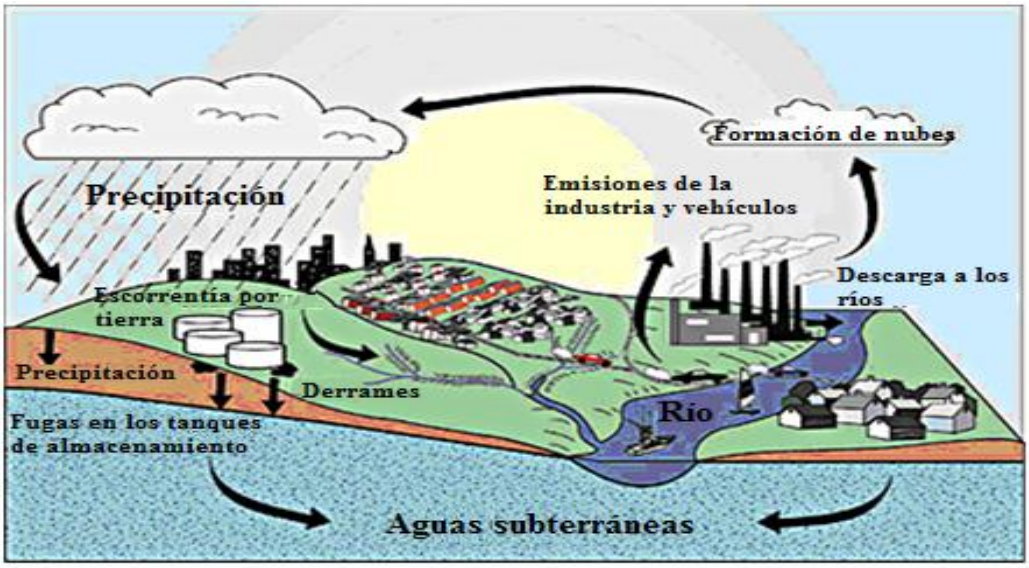

Figura 3.1. Movimiento del MTBE en el medioambiente. Fuente: Adaptada de MTBE Project. http://www.cvwd.com/Documents-MTBE-Project.aspx

En aguas superficiales, más del 80% del MTBE se llega a volatilizar en 2 o 3 semanas tras la descarga del compuesto. Sin embargo, la tasa de pérdida disminuye a medida que las aguas se transforman en más profundas (Keller y colaboradores, 1998).

El Servicio Geológico de EEUU (USGS) a través del Programa Nacional de Evaluación de la Calidad del Agua (NAWQA) (Squillace y colaboradores, 1999), realizó un estudio para evaluar la presencia de compuestos orgánicos volátiles en las aguas subterráneas en ese país, y concluyó que el MTBE se podía detectar en el 16,9% en los pozos de las zonas urbanas y en un 3,4% en los pozos en las zonas rurales. El caso más notorio de contaminación de agua subterránea con este compuesto oxigenante se produjo en la ciudad de Santa Mónica (California) dónde el agua subterránea era utilizada como abastecimiento para la población. La concentración de MTBE aumentó a 610 mg / L en

abril de 1996 desde agosto de 1995, donde ya se detectó presencia de MTBE. Se procedió al cierre de cinco pozos y un total de 25 tanques de almacenamiento subterráneos de gasolina fueron identificados como participantes en la contaminación (US EPA, 2000).

En el año 2003, Rosell y colaboradores detectaron valores de MTBE en acuíferos españoles entre 20 y 670 µg/ L. En el año 2006, los mismos autores, llevaron a cabo un amplio estudio sobre la distribución que presentaba el MTBE en diversas zonas de Cataluña (España). La persistencia del MTBE en los acuíferos (debido a sus características físico-químicas), ha favorecido el hecho que sea considerado un buen indicador de contaminación por oxigenantes de las gasolinas.

La contaminación de las aguas subterráneas españolas es una problemática real, grave y compleja en amplias zonas del país, agravándose aún más en la última década. Tristemente, se determina un tiempo de 20 años, como el periodo máximo de utilización de los acuíferos de la cuenca mediterránea debido a la contaminación que presentan en la actualidad.

La climatología de la Cuenca Mediterránea (especialmente España), es una variedad del clima templado o del clima subtropical, caracterizándose por sus veranos calurosos y secos y por sus inviernos templados. Es propio que por sus temperaturas relativamente suaves en invierno y muy calurosas en verano, en las estaciones intermedias (meses de primavera y otoño), se concentre la pluviosidad siendo ésta bastante escasa. Por este motivo, las aguas subterráneas de muchas zonas de España (Andalucía especialmente), se muestran como una de las principales fuentes de suministro para riego y para uso doméstico, lo que hace necesario su control.

4. Biodegradabilidad del MTBE

La contaminación por MTBE ha sido detectada en el aire urbano (Grosjean y colaboradores,1998; Vainiotalo y colaboradores, 1998), en aguas superficiales (Reuter y colaboradores, 1998) así como en el agua de lluvia y en masas de agua subterránea (Landmeyer y colaboradores 1997, 1998; Lince y colaboradores, 1998).

Aunque hay muchos métodos físico-químicos utilizados para eliminar los contaminantes del agua, a menudo la biodegradación es el método que más limita el destino de un producto químico, el transporte, y los efectos nocivos en el medio ambiente. Inicialmente, la idea predominante era que el MTBE fuese recalcitrante. Autores como Fujiwara y colaboradores (1984), Jensen y Arvin (1990) y Yeh (1992) aseveraron mediante sus estudios que el MTBE era resistente al ataque microbiano en condiciones aeróbicas y anaeróbicas. Sin embargo, numerosas investigaciones posteriores indicaron que el MTBE podía biodegradarse aeróbica y anaeróbicamente.

Hasta la fecha, aunque este área de investigación se mantiene activa, el número de informes que presentan una exitosa degradación de MTBE en condiciones anaeróbicas ha sido escaso (Somsamak y colaboradores, 2001; Bradley y colaboradores, 2001a y 2001b; Finneran y Lovley 2001; Kolhatkar y colaboradores, 2002).

Sin embargo, ha sido demostrado a nivel de laboratorio que la degradación aeróbica es posible, y en algunos casos muy eficiente, mediante cultivos puros (Hatzinger y colaboradores, 2001; Francois y colaboradores, 2002) y mixtos (Salanitro y colaboradores, 1994). Estos microorganismos pueden utilizar el MTBE como única fuente de carbono y energía (Deeb y colaboradores, 2001; Mo y colaboradores, 1995) o ser efectiva esta degradación, gracias a procesos cometabólicos (Hyman y colaboradores, 1998, Liu y colaboradores, 2001).

La **Tabla 1** muestra las tasas de biodegradación aeróbica de MTBE, así como los rendimientos celulares por parte de algunas cepas bacterianas.

La problemática existente en estos tipos de degradación es la baja tasa que presentan estos organismos en el rendimiento de biomasa, en densidad óptica, en ensayo de proteína, o en el número más probable. Se especula que puede ser debido a que un compuesto intermedio sea resistente a la degradación total, disminuyéndose por lo tanto la cantidad de energía generada. Pero investigadores como François y colaboradores (2002), estudiaron microorganismos (en concreto *Mycobacterium austroafricarum* IFP 2012) con un crecimiento aeróbico en MTBE relativamente rápido.

Tabla 1. Estudios de degradación aeróbica de MTBE con diferentes cultivos bacterianos tanto puros como mixtos. Fuente: Adaptada de Hanson y colaboradores, 1999.

Referencias	Organismo	Tipo de Metabolismo	Tasa de Degradación de MTBE	Rendimiento Celular
Salanitro y col.,1994	MC-100, cultivo mixto o BC1	Aerobio Heterótrofo	34 mg/g células/h	0.21-028 g peso seco/g MTBE
Cowan and Park, 1996	Cultivo mixto (Biolodos de refineria)	Aerobio Heterótrofo		0.33-.44 mg biomasa COD/mg MTBE consumido
Steffan y col.,1997	ENV 425	Cometabolismo con propano	4.6 nmol/min/mg proteina celular	
Steffan y col.,1997	ENV 421	Cometabolismo con propano	9.2 nmol/min/mg proteina celular	
Hardison y col.,1997	*Graphium sp.*	Cometabolismo con n-butano	1.9 nmol/h/mg peso seco sin incubación con butano; 0.6 nmol/h/mg peso seco con butano	
Hyman y col., 1998	*Xanthobacter sp.*	Cometabolismo con propano	51 nmol/min/mg proteina	
Fortin y Deshusses, 1999	Filtro biopercolador de cultivo mixto	Aerobio Heterótrofo	5-10 mg MTBE/g peso seco/h	0.1 peso seco/g MTBE
Garnier y col.,1999	*Pseudomonas aeruginosa*	Cometabolismo con pentano	3.9 nmol/min/mg proteina	
Hanson y col.,1999 Deeb y col.,2001	Rubrivirax gelatinosus PM1	Aerobio Heterótrofo, 2 E6 células/mL	0.07, 1.17, 3.56 g/mL/h para MTBE= 5, 50, 500 mg/L 50 mg/g células/h; 19 nmol/min/mg proteina	0.18 mg células/ mg MTBE
Liu y col.,2001	*Anthrobacter* (ATCC 27778)	Cometabolismo con butano	6.78 nmol/min/mg TBA -0.07 nmol/min/mg	
Kane y col.,2001	Cultivo mixto (material de acuifero)	Aerobio Heterótrofo	0.4 mg/L/dia	
Salanitro y col.,2001	SC-100, *Rhodococcus sp. nov.*	Aerobio Heterótrofo		
Hatzinger y col.,2001	*Hydrogenophaga flava* F55	Aerobio Heterótrofo	86 nmol/min/mg proteina, 30°c, cultivado en extracto de levadura	0.4 mg/g MTBE
Vainberg y col.,2002	Cultivo mixto	Aerobio Heterótrofo	0.46 g/g TSS/dia	0.55 g TSS/g TOC
Francois y col.,2002	*Mycobacterium austroafricanum*	Aerobio Heterótrofo	0.6 nmol/h/g peso seco; 20 nmol/min/mg proteina	0.44 g/g MTBE
Sedran y col.,2002	Cultivo mixto (enriquecido con MTBE)	Aerobio Heterótrofo	0.01 mm/h TBA- 0.0185 mm/h	
Wilson y col.,2002a	Cultivo mixto (mezcla de licores de refineria y municipal, material de lavado de un aquifero)	Aerobio Heterótrofo	$3.4.10^{-4}$ nmol/min/mg VSS	
Kharoune y col.,2002	Cultivo mixto (suelo contaminado con gasolina)	Aerobio Heterótrofo	1.1 mg/L/h	

Por tanto, la biodegradabilidad del MTBE, en cuanto a la limitación que presenta su transporte en el medio ambiente dentro de las aguas subterráneas y especialmente bajo diferentes condiciones ambientales, es probable que continúe debatiéndose durante años. El gran problema es que la contaminación por MTBE se mantiene durante largos periodos de tiempo en el medioambiente.

La búsqueda y caracterización de nuevas cepas bacterianas con capacidad para degradar/biotransformar MTBE, adquiere una gran importancia, ya que cepas bacterianas con propiedades de degradación y transformación de compuestos xenobióticos debido a la expresión de genes metabólicos, podrían ser de gran utilidad y significación en el desarrollo de nuevas tecnologías de biorremediación.

Son numerosos los estudios que se han centrado en mejorar esta búsqueda y posterior caracterización intentando progresar en los procedimientos de aislamiento y métodos de enriquecimiento bacteriano de diferentes muestras ambientales, probando la capacidad para crecer en medios minerales adicionados de oxigenante como única fuente de carbono o aislando e identificando cepas bacterianas capaces de usar MTBE, ETBE, TAME como fuente de carbono y energía, observando las características de crecimiento y el consumo de estos compuestos oxigenados (Fayolle y colaboradores, 1998; Rohwerder y colaboradores, 2006; Lyewet y colaboradores, 2007; Purswani y colaboradores, 2008).

Actualmente, los procesos de selección de bacterias tienden a ser bastante limitados, por lo que no se proporciona mucha información sobre las cepas bacterianas antes de ser utilizadas en ensayos posteriores más complejos y costosos.

En la presente Tesis Doctoral, se describe una metodología precisa para el aislamiento y selección de cepas bacterianas capaces de usar MTBE como única fuente de carbono y energía basándose en la detección fisiológica y genética. Además se presenta también la caracterización taxonómica de una cepa bacteriana (*Paenibacillus* sp. SH7) aislada y seleccionada, con capacidad para crecer (bajo las condiciones de este estudio) en

medios adicionados de MTBE, para la cual se ha propuesto la denominación de *Paenibacillus oxygenati* sp. nov.

5. Caracterización de cepas bacterianas

La descripción de nuevas especies bacterianas contribuye a incrementar el conocimiento no sólo de las propias células sino también sobre innumerables procesos y la participación en ellos. Se suelen utilizar tanto técnicas genotípicas como fenotípicas las cuales proporcionan información a nivel de ADN y ARN celular en el primer caso así como información sobre características morfológicas, fisiológicas, bioquímicas e información quimiotaxónomica en el segundo (Sarmiento, 2008).

5.1. Técnicas fenotípicas y genotípicas

Las técnicas fenotípicas se basan en detectar los caracteres típicos expresados por el microorganismo, abarcando su caracterización cultural, morfológica, fisiológica y bioquímica, correspondiéndose a las técnicas clásicas de identificación. Así, aspectos tales como las características microscópicas, macroscópicas, culturales (requerimientos de crecimiento) o pruebas bioquímicas y resistencia a determinadas sustancias son incluidas en estas determinaciones y consideradas como técnicas "clásicas" dentro de la taxonomía bacteriana. Por otra parte las diferencias en la composición de distintos componentes celulares (composición de la pared celular, ácidos grasos o quinonas respiratorias) y considerada como información quimiotaxonómica es también utilizada para una completa clasificación.

Ácidos grasos

Los ácidos grasos están principalmente constituyendo los grupos acilo de los fosfolípidos, localizándose fundamentalmente en las membranas de las células bacterianas. Debido a que la mayor parte de bacterias los contienen, adquieren una gran importancia desde el punto de vista taxonómico (Tindall y colaboradores, 2010). Mediante un enlace éster, los ácidos grasos están unidos a moléculas de glicerol, formando una molécula de fosfolípido. Según autores como Shaw (1974) la longitud que presentan con más frecuencia los ácidos grasos en bacterias está entre C_{10} y C_{20}, siendo los de C_{15} y C_{19} los más habituales.

Azúcares de la pared celular

La descripción de una cepa bacteriana a nivel taxonómico, es completada gracias a la composición de los azúcares mostrados en la pared celular. Según Cummins y Harris, 1956, el modelo de los componentes aminoacídicos que están presentes en la pared celular, son utilizados para distinguir grupos taxonómicos grandes como lo son los géneros, mientras que las especies dentro de ese grupo, pueden ser distinguidas por el tipo de azúcares y amino azúcares de sus paredes celulares.

Peptidoglicanos

La mureína (sáculos de glucopéptido), es un elemento esencial y único en la pared de la mayoría de las células bacterianas. Según Schleifer y Kandler (1972), en las diferentes especies de bacterias Gram-positivas, existe una gran heterogeneidad en la secuencia y composición de los péptidos que conforman los peptidoglicanos. Gracias al estudio y

conocimiento de las diversas estructuras de los peptidoglicanos se ha contribuido a la clasificación de las bacterias Gram-positivas.

Quinonas

A nivel bacteriano, las quinonas son clasificadas en dos grandes grupos estructurales: ubiquinonas (coenzima Q) y las menaquinonas (vitamina K) (Collins y colaboradores, 1977). Éstas, forman parte de la constitución de las membranas plasmáticas bacterianas, jugando un gran papel en la fosforilación oxidativa y en el transporte de electrones (Pennock, 1966 y Brodie y Wataabe, 1966).

Los patrones de hidrogenación (saturación) que presentan las cadenas laterales de isoprenoides de las bacterias Gram positivas son bastante diversos. Según Tindall (2004) la posición y grado de saturación puede relacionar grupos como los definidos a través de la secuenciación del gen ARNr 16S.

La caracterización genotípica por su parte, se desarrolla gracias a diferentes técnicas moleculares, las cuales ofrecen información necesaria para una correcta clasificación del microorganismo en cuestión. En taxonomía bacteriana, el análisis de la secuencia génica del ARNr 16S es el marcador más ampliamente utilizado, no afectándose por las condiciones del cultivo (Sanz y colaboradores, 1998). En la actualidad, los estudios basados en la secuenciación del ARNr 16S han sido sustituidos por otros equivalentes basados en ADNr 16S (Goebel y colaboradores, 1987). También la hibridación ADN-ADN, contenido en G+C (% mol) o métodos basados en la reacción en cadena de la polimerasa, PCR (polymerase chain reaction), son técnicas cuyos resultados son útiles para la clasificación.

El gen ARNr 16S

Dos subunidades forman el ribosoma bacteriano; la subunidad 30S y la subunidad 50S. En concreto, la subunidad 30S contiene la molécula de ARNr 16S, y el análisis de este molécula, es considerado el método más utilizado en los estudios de procariotas, de parentesco y evolución microbiana (Sarmiento, 2008). El gen ARNr 16S, se encuentra presente en todos los microorganismos, obteniendo un mismo papel en todos los ribosomas bacterianos, conteniendo secuencias variables, hipervariables y conservadas. Este gen es amplificado y secuenciado, y posteriormente es comparado con secuencias almacenadas en bases de datos. Los microorganismos que presentan una similitud igual o superior al 97% de los genes que codifican el ARNr 16S, pueden pertenecer a la misma especie. Por tanto, esta técnica favorece la caracterización de bacterias aisladas desconocidas ofreciendo la posición filogenética.

Hibridación ADN-ADN

Esta técnica se basa en la homología de los ácidos nucleicos de dos microorganismos. Con su uso puede conocerse la similitud entre genomas completos de dos microorganismos diferentes, es decir, determinar si dos cepas bacterianas son de las misma especie (Sarmiento, 2008). En taxonomía, una especie bacteriana se define como el conjunto de cepas que comparten una similitud mayor o igual al 70% en experimentos de hibridación ADN-ADN (Stackebrandt y Goebel, 1994).

Contenido G+C (mol%)

El contenido G+C denota el contenido de guanina y citosina respectivamente expresado como % de la cantidad de bases nitrogenadas que son guanina o citosina que presenta el genoma o molécula de ADN objeto de estudio.

El contenido de G+C es variable dependiendo del organismo; por ejemplo las Actinobacterias se caracterizan por poseer altos contenidos de G+C; en cambio un 38% es el contenido en levaduras. Debido a los problemas surgidos en las especies procariotas se ha recomendado el uso de las relaciones G+C a nivel de una clasificación jerárquica más elevada.

Taxonomía del género *Paenibacillus*

Ash en 1993, describió los miembros del género *Paenibacillus* como bacilos Gram positivos, Gram negativos o Gram variable, móviles por flagelos periticos, con esporas elipsoidales con esporangio hinchado. Anaerobios facultativos o aerobios estrictos. La mayoría de las especies son catalasa positiva y oxidasa variable. La reacción de Voges-Proskauer (producción de acetilmetil-carbinol) es variable y el pH en dicho medio es menor de 6,0. No producen sulfhídrico y el indol es generado sólo por algunas especies. La reducción de nitrito es variable, así como la hidrólisis de la caseína, almidón, urea y la descomposición de la tirosina.

El crecimiento óptimo de la mayoría de las especies se produce a 28-30º C y éste se ve inhibido al 10% de NaCl. Algunas especies no crecen en medio que contenga 0.001% de lisozima. Son productores de ácido a partir de diversos azúcares y algunas especies son capaces de descomponer polisacáridos. *P. polymyxa*, *P. periorae*, *P. azotofixans* y *P. macerans* producen gases a partir de varios azúcares. El ácido graso mayoritario es el ácido

anteiso-$C_{15:0}$. El intervalo de la composición de bases G+C (% mol) varía entre 44-54% y los niveles de similitud en el gen ARNr 16S son mayores del 89.6% para los miembros del género. La especie tipo es *Paenibacillus polymyxa*.

Especies de este género se han localizado en numerosos ambientes, desde hemocultivos hasta en alimentos, suelos y raíces de las plantas (Roux y Raoult, 2004; Berge y colaboradores, 2002; Guisado y colaboradores, 2015). Este género, ha demostrado capacidad para producir enzimas extracelulares (degradadoras de polisacáridos y proteasas) (Rai y colaboradores, 2010), que pueden ser utilizadas en aplicaciones industriales y biotecnológicas. A nivel medioambiental, la característica más importante que pueden tener varias especies del género *Paenibacillus* es la degradación de contaminantes ambientales (Sirota-Madi y colaboradores, 2010; Khianngam y colaboradores, 2011).

6. Vías de degradación de MTBE: Intermediarios metabólicos y enzimas implicadas en su degradación

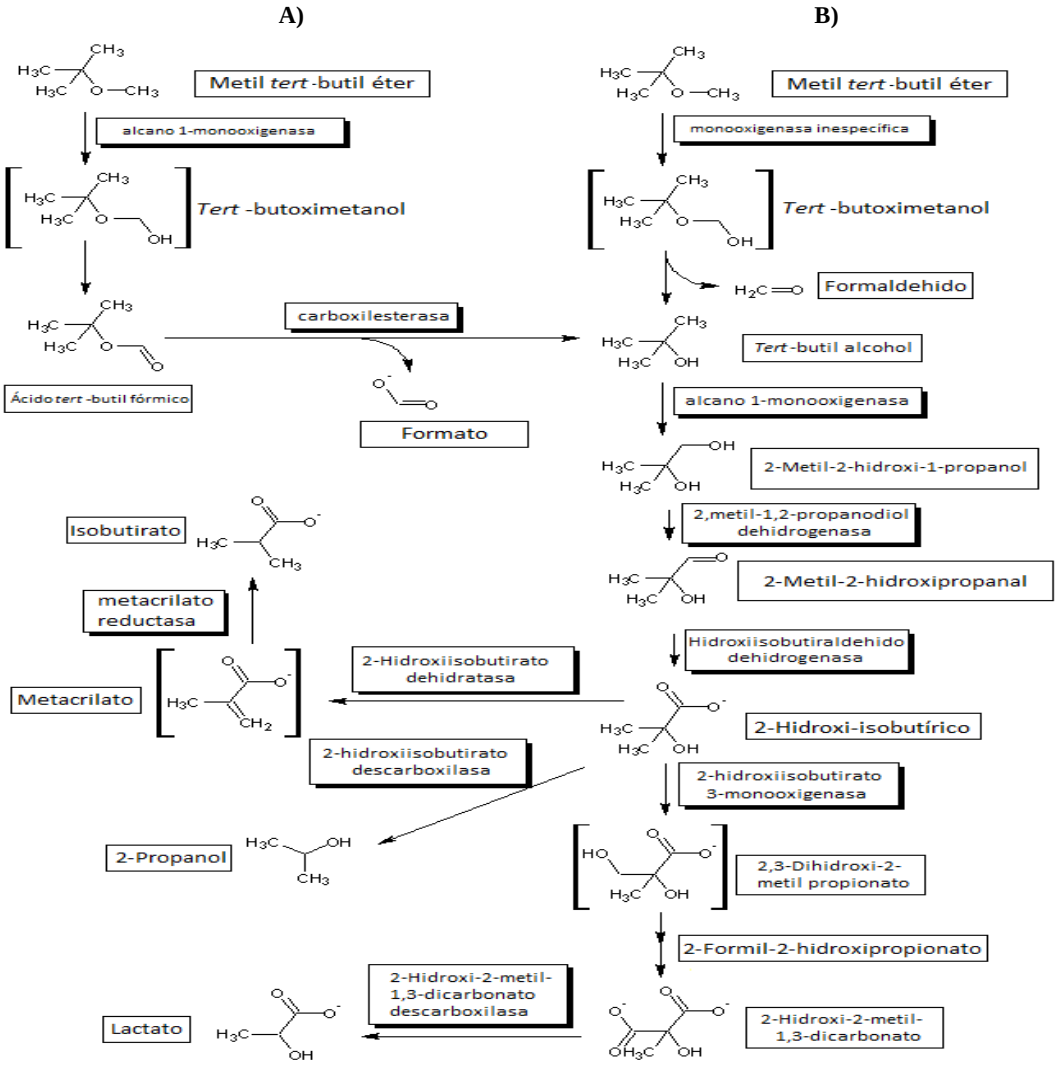

Figura 6.1. Mapa de la ruta de degradación del MTBE. A) Ruta llevada a cabo por *Nocardia* sp. ENV425 (Steffan y colaboradores, 1997). B) Ruta llevada a cabo por *Mycobacterium vaccae* JOB5 (Smith y colaboradores, 2003) y M*ycobacterium austroafricanum* IFP 2012 (Francois y colaboradores, 2002). Fuente: Adaptada de C. Rosendahl Pedersen, C. Essenberg and M.Turnbull, personal page. University of Minnesota, USA.

El mecanismo de degradación del MTBE por parte de los microorganismos es un campo aún poco conocido. Son varios los autores que han intentado clarificar los mecanismos de degradación que llevan a cabo diversas cepas bacterianas. Algunas de ellas son capaces de mineralizar el MTBE, mientras otras sólo pueden utilizar metabolitos secundarios tales como *tert*-butil alcohol (TBA), ácido *tert*-butil fórmico (TBF) o formaldehido.

Es extraordinario cómo los microorganismos pueden romper el enlace éter del oxigenante, ya que se requiere el empleo de una gran cantidad de energía y a veces la cantidad disponible de carbono es muy baja (White y colaboradores, 1996). En el año 1997, Steffan y colaboradores utilizaron la cepa bacteriana *Nocardia* sp. ENV425 para estudiar la degradación de MTBE. Esencialmente la ruta de degradación de este oxigenante es la representada en la **Figura 6.1**, y basada en la ruta descrita para este microorganismo.

En 2002, Francois y colaboradores, estudiaron la ruta de degradación llevada a cabo por la cepa bacteriana *Mycobacterium austroafricanum* IFP 2012, y observaron ligeras diferencias al inicio de la misma, en concreto en el paso de MTBE a TBF, el cuál era luego hidrolizado a TBA y formiato. Igualmente ocurría con la cepa bacteriana *Mycobacterium vaccae* JOB5 (Smith y colaboradores, 2003).

Entre los posibles mecanismos para la división del enlace éter por parte de los microorganismos podemos citar la ruptura por acción de enzimas monooxigenasas (de manera general); la oxidación por parte del citocromo P450 (P450 monooxigenasa); cambio de grupos hidroxilo, mecanismos reductores, por hidrólisis, por intervención de liasas carbono oxigenadas y por ruptura anaeróbica de los éteres metílicos-arilo (White y colaboradores, 1996).

Las enzimas mostradas en la **Figura 6.1** para la ruta de degradación del MTBE fueron propuestas por Smith y colaboradores en 2003.

Años más tarde, Lopes-Ferreira y colaboradores (2006) realizaron estudios sobre caracterización y clonación de varios genes implicados en la parte final de la ruta de degradación del MTBE, en concreto del paso de 2-metil-2-hidroxi-1-propanol al ácido 2-hidroxi-isobutírico (HIBA). Los genes referidos pertenecían a la cepa bacteriana *M. austroafricanum* IFP 2012. La ruta de degradación del 2-hidroxiisobutirato es bastante incierta, siendo el metacrilato, el 2-propanol, y el 2,3-dihidroxi-2-metil propionato los productos de degradación propuestos. El metacrilato puede ser reducido a isobutirato de una forma aeróbica por la cepa bacteriana *Wolinella succinogenes* (Gross y colaboradores, 2001).

Varios autores como François y colaboradores (2002) y Fayolle y colaboradores (2003), concuerdan en la ruta de degradación propuesta por Steffan y colaboradores, 1997. En 1998, Hyman y colaboradores describieron el papel de monooxigenasas inducidas en una cepa de *Xanthobacter* y en *Mycobacterium vaccae* JOB5, las cuales crecían en presencia de propano. Sin embargo, estas monooxigenasas que oxidaban alcanos, no eran del tipo citocromo P-450. Comprobaron que el primer intermediario de la ruta de degradación del MTBE era el TBF, mientras que el TBA era sustrato para las monooxigenasas del propano.

6.1 Enzimas involucradas en la vía de degradación de MTBE.

Gracias al estudio y comprensión del mecanismo degradativo que realizan los microorganismos, se han podido identificar las diferentes enzimas involucradas en la degradación aeróbica de MTBE. A continuación se describen en detalle los grupos a los que pertenecen.

Citocromo P450

El principal responsable del metabolismo oxidativo de los compuestos xenobióticos ambientales se denomina citocromo P450 (Chauvaux y colaboradores, 2001). En realidad, es una familia de hemoproteínas presentes en numerosas especies, habiéndose identificado más de 2000 isoformas diferentes **(Figura 6.1.1)**. Según Urlacher y colaboradores (2012), constituyen una gran familia de monooxigenasas para llevar a cabo reacciones oxidativas difíciles como: *O*- desalquilación, *N*-desalquilación, *S*-oxidación, epoxidación de compuestos exógenos y endógenos o hidroxilación del enlace CH. Lopes-Ferreira y colaboradores (2006) indicaron que el citocromo P450 era codificado e inducido por los genes *eth* en cepas bacterianas como *Rhodococcus ruber* IFP2001, *Rhodococcus zopfii* IFP2005 y *Mycobacterium sp.* IFP 2009 estando implicado en el paso inicial de la degradación de MTBE **(Figura 6.1.2.)**.

Otros estudios, han demostrado que las monooxigenanas también están involucradas en la oxigenación del TBA, como por ejemplo en la cepa *Hydrogenophaga flava* ENV735 (Hatzinger y colaboradores, 2001).

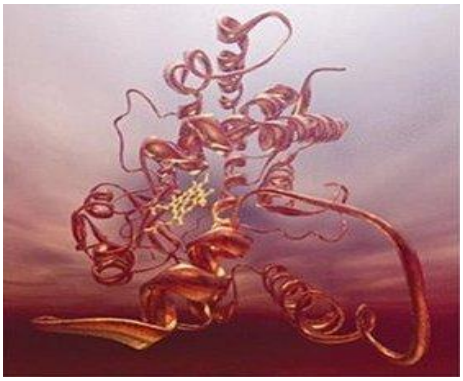

Figura 6.1.1 Estructura del citocromo P-450. Fuente: "Cytochrome P450: Role in the Metabolism and Toxicity of Drugs and other Xenobiotics (Issues in Toxicology). RSC Publishing 2008.

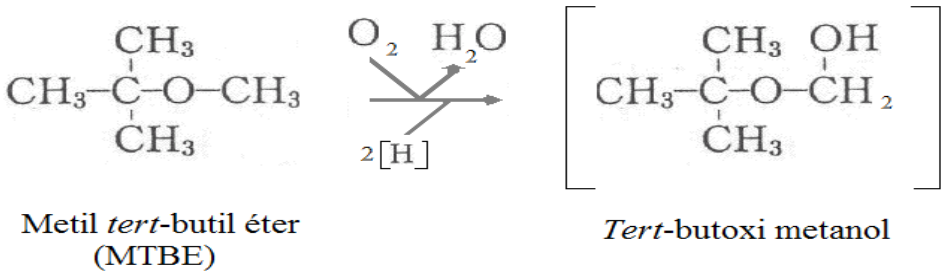

Figure 6.1.2 Oxidación del MTBE por el citocromo P450 para producir *tert*-butoxi metanol. Fuente: Adaptada de Lopes-Ferreira y colaboradores, 2006.

Otras monooxigenasas implicadas en la degradación de éteres

Alcano monooxigenasas

Fueron identificadas por primera vez en la cepa bacteriana *Pseudomonas putida* GPO1(Egglink y colaboradores, 1987,1998). Son inducidas en bacterias del género *Pseudomonas* durante el metabolismo de alcanos utilizados para el crecimiento (Morales y colaboradores, 2009) y también entre otras especies bacterianas (Lopes Ferreira et al., 2006; Hyman et al., 2013; Bravo y colaboradores, 2015). **AlkB** (monooxigenasa no hemo), con localización en la membrana, permite a las bacterias utilizar alcanos como fuente de carbono y energía, resultando de gran interés biotecnológico en procesos de biorremediación. Esta enzima cataliza la siguiente reacción química:

alcano + rubredoxina $_{(reducida)}$ + O_2 $\rightleftharpoons$ un alcohol primario + rubredoxina $_{(oxidada)}$ + H_2O

Actúa sobre alcanos de entre 6 a 12 carbonos. El componente catalítico de las alcano monooxigenasas es una proteína integral de membrana que engloba tres átomos de hierro no-hemo en estado de oxidación $^{+2}$. Este sistema transfiere un átomo de oxígeno desde el oxígeno molecular al carbono terminal de hidrocarburos alifáticos de entre 6 a 12 carbonos, encaminando a la formación de un alcohol primario. El otro átomo de oxígeno se reduce por medio de electrones suministrados por una reductasa de rubredoxina dependiente de NAD, vía transportador de electrones rubredoxina 2. Este sistema enzimático puede oxidar un gran abanico de sustratos. En concreto, son varios los autores que han encontrado la presencia de estas enzimas en especies de cepas bacterianas utilizadas en la biodegradación del MTBE (Hanson, 1999; Deeb, 2000; Kane, y colaboradores 2007). Por ejemplo, *Pseudomonas citronellolis* UAM-Ps1, co-transforma metabólicamente MTBE gracias a la acción de una alcano monooxigenasa (Bravo y colaboradores, 2015).

Esterasas

Las esterasas son enzimas catalizadoras de reacciones de hidrólisis de ésteres carboxílicos (carboxiesterasas), amidas (amidasas), ésteres de fosfato (fosfatasas), etc.

Uno de los compuestos intermediarios con mayor importancia que presenta la ruta degradativa del MTBE, es el TBA. Como la **Figura 6.1** recoge, las esterasas actúan en las primeras etapas de la degradación de MTBE, hidrolizando TBF a TBA (Lopes-Ferreira y colaboradores, 2006). Este metabolito se suele detectar en numerosas aguas subterráneas contaminadas con MTBE debido a la degradación parcial de éste, exhibiendo una gran toxicidad (Babe y colaboradores, 2007).

Dehidrogenasas y otras enzimas

En todos los seres vivos, las enzimas alcohol deshidrogenasas (ADH) actúan sobre los alcoholes primarios, secundarios y hemiacetales. Son un grupo de siete enzimas que facilitan la interconversión entre alcoholes y aldehídos o cetonas con la reducción de NAD^+ a NADH. En las bacterias, algunas alcohol deshidrogenasas catalizan la reacción opuesta como parte de la fermentación alcohólica. La reacción que catalizan es:

$$\text{Alcohol} + NAD^+ \rightleftharpoons \text{Aldehído o Cetona} + NADH$$

MpdB y MPDC (alcohol deshidrogenasas), son enzimas que participan regulando la conversión de 2-metil 1,2 propanodiol (2-M1,2-PD) a ácido 2 hidroxi-isobutírico (HIBA) en la ruta metabólica. En el estudio llevado a cabo por Lopes-Ferreira y colaboradores (2006) fueron aisladas de *M. austoafricanum* IFP 2012, y posteriormente clonadas y expresadas en *M. smegmatis* mc2 155; más tarde se observó que podían inducirse durante el crecimiento de la cepa IFP2012 en MTBE. En 2006, Rohwerder y colaboradores describieron un mutasa dependiente de cobalamina implicada en la degradación de 2-HIBA al 3-hidroxi butiril CoA.

Schafer y colaboradores en el año 2007, encuentran una posible enzima que degrada el TBA a 2-HIBA, la cual se asemeja a una ftalato dioxigenasa. No obstante, debido a la reacción que cataliza esta enzima, se describió como una monoxigenasa (Rohwerder, 2009. Comunicación personal).

7. Técnicas de biorremediación en aguas contaminadas con MTBE

Se define el término "biorremediación" como "grupo de tratamientos que aplican sistemas biológicos para catalizar la transformación de compuestos químicos en otros menos tóxicos o llevar a cabo su mineralización" y se muestra como una óptima opción, la cual "utiliza organismos vivos fundamentalmente bacterias, pero también hongos, levaduras, plantas y algas para absorber, degradar o transformar los contaminantes presentes en el medio ambiente, retirándolos, inactivándolos o atenuando su efecto en suelo, agua y aire" (Atlas y Unterman, 1999; Hughes y colaboradores, 2000). De hecho, es considerado el mejor método para eliminar los compuestos oxigenantes de las masas de agua, ya que las técnicas convencionales no son ni eficientes ni rentables (Lopes-Ferreira y colaboradores, 2006). En este sentido, Eixarch y Constanti (2010) afirmaron que la principal ventaja de la biorremediación es que es una técnica no invasiva, además de rentable.

Los resultados obtenidos en estudios de campo han concluido que la atenuación natural del MTBE es un proceso muy lento y en algunos casos indetectable (Johnson y colaboradores, 2000). En cambio, otros estudios de campo sobre la aplicación de tratamientos de biorremediación *in situ*, publicados por la Agencia de Protección Ambiental (EPA), han demostrado cierta efectividad. Así, Wilson y colaboradores (2002) indicaron resultados positivos al aplicar biorremediación *in situ* por bioestimulación mediante la difusión de oxígeno en aguas subterráneas contaminadas por una pluma anaeróbica de MTBE. Otras investigaciones han demostrado, por ejemplo, que el uso en suelos contaminados de biobarreras creadas por inoculación con grandes cantidades de cultivos bacterianos procedentes de ambientes contaminados con MTBE y una buena oxigenación, son efectivos.

Por otro lado para mejorar y promover una mejor biorremediación, se fomentó la adición en campo de microorganismos que se adaptaban y degradaban MTBE y con ello aumentar la población microbiana (*bioaumentación*). También fue probada la oxigenación asociada con la bioaumentación, pero sólo mejoró la biodegradación cuando la oxigenación estaba presente, sin ningún tipo de bioaumentación (Salanitro y colaboradores, 2000).

Otra estrategia fue promover la biodegradación cometabólica de MTBE añadiendo un alcano, tal como propano (co-sustrato) bajo condiciones aeróbicas. Sin embargo, estos procesos mencionados tuvieron sus críticas; los argumentos dados fueron que la adición de propano u otro alcano, incrementaba la demanda de oxígeno junto con los costos y que la bioaumentación no era necesaria ya que solamente había que darles tiempo a los microorganismos ya presentes en el suelo para su adaptación a las nuevas condiciones.

La *fitorremediación* es otra tecnología biológica que puede aplicarse para la remediación de masas de agua contaminada con MTBE aunque en la mayoría de los estudios llevados a cabo, el oxigenante no sufre degradación, sino que son las plantas las que actúan como "bombas extractoras", existiendo correlación positiva entre la cantidad de MTBE eliminado, el agua transpirada y la superficie foliar existiendo sólo un transvase del contaminante de un compartimento medioambiental a otro (Rubin y Ramaswami, 2001). Chard y colaboradores (2001), observaron que un grupo de árboles maduros absorbían este oxigenante y atenuaban y controlaban el movimiento de una columna de agua subterránea de MTBE.

El tratamiento biológico de aguas tanto superficiales como subterráneas contaminadas con MTBE también es posible mediante el uso de biorreactores con biomasa fija y en suspensión, aunque la mayor efectividad de los primeros se ha puesto de manifiesto en numerosos estudios (Fortin y colaboradores, 1999; Acuna-Askar, 2000; Kharoune y colaboradores, 2001; Moreels y colaboradores, 2004; Hu y colaboradores, 2004; Maciel y colaboradores, 2008).

8. Biopelículas y Exopolisacáridos (EPS). Estudios e Importancia

En la Naturaleza, las bacterias existen bajo dos formas o estados diferentes; bacterias adheridas a una superficie establecidas como biopelícula ó *biofilm*, y como bacterias planctónicas, de libre flotación. Sólo el 1% de todas las células bacterianas que existen, están en estado planctónico; el 99% restante lo hacen en calidad de biopelícula (Sanclement y colaboradores, 2005; Ramadan, 2006).

Las biopelículas bacterianas representan una estrategia de supervivencia, ya que dicha formación les confiere protección frente a fluctuaciones medioambientales de temperatura, humedad, pH y presencia de agentes tóxicos, al igual que concentra nutrientes y facilita la eliminación de desechos (Post y colabradores, 2004).

Los biopelículas se definen como "estructuras complejas formadas por agregados de células embebidas en una matriz polimérica de naturaleza orgánica y origen microbiano, adheridas a un material o interfase que puede ser de naturaleza abiótica (rocas, metales, vidrios, plásticos, etc.) o biótica (mucosa intestinal, plantas, etc.,). En ellas, las células exhiben un estado metabólico alterado comparadas con el crecimiento planctónico correspondiente, especialmente con respecto a la trascripción de genes y a las interacciones".

La estructura que presenta una biopelícula puede ser en general considerada como universal, pero el conjunto de cada agrupación formada por microorganismos en una biopelícula es exclusiva de su género. Además, en estado planctónico, las células bacterianas tienden a la división, en cambio en el interior de una biopelícula no se produce, atribuyéndose este fenómeno a la adquisición de un fenotipo alterado (Chole y colaboradores, 2003).

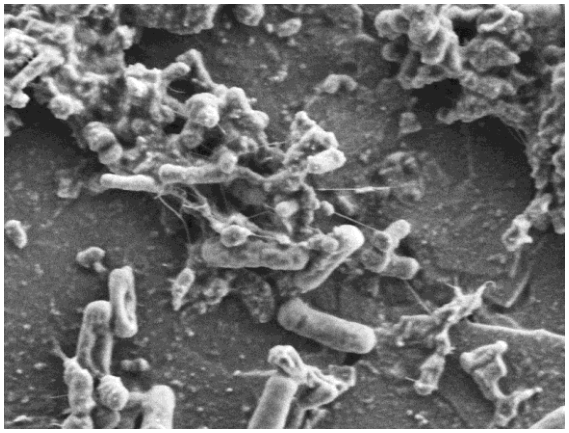

Figura 8.1. Biopelícula de la cepa bacteriana *Agrobacterium sp.* MS2. Fuente: esta Tesis Doctoral.

Aparte de biomasa celular, una biopelícula está constituida por una matriz polimérica y sustancias inorgánicas (Donlan y colaboradores, 2002).Aproximadamente el 97% del peso de la matriz polimérica es agua, presentándose como solvente o unida a las células. También, la matriz está formada por sustancias poliméricas extracelulares (EPS), "exopolisacáridos" o "exopolímeros" (Wingender y colaboradores, 1999), las cuales son secretadas por los mismos microorganismos que integran la biopelícula bien al medio o bien quedan adheridos a la célula en forma de cápsula (Margesin y Schinner, 2001; Mancuso Nichols y colaboradores, 2005), para así, poder resistir o superar adversidades como condiciones extremas, cambios fisicoquímicos del medio en donde habitan, etc.

La calidad nutricional del medio circundante en el que se desarrolla una biopelícula influye en la producción de EPS, ya que ha sido observado cómo se produce un aumento en el número de células bacterianas adheridas cuando hay un incremento en la concentración de nutrientes. Thomas y Nakaishi (2006) observaron que se promueve la síntesis de EPS cuando hay una disponibilidad excesiva de carbono y/o limitación de nitrógeno, potasio o fosfato.

En general, la estructura y formación de una biopelícula depende de los aspectos medioambientales y del sustrato al cual se adhiere. Por tanto, bajo condiciones de estrés, debido por ejemplo a la presencia de inhibidores del crecimiento microbiano, al estrés nutricional o a la presencia en nuestro caso de compuestos xenobióticos (oxigenantes de las gasolinas), la composición y cantidad del EPS puede verse alterado (Sponza, 2002).

8.1. Composición química de los exopolisacáridos

Los exopolisacáridos están compuestos fundamentalmente por carbohidratos, formando homo o heteropolímeros, conteniendo además múltiples sustituyentes inorgánicos e orgánicos y proteínas. Normalmente, los EPS heteropolisacáridos están compuestos por unidades duplicadas que cambian en el tamaño; desde disacáridos hasta 24 tipos de monosacáridos o más, conteniendo grupos acilos adicionales (Sutherland, 2001). En cambio, sólo un tipo de monosacárido componen los homopolisacáridos, aunque se diferencien en su propiedades y estructura.

Carbohidratos

Los carbohidratos constituyentes de los polisacáridos microbianos, tienen una gran diversidad. Según Lindberg (1990), D-galactosa, D-glucosa, y D-manosa, son los azúcares más representados en los EPS bacterianos, encontrándose también asiduamente los monómeros L-ramnosa, L-fucosa, y las 6-desoxihexosas. Existen polisacáridos que contienen azúcares más extraños, como por ejemplo: L-hexosas, galactosa y glucosa en su configuración furanosa o N-acetil D-galactosamina y N-acetil-D- glucosamina. Por otro lado, la presencia en los EPS, en algunos casos, de ácidos urónicos (componentes

frecuentes de moléculas polisacarídicas), les confieren una naturaleza polianiónica. Según autores como Sutherland (2001), el ácido D-glucurónico, es el más habitual.

Sustituyentes orgánicos e inorgánicos

Dentro de los **sustituyentes inorgánicos** de la estructura de los EPS, se encuentran los cationes presentes fundamentalmente en polímeros polianiónicos. Cationes divalentes como Sr^{2+}, Ba^{2+} y Ca^{2+}, se encuentran unidos fuertemente a algunos alginatos, los cuales se incorporan durante la producción del polisacárido; aunque pueden quedar desplazados como por ejemplo por procesos de electrodiálisis, intercambio iónico, etc. Por otro lado, se encuentran los grupos fosfato, cuya presencia es más generalizada y localizada en polisacáridos producidos por bacterias Gram positivas. También, diversos grupos sulfato han sido hallados en algún exopolisacárido microbiano, aunque inicialmente se consideró que la existencia de estos grupos era exclusivo de polisacáridos de organismos eucariotas.

Sustituyentes orgánicos también aparecen en la composición de los EPS, como son los restos acetato; no contribuyen a la carga total de la macromolécula pero están implicados en su conformación molecular. Gracias a estos sustituyentes orgánicos, el polisacárido adquiere un naturaleza lipofílica, la cual le permite ser de gran utilidad en concretas aplicaciones industriales (Calvo y colaboradores, 2004; 2009). Los grupos acetilo en muchos EPS se encuentran en proporción estequiométrica con los monosacáridos presentes. Los ácidos urónicos unidos a los restos piruvato, otorgan al exopolisacárido una naturaleza aniónica; normalmente se encuentran unidos a una hexosa neutra, ya que se presentan en una proporción estequiométrica con los azúcares componentes del polímero. Lindberg y colaboradores, 1976, los localizó unidos a una metilpentosa o a un resto urónico.

Proteínas

Según autores como Sheng y colaboradores, 2010, las proteínas presentes en los EPS provienen generalmente de la lisis celular y de la secreción bacteriana. Desarrollan un papel fundamental en los procesos de agregación; también en funciones estructurales tales como formación y estabilización de la red de polisacáridos de la matriz debido a los péptidos capsulares y/o lectinas; y además, según Higgins y Novak, (1997) están implicadas en diversas funciones biológicas y enzimáticas, favoreciendo la adhesión bacteriana para la formación de las biopelículas. Las proteínas son la fracción de mayor importancia en la composición de los EPS, pero esta predisposición, cambia cuando las condiciones ambientales no son favorables (fuente de carbono escasa o inadecuada, o una alta temperatura) (Gao y colaboradores, 2013).

8.2. Biopelículas y biorremediación

En las últimas décadas han surgido numerosos estudios basados en el uso de biopelículas para restaurar ecosistemas contaminados con xenobióticos (Timberlake y colaboradores, 1988; Pavasant y colaboradores, 1996; Decho 2000; Singh y colaboradores, 2006). La protección que confiere la matriz polimérica de la biopelícula permite una mejor adaptación y supervivencia microbiana lo que conlleva un aumento de la degradación de los tóxicos, ya que en ésta la presencia de enzimas implicadas en la degradación de contaminantes es mayor (Eweis y colaboradores, 1997; Fortin y colaboradores, 1999). Además, la ausencia de corrosión y deterioro de las superficies y materiales que conforman los soportes o membranas inertes donde los microorganismos se adhieren, es también una característica fundamental y adicional para la utilización de las biopelículas en procesos de biorremediación.

Dentro de la biorremediación de aguas subterráneas contaminadas con diversos compuestos orgánicos semi-volátiles (como compuestos halocarbonados, nitroaromáticos u oxigenantes de las gasolinas, entre otros) ha cobrado un especial interés dentro de las tecnologías de biopelícula fija, el uso de biofiltros percoladores, biofiltros sumergidos, biorreactores de membrana y biorreactores de membrana extractiva (Livingston, 1991,1993; Freitas dos Santos y Livigstone, 1994; Eweis y colaboradores, 1997, 1998; Fortin y Deshusses, 1999a,b; Casey y colaboradores, 1999; Nicolella y colaboradores, 2000; Stefan y colaboradores, 2000; Hu y colaboradores, 2004; Purswani y colaboradores, 2011).

Un biofiltro aireado sumergido consiste en un lecho de medio de soporte sumergido sobre el que se desarrollan las bacterias y a través del cual se hace circular el agua contaminada a tratar, ya sea en un flujo descendente o ascendente en presencia de oxígeno. Son varias las ventajas que presenta esta tecnología frente a otros procesos, pero dentro de los inconvenientes que presenta, en concreto para el tratamiento de aguas subterráneas contaminadas con oxigenantes de las gasolinas como MTBE, ETBE o TAME, se pueden nombrar el bajo crecimiento bacteriano (aunque sigue siendo superior la densidad bacteriana en comparación con procesos de células inmovilizadas) y el denominado fenómeno de *air stripping* (pérdida por volatilización del compuesto oxigenante) cuando el flujo de aire es inyectado dentro del biorreactor (Purswani y colaboradores, 2011, 2014).

Los sistemas de biorreactores de membrana extractiva (*EMBR:* Extractive membrane bioreactor) como su nombre indica, utilizan una membrana, la cual resiste selectivamente la transferencia de diferentes componentes de un fluido, efectuando así una separación física de los constituyentes. Por tanto, esta membrana adquiere la característica de ser permeable, además de poseer un alto grado de selectividad tanto para el compuesto contaminante como para el oxígeno, evitándose el problema de *air stripping* comentado anteriormente, ya que éste será utilizado a demanda por el biocultivo a través de esta

membrana extractiva. La capacidad de rendimiento del tratamiento biológico mediante la explotación de la capacidad que tiene la membrana, hace que se consiga un elevado grado en la separación, al mismo tiempo que permite el transporte de los componentes de una fase a otra. Estos biorreactores por tanto, consisten esencialmente en la combinación de los sistemas de reactores biológicos y en los sistemas de membrana.

Por ejemplo, esta tecnología ha sido usada con éxito para el tratamiento de agua contaminada con diversas sustancias volátiles (compuestos organoclorados, fundamentalmente) (Livingston, 1991,1993; Freitas dos Santos y Livigstone, 1994). Estos últimos autores, por ejemplo, utilizaron un biorreactor de membrana extractiva inoculado selectivamente con cepas bacterianas, para evaluar la eliminación de 2-dicloroetano (DCE). Se eliminó el 99% del compuesto xenobiótico tras el tiempo de operación y además se redujo la pérdida del mismo por *air stripping*.

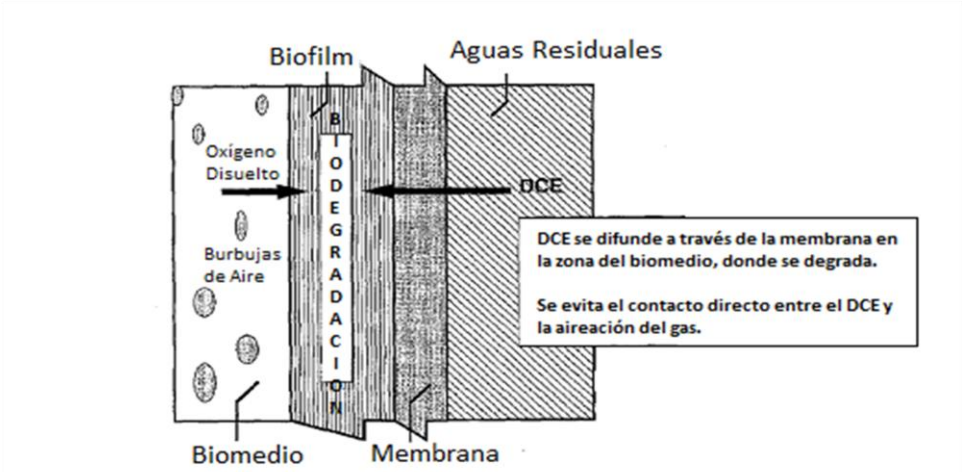

Figura 8.2. Adaptada de Freitas dos Santos and Livigstone, 1994.

8.3. Reactores de membrana extractiva con desarrollo de biopelícula

Los reactores de membrana extractiva con desarrollo de biopelícula (*EMBFR*, Extractive Membrane Biofilm Reactors) son una modificación de la anterior tecnología basada en el uso de membranas semipermeables, a través de las cuales los contaminantes migran al compartimento biológico en el que los microorganismos con capacidades para biotransformar y mineralizar un determinado contaminante, crecen en un medio rico en sales minerales, formando una biopelícula en la superficie de la membrana. Este sistema de tratamiento utiliza una barrera física (membrana hidrófoba), que separa el sistema biológico para el tratamiento de agua contaminada con xenobióticos y permite el paso selectivo del compuesto de estudio. Esta configuración del sistema, ofrece la posibilidad de controlar las condiciones de crecimiento de microorganismos en el compartimento biológico para así asegurar una degradación eficiente de los compuestos contaminantes, independientemente de las condiciones prevalecientes en el efluente a tratar. En los sistemas aeróbicos, el oxígeno puede ser suministrado también a través de membranas semipermeables evitando así la pérdida del contaminante por volatilización.

Este sistema ha sido utilizado por ejemplo por Katsivela y colaboradores (1999) para la eliminación de 1,3-dicloropropeno (DCPE) presente en aguas residuales industriales y altamente reactivo y tóxico. En este sistema, la membrana extractiva separaba el biomedio del agua residual industrial, en términos de la concentración de productos químicos tóxicos, fuerza iónica y en términos de pH. El biofilm formado en la superficie de la membrana de silicona en contacto con el biomedio fue capaz de mineralizar el tóxico (DCPE) después de haber sido difundido a través de la membrana.

Sin embargo, Ferreira y Livingston (2000), utilizaron también esta tecnología para el tratamiento de aguas contaminadas con monoclorobenzeno y 1,2-dicloroetano pero ellos finalmente concluyeron que la formación de una biopelícula sobre las membranas

semipermeables disminuyó la eficiencia del sistema, en términos de eliminación de contaminante, al limitar el paso de éste a través de la membrana semipermeable. Para estos autores la biopelícula sólo era importante para mantener activas las poblaciones microbianas en el caso de alternancia de contaminante y por la tanto en periodos en los cuales no estuviera presente el compuesto a degradar.

Basándonos en los resultados previos obtenidos por nuestro Grupo de investigación sobre el uso de biofiltros sumergidos aireados para el tratamiento de aguas subterráneas contaminadas con MTBE, ETBE y TAME (Purswani y colaboradores, 2011; 2014), en esta Tesis Doctoral se ha estudiado la eficiencia de la tecnología *EMBFR* para la biorremediación de aguas subterráneas contaminadas con MTBE, siendo la primera vez que se aplica para la eliminación de este tipo de contaminantes.

9. Métodos para la determinación de la toxicidad de aguas contaminadas por oxigenantes de las gasolinas

Durante los últimos años, los ensayos de ecotoxicidad han adquirido un papel importante en la evaluación de la toxicidad potencial de muestras medioambientales. Previamente, algunos autores (Pedersen y colaboradores 1988; Ruíz y colaboradores, 1997; Boluda y colaboradores, 2002) ya indicaron que para evaluar ésta y establecer la naturaleza del contaminante, era necesaria la combinación de ensayos ecotoxicológicos y químicos, ya que con ello, se podría predecir cómo pueden llegar a comportarse las sustancias tóxicas en el medioambiente.

Normalmente, en los ensayos de ecotoxicidad son utilizados organismos representativos para la detección de los efectos crónicos y agudos que puedan provocar la presencia de un determinado contaminante. Pulgas de agua (*Daphnia magna*), algas (*Zooglea* sp.) o bacterias marinas luminiscentes (*Allivibrio fisheri*, antes *Vibrio fischeri*)

son algunos de los organismos utilizados en los ensayos. Estos microorganismos presentan ventajas como: 1) responder de manera rápida a los cambios producidos en el medio ambiente, 2) poseer similares rutas bioquímicas a las de los organismos superiores y 3) presentar cortos ciclos de vida (García, 2004).

La **Tabla 2,** recoge los efectos del MTBE sobre diversos organismos de agua dulce (bacterias, microalgas, invertebrados, peces y anfibios)**.** Curiosamente, los organismos más grandes, incluyendo vertebrados e invertebrados, suelen ser menos sensibles a la exposición de este compuesto que los microorganismos.

Tabla 2. Efectos del MTBE en organismos de agua dulce. Fuente: Adaptada de Werner y colaboradores, 2001.

Medidas de los efectos del MTBE en organismos de agua dulce

Especies	Efecto	Concentración efectiva	Referencia
Bacterias			
Salmonella typhimurium	Reducción del números de células (48 h)	7.4 mg/L	Kado y colaboradores, 1998
Vibrio phosphoreum	Reducción de la luz de emisión (15 min)	41.8 mg/L	Gupta and Lin, 1995
Microalgas			
S. capricornutum	EC50	184 mg/l (medido)	BenKinney et al., 1994
Synechococcus leopoliensis	Disminución en el crecimiento (3 días)	2400 mg/l (nominal)	Rousch and Sommerfeld, 1998
Invertebrados			
Brachionus calyciflorus	LC50 (24 h)	960 mg/l (medido)	Werner and Hinton, 1998
Daphnia magna	LC50 (96 h)	681 mg/l (medido)	BenKinney et al., 1994
Anfibios			
Rana temporaria (tadpoles)	LC50 (48 h)	2500 mg/l	Paulov, 1987
R. temporaria (tadpoles)	Aumento de peso, la estimulación de la metamorfosis	100 mg/l	Paulov, 1987
Peces			
Lepomis macrochirus	LC50 (96 h)	1054 mg/l	Stubblfield et al., 1998
O. mykiss	LC50 (96 h)	1237 mg/l	BenKinney et al., 1994

*.LC, concentración letal; EC, concentración de efecto.

Por otro lado, también se han llevado a cabo ensayos de ecotoxicidad utilizando semillas de *Lactuca sativa*; con ellas se ha evaluado en concreto, el efecto que provoca el MTBE en el crecimiento de sus raíces o en la tasa de germinación. (Vosahlikova y colaboradores, 2006). Mortelmans y Zeiger (2000) evaluaron el potencial mutagénico también de MTBE, mediante el denominado test de Ames, utilizando la cepa bacteriana *Salmonella typhimurium his* la cual no demostró genotoxicidad a esta sustancia.

Otros autores han utilizado bacterias luminiscentes como sensibles indicadores a la contaminación con MTBE como por ejemplo, *Aliivibrio fischeri* (anteriormente *Vibrio fischeri)*, cepas de *Escherichia coli* ó *Pseudomonas fluorescens* (Kragelund y colaboradores, 1995; Unge y colaboradores, 1999; Petersen y colaboradores, 2001). También han sido utilizadas cepas de la levadura, *Saccharomyces cerevisiae* (Eldridge y colaboradores, 2011) o biomasa microbiana mixta (Roslev y colaboradores, 2015).

9.1. El bioensayo Microtox®

El ensayo Microtox® ha sido utilizado como bioensayo de toxicidad en los últimos años para la evaluación de la toxicidad de muestras medioambientales. Diversos campos de estudio lo han aplicado en sus investigaciones, como en la identificación de fuentes de contaminación en el monitoreo de aguas superficiales, en la detección de la contaminación en aguas de bebida, en suelos contaminados en procesos de remediación y en efluentes de plantas de tratamiento de aguas, entre otros (García, 2004). También es aplicable a suelos y a sedimentos (muestras sólidas) como a sus extractos acuosos (Onorati y colaboradores, 2004), aunque inicialmente fue aplicado para evaluar la toxicidad aguda de aguas residuales y naturales. Este ensayo es menos costoso y se realiza con mayor rapidez en comparación con otros ensayos existentes; además, es ampliamente utilizado por su sensibilidad, aplicación para contaminantes inorgánicos e orgánicos, y por la

reproducibilidad que posee (Riisberg y colaboradores, 1996; Salizzato y colaboradores, 1998; Onorati y colaboradores, 2004).

El fundamento del bioensayo Microtox®, se basa en la monitorización de los cambios en las emisiones de luz natural que presenta una bacteria marina luminiscente *Allivibrio fischeri* (anteriormente *Vibrio fischeri*) en presencia de agentes contaminantes. Es una bacteria de la familia *Vibrionaceae*, Gram negativa, anaerobia facultativa, siendo la bioluminiscencia (producción de luz por parte de ciertos organismos vivos) su característica más relevante.

La luminiscencia es producida, por procesos metabólicos normales que tienen lugar en la propia bacteria. A partir de energía química, la enzima luciferasa cataliza la reacción de producción de energía luminosa, estando unida al metabolismo respiratorio del microorganismo. La reducción de la bioluminiscencia, es producida por la inhibición del metabolismo de la bacteria al estar en contacto con los tóxicos, y es directamente proporcional a la presencia de contaminantes en la muestra. Los resultados obtenidos de toxicidad en Microtox®, son expresados como la concentración del agente tóxico, que produce una reducción del 50% de la luminiscencia inicial de *Vibrio fischeri* (EC_{50}) (Onorati y colaboradores, 2004).

En 1984, la EPA recomendó el bioensayo Microtox® para la evaluación de la toxicidad aguda de muestras de agua ya que este bioensayo mostró con resultados anteriores obtenidos en *Daphnia* y en peces, buena correlación además de una mayor sensibilidad.

Los resultados obtenidos de la aplicación del bioensayo Microtox® para evaluar la toxicidad de MTBE han sido, en general, de gran utilidad. Vosahlikova y colaboradores (2005), evaluaron mediante este bioensayo la toxicidad de una fuga de MTBE al medioambiente. Otros autores como Roslev y colaboradores (2015), también lo utilizaron para evaluar y vigilar no sólo la contaminación por parte de MTBE sino también la

generada por productos de degradación como es el caso del TBA y el formaldehído. Los efectos inhibitorios que se produjeron, pudieron ser observados en biomasa microbiana mixta, seis cepas bacterianas y en levaduras.

En la presente Tesis Doctoral, el bioensayo Microtox® ha sido utilizado para revelar la toxicidad exhibida por las cepas bacterianas seleccionadas (resultados complementarios a los obtenidos de los ensayos de degradación y *screening* génico), al objeto de poder realizar una selección más real de microorganismos para su posible uso como inóculos selectivos, y para la evaluación de la ecotoxicidad del biomedio y efluente tratado mediante reactores de membrana extractiva con desarrollo de biopelícula (EMBFR) inoculados con las cepas bacterianas seleccionadas.

II. OBJETIVOS

El metil *tert*-butil éter (MTBE) ha sido el compuesto oxigenante más utilizado en la gasolina reformulada debido a su fácil producción, favorables características de mezcla y transferencia, así como por su bajo costo; sin embargo, este compuesto es un importante contaminante de las aguas subterráneas y constituye una amenaza veraz y ascendente para la calidad de las mismas.

Por ello, el desarrollo de nuevas tecnologías para la eliminación de compuestos semi-volátiles (entre ellos, MTBE) presentes en las masas de agua, constituye un elemento muy importante para restaurar la calidad de las mismas y por consiguiente, el medioambiente.

El **objetivo principal** de esta Tesis Doctoral, ha sido el estudio técnico y biológico de un sistema de biorreactor de membrana extractiva con desarrollo de biopelícula para el tratamiento de aguas contaminadas con metil *tert*-butil éter (MTBE).

Para alcanzar este objetivo principal, se establecieron los siguientes **objetivos específicos**:

1. Selección y aislamiento de cepas bacterianas con capacidad para utilizar MTBE como única fuente de carbono y energía a partir de muestras ambientales.

2. Estudio de la capacidad de las cepas seleccionadas para adherirse sobre las membranas extractivas tubulares, componentes de los biorreactores de membrana extractiva con desarrollo de biopelícula.

4. Estudio de la eficiencia de la tecnología de biorreactores de membrana extractiva con desarrollo de biopelícula para la eliminación de MTBE de aguas contaminadas, bajo distintas condiciones de operación.

5. Evaluación de la ecotoxicidad exhibida por los efluentes tratados.

Esta Tesis Doctoral ha sido financiada y desarrollada en el ámbito del proyecto titulado: "Biorreactores de membrana extractiva como alternativa para el tratamiento de aguas contaminadas con oxigenantes y compuestos aromáticos (BTEX) presentes en la formulación de las gasolinas", concedido por la Junta de Andalucía a través del Programa Proyectos de Excelencia (Referencia del proyecto P10-RNM-6153) y cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER).

III. RESULTADOS

RESULTS

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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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CHAPTER 1

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 that 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 monoxygenase 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.

Keywords: MTBE; bioremediation; monooxygenases; ecotoxicity; BTEX.

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 physicochemical 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 et al., 2000; Rohwerder et al., 2006; Lyewet 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).

The biodegradation of ether oxygenates and *tertiary* alcohol metabolites occurs preferentially under aerobic conditions (Deeb et al., 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 ErbaS.p.a, DivAnalitica 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 PanreacQuimica 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}; lipoicacid 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 a 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, 500ml of the sample was filtered through a 0.45µm 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, 1ml 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 a MTBE-saturated atmosphere at 28°C for 30 days. Different colony morphologies were selected andstreaked 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 (30ºC) and agitation (150 rpm) for 4 days, followed by centrifugation of 1 ml of culture (for 1min at 14,000rpm). 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 28ºC in a 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 strains in modified FTW medium without MTBE and/or BTEX was evaluated and the data obtained were considered as “biotic controls”.

MTBE biodegradation assays

Those bacterial strains that showed positive growth on MTBE and/or BTEX in the preceding assay were tested for MTBE biodegradation. For this, each strain was inoculated into a sterile glass tube with 5 ml of modified FTW medium supplemented with 150 mg l^{-1} MTBE and incubated for 4 days at 28ºC under controlled agitation conditions. A volume of 500 µl of these bacterial pre-grown cultures was inoculated in glass vials (125 ml total capacity) with 25 ml of modified FTW medium plus 150 mg l^{-1} MTBE, sealed with polytetrafluoroethylene (PTFE) stoppers and incubated for 8 days as described above. Controls glass vials [non inoculated (abiotic control) and inoculated in mineral medium without MTBE (biotic control)] were included in this assay. After 12, 24, 48, 72, 96 and 192 h incubation, growth (as OD_{600nm}) and the MTBE concentration was determined. All the experiments were performed in triplicate.

MTBE analysis

The gas chromatography mass spectrometry (GC/MS) headspace technique was used to quantify the remaining concentrations of MTBE in the samples following the methodology described by Purswani et al., (2011). A volume of 1.5 ml of the unfiltered inoculated and control samples was placed into 2ml vials and clamped. The samples were heated at 90°C for 90 min, followed by the injection of 50 µl of the gas phase into the GC/MS equipment (Hewlett-Packard 6890 GC coupled to a MS Hewlett-Packard 5973 mass selective detector, Palo Alto, CA, U.S). 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: 40ºC (3.5 min), 10ºC min^{-1} up to 85ºC, 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). This same headspace technique allowed the detection of MTBE and TBA simultaneously. All samples were performed in triplicate and the mean and standard deviations calculated.

DNAextraction

Cells were pregrown on TSB medium for 24 or 96 h. Cells from a 1.5 ml aliquot were harvested by centrifugation. Extraction of DNA was performed using the DNeasyBlood & Tissue DNA kit (QIAGEN®).

Phylogenetic identification of gasoline oxygenate-degrading strains

Selected bacterial isolates were identified by sequence analysis of the gene encoding 16S rRNA (16S rDNA). Primers fD1 (5´-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3´) and rD1 (5´-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3´), (Weisburg et al., 1991), synthetized by Sigma Genosys (Dorset, UK), were used to amplify and sequence part of the 16S rRNA gene as described previously by Pozo et al., (2002). The DNA sequences were processed, assembled, and edited for sequencing errors. Closely matching sequences were found in the GenBank database using the BLASTn algorithm, (Altschul et al., 1997). Multiple sequence alignment was performed using ClustalW (Thompson et al., 1994), and the phylogenetic tree was constructed using QuickTree (Howe et al., 2002). The stability of relationships was assessed by means of a bootstrap analysis of 1,000 datasets. The tree was illustrated using iTol (http://itol.embl.de/).

Presence of monooxygenase genes

Polymerase chain reaction (PCR) amplification of known ether-degrading monooxygenases such as EthB and AlkB were tested on total genomic DNA extracted from isolated bacterial strains using a thermocycler (Mastercycler® pro, Eppendorf, Germany). The PCR conditions for the *alkB* gene were: 96ºC for 7 min; 30 cycles of 96ºC for 2 min, 55ºC for 30 s, 40ºC for 40s, and 72ºC for 1 min; and an extension of 10min at 72ºC. The PCR conditions for the *ethB* gene were: 96ºC for 7 min; 30 cycles of 96ºC for 30 s, a 40-60ºC gradient for 40 s and 72ºC for 1 min; and an extension of 10 min at 72ºC. The primers for *ethB* amplification were (Jechalke et al., 2011): ethB_for: 5´-AGGAGGAATCTATGACACTG-3´ and ethB_rev: 5´-GGCATCGGCATCACTTCGGGTAG-3´. The primers for *alkB* were: alk-H1F – CIGIICACGAIITIGGICACAAGAAGG and alk-H3R – IGCITGITGATCIIIGTGICGCTGIAG (where I = Inosine) (Chénier et al., 2003). An extra set of primers for P450 monooxygenase was used (Hyun et al.,1998): P450-Actino-F: TSCTSCTSATCGCSGGSCACGAGAC and P450-Actino-R: GCSAGGTTCTGSCCSAGGCACTGGTG (where S = G+C). For this last set of primer pairs, the following PCR conditions were used: 94ºC for 3 min; 30 cycles of 94ºC for 35 s, 54ºC for 35 s, 72ºC for 30s; and an extension of 7 min at 72ºC.

Toxicity analysis

The Microtox® bioassay was used to measure acute toxicity (as EC_{50}) of the samples at 96 and 192 h after inoculation in each experiment. The toxicity test is based on the bioluminescence reduction of the microorganism *Vibrio fischeri* after 5 min exposure to the medium (Onorati et al., 2004) using the Microtox® Model 500 toxicity analyser

(Instrumentación Analítica S.A. Madrid, Spain). The effective concentration for 50% inhibition of luminescence (EC_{50}) after 5 min incubation was calculated with data reduction software using the methodology proposed by the manufacturer.To evaluate the toxicity (as EC_{50}) of MTBE at different concentrations (10 to 150 mg l^{-1}), a standard curve was constructed (data not shown).

Nucleotide accession numbers

The partial 16S rRNA sequences of the isolates identified in the present study have been deposited in the GenBank nucleotide sequence database under accession numbers: *Rhodococcus ruber* DD1 (KC625546.1), *Rhodococcus ruber* EE1 (KC625549.1), *Rhodococcus ruber* A5 (KC625544.1), and *Rhodococcus ruber* FF2 (KC625552.1), *Gordonia alkanivorans* FF5 (KC625553.1), *Nocardia nova* A6 (KC625545.1), *Rhodococcus qingshengii* DD6 (KC625547.1), *Corynebacterium* sp. SS5 (KF021240.1), *Micrococcus luteus* SS6 (KF021241.1), *Bacillus* simplex SH3 (KC625557.1), *Bacillus simplex* SH8 (KC625559.1), *Paenibacillus* sp.SH7 (KC625558.1), *Methylobacterium extorquens* MS1 (KC625555.1), *Agrobacterium* sp. MS2 (KF021239.1), *Sphingobium yanoikuyae* MS3 (KC625556.1), *Rhodococcus ruber* EE6 (KJ792868), *Cupriavidus basilensis* MA1 (KC625554.1), *Pseudoxanthomonas mexicana* EE5 (KC625550.1), *Enterobacter* sp. A4 (KF021237.1) and *Pseudomonas aeruginosa* MA2 (KF021238.1).

RESULTS

Pre-screening for MTBE-degrading microorganisms

Following enrichment and isolation steps of oxygenate-degrading microorganisms from the environmental samples as described in the Materials and Methods section, a total of 36 strains were isolated via direct plating and enrichment procedures after incubation on agar plates washed with fuel oxygenate-saturated atmosphere supplemented with MTBE. These strains were further grown on 96-well plates on minimal medium supplemented with fuel oxygenate, upon which only 13 bacterial strains showed any signs of growth (as $OD_{600\ nm}$).

Strains A4, A5 and A6 were directly isolated from hydrocarbon-contaminated groundwater. Strains SH3, SH7, SH8, SS5 and SS6 were from the soil of a pilot plant artificially contaminated with hydrocarbons (Silva-Castro et al., 2013). Furthermore, bacterial strains were isolated after MTBE enrichment steps using samples from hydrocarbon-contaminated soil (MS1, MS2 and MS3) and groundwater (MA1 and MA2).These bacterial strainswere tested for growth (OD_{600nm}) on modified FTW medium supplemented with MTBE and/or BTEX as a sole carbon and energy source, using sterile 96-well plates. Those that showed positive growth values in the preceding assay, in addition to bacterial strains DD1, DD6, EE1, EE5, EE6, FF2 and FF5 isolated from a previous study (Purswani et al., 2014), were pre-screened for their MTBE biodegradation ability in batch experiments, analysed for their monooxygenase genes, and were taxonomically identified using their 16S rRNA gene sequence.

Fig. 1 shows the classification of the 20 bacterial strains and closely related sequences from the GenBank nucleotide sequence database. Most of the bacterial strains

were Gram-positive bacteria belonging to the phyla *Actinobacteria* and *Firmicutes*, representing 45% and 15% of the total samples respectively. The remaining bacterial strains were grouped into the phylum *Proteobacteria*, under the classes *Alphaproteobacteria* (20%), *Betaproteobacteria* (5%) and *Gammaproteobacteria* (15%). Sequence comparison with those in the databases demonstrated the affiliation of strains DD1, EE1, EE6, A5, and FF2 to *Rhodococcus ruber* (≥99% identity), FF5 to *Gordonia alkanivorans* (100% identity), A6 to *Nocardia nova* (99% identity), DD6 to *Rhodococcus qingshengii* (99 % identity), SS5 to *Corynebacterium* sp (98% identity), SS6 to *Micrococcus luteus* (99% identity), SH3 and SH8 to *Bacillus simplex* (99% identity), SH7 to *Paenibacillus* sp.(99% identity), MS1 to *Methylobacterium extorquens* (100% identity), MS2 to *Agrobacterium* sp. (100% identity), MS3 to *Sphingobium yanoikuyae* (99% identity), MA1 to *Cupriavidus basilensis* (99% identity), EE5 to *Pseudoxanthomonas mexicana* (99% identity), A4 to *Enterobacter* sp. (99% identity) and MA2 to *Pseudomonas aeruginosa* (99% identity).

Growth and biodegradation pre-screening assays are also shown in **Fig. 1.** Within the phylum *Actinobacteria*, the bacterial strain A6 was the only strain that grew in medium supplemented with MTBE, BTEX and MTBE+BTEX, and exhibited a significant MTBE degradation potential of up to 60 mg l^{-1}. The phylum *Firmicutes* included three bacterial strains isolated from soil samples of a pilot plant (SH3, SH7 and SH8) that showed moderate MTBE degradation ability (30–35 mg l^{-1}). No growth in medium supplemented with BTEX was detected, however on BTEX+MTBE-supplemented medium, these strains were highly capable of growth.

The phylum *Proteobacteria* included those microorganisms capable of growth in medium supplemented with MTBE and aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylene (alone or together).Part of the pre-screening procedure involved

genetic screening for the presence of monooxygenase ether-degrading genes (alkane monooxygenases or cytochrome P450 monooxygenases) using various primer-pairs on the identified bacterial strains.

From the 20 microorganisms, only six (A5, A6, DD6, EE1, EE6, FF2) expressed some of these genes **(Fig. 2).** All of the microorganisms that contained monooxygenase genes were Gram-positive.Three of the six strains contained both alkane and cytochrome monooxygenases, i.e., strains A6, FF2 and EE6. Furthermore, amplification of different primers for cytochrome monooxygenases resulted in positive amplification and the presence of two different cytochrome monooxygenases in strain EE1. The primers described in Hyun et al., (1998) were specific for Actinobacteria, and in fact, those bacterial strains containing a monooxygenase gene that were amplified by these primers were Actinobacteria.

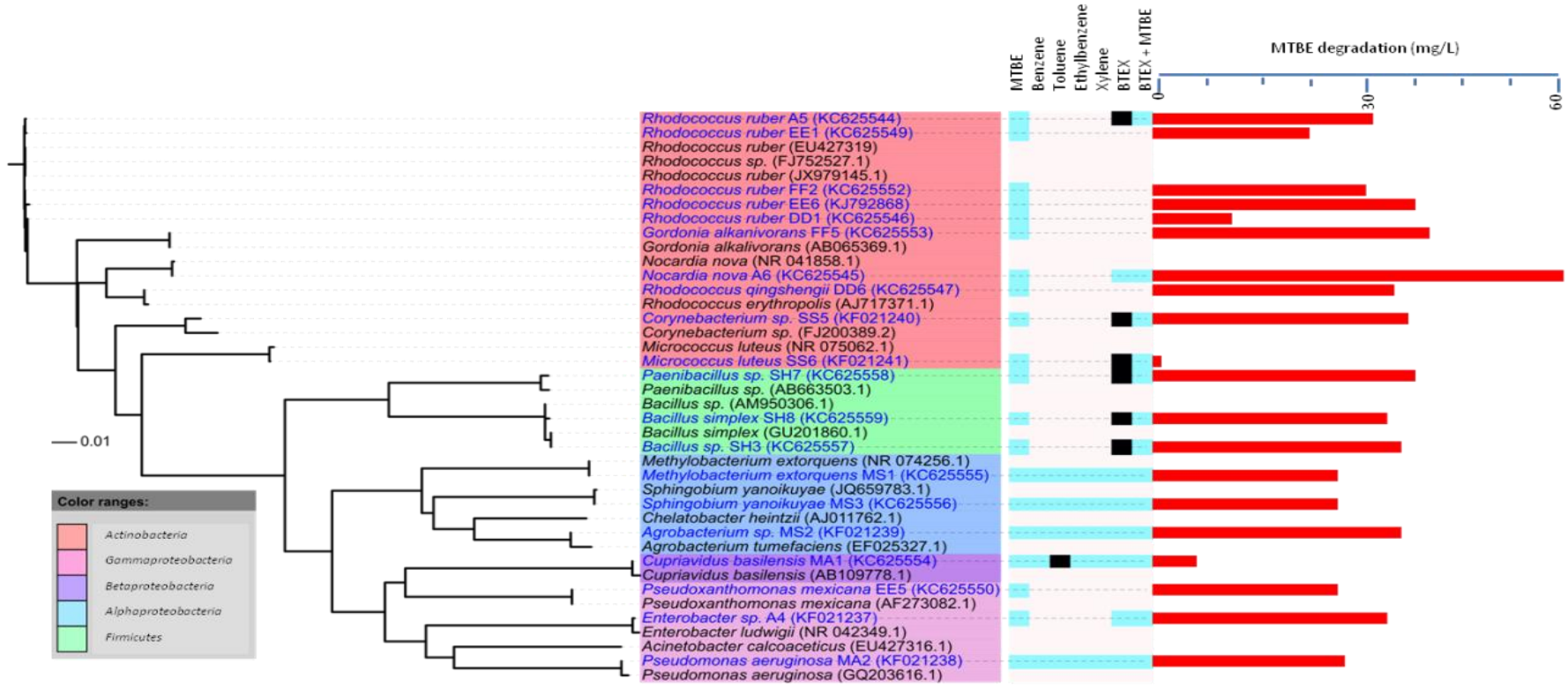

Fig.1. Phylogenetic neighbor-joining tree of the 16S rRNA gene sequences from the bacterial isolates and preliminary growth and degradation of xenobiotic compounds. Sequences are indicated by their corresponding NCBI accession numbers. Bacterial strains identified in the present study are marked in blue. The positive/negative growth assay was assessed for all strains on different compounds, whereby: ■–Positive growth, ■– No growth, and □– Not tested. MTBE degradation values (mg l^{-1}) from preliminary tests are represented as red lines.

Fig. 2. Phylogenetic tree of the closely related alkane monooxygenase and cytochrome P450 gene sequences. Coloured boxes indicate the primers used to amplify the monoxygenase sequences. –Primers described by Jechalke et al.,(2011) for EthB – Primers described by Chénier et al., (2003) for alkane monoxygenases –Primers described by Hyun et al,. (1998) for cytochrome P450 monooxygenases.

Growth and MTBE biodegradation curves

Based on results from growth and degradation on oxygenate/aromatic hydrocarbon pre-screening experiments of the isolated microorganisms as well as on ether-degrading gene-screening data, five bacterial strains (A5, EE1, EE6, MS2 and SH7) were selected. Note that among these microorganisms, two bacterial strains (MS2 and SH7) that did not express either ether-degrading gene but showed remarkable MTBE degradation values under several conditions were selected.

The ability of the selected microorganisms to grow and biodegrade MTBE was tested by culturing them for 8 days on modified FTW media supplemented with 150 mg l^{-1} MTBE under agitation and controlled temperature. **Fig. 3, 4** and **5** show the growth (as OD_{600nm}) and MTBE degradation (as MTBE amount remaining) by the selected bacterial strains during the incubation.

The results obtained display several trends, which allowed to assign these bacterial strains to three groups:

1. Bacterial strains that degrade MTBE and whose gene screening showed the presence of genes that code for known degrading enzymes: strains EE1 and EE6.
2. Bacterial strains that degrade small amounts of MTBE and whose gene screening showed the presence of genes that code for known degrading enzymes: strain A5.
3. Bacterial strains that degrade MTBE and did not show the presence of genes encoding known degrading enzymes: strains MS2 and SH7.

Bacterial strains EE1 (**Fig. 3a**) and EE6 (**Fig. 3b**) degraded 60 mg l^{-1} MTBE within 24 h incubation and 36 mg l^{-1} MTBE within 192 h incubation, respectively. Strain EE6 reached maximum growth after 48 h, showing the highest growth rate of all the strains studied (0.63 generations/h) and produced the highest number of generations during the growth period (6.3 generations). After 24 h incubation, bacterial strain A5 (**Fig. 4**) degraded only 10 mg l^{-1}MTBE, showing a maximum growth after 96 h incubation. **Fig. 5** (a,b) displays the growth and MTBE degradation of strains MS2 and SH7. It is important to highlight that the greatest degradation was observed by bacterial strain SH7 (100 mg l^{-1}) and this was accomplished within 2.5 generations, but this bacterial strain did not test positive for any of the monooxygenase primers tested. Accumulation of *tert*-butyl alcohol (TBA), the primary metabolite of MTBE biodegradation, was also not detected during the assay. Strain MS2 showed a degradation value of 85 mg l^{-1}, accomplished within 2.2 generations.

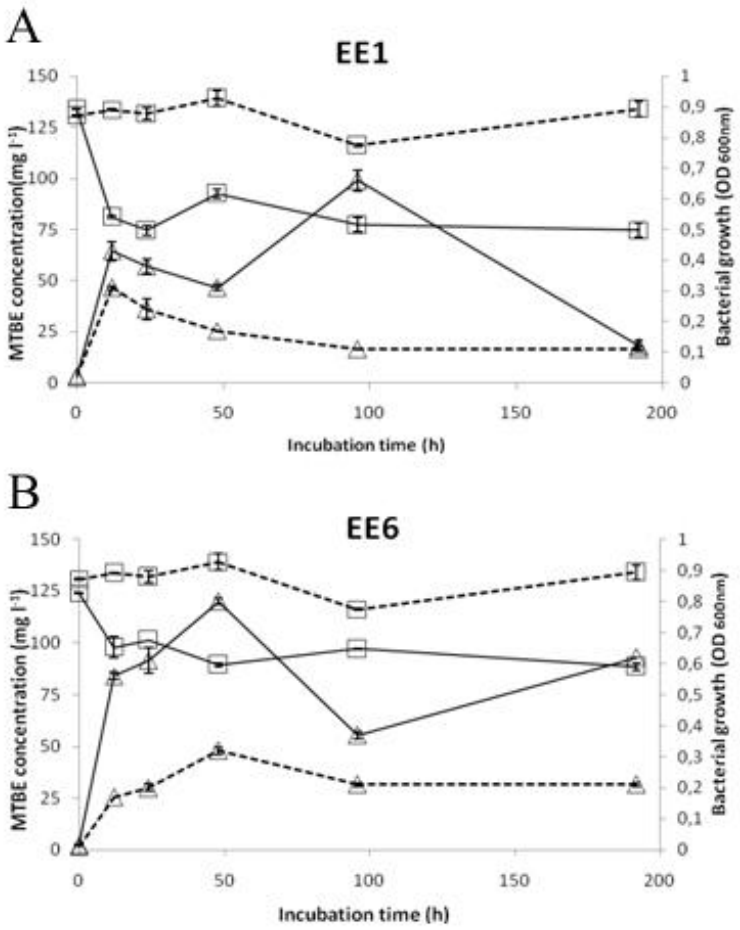

Fig.3. Growth (as OD_{600nm})and MTBE degradation (amount, mg l^{-1}) of bacterial strains EE1 **(A)** and EE6 **(B)** in the presence of MTBE.Values represent means of triplicate samples, and error bars represent standard deviation of the mean. □– MTBE concentrations. △–Bacterial growth. Dotted lines represent the abiotic (□) control and the biotic control without MTBE (△).

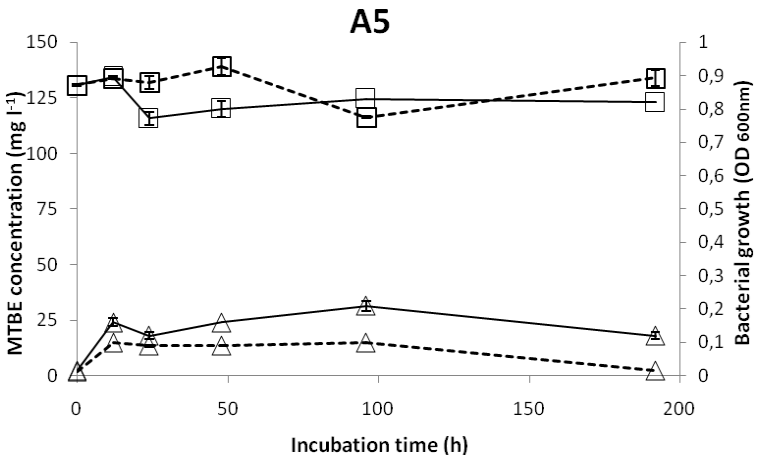

Fig. 4. Growth (as OD_{600nm})and MTBE degradation (amount, mg l^{-1}) of bacterial strain A5 in the presence of MTBE. Values represent means of triplicate samples, and error bars represent standard deviation of the mean. □– MTBE concentrations. △–Bacterial growth. Dotted lines represent the abiotic (□) control and the biotic control without MTBE (△).

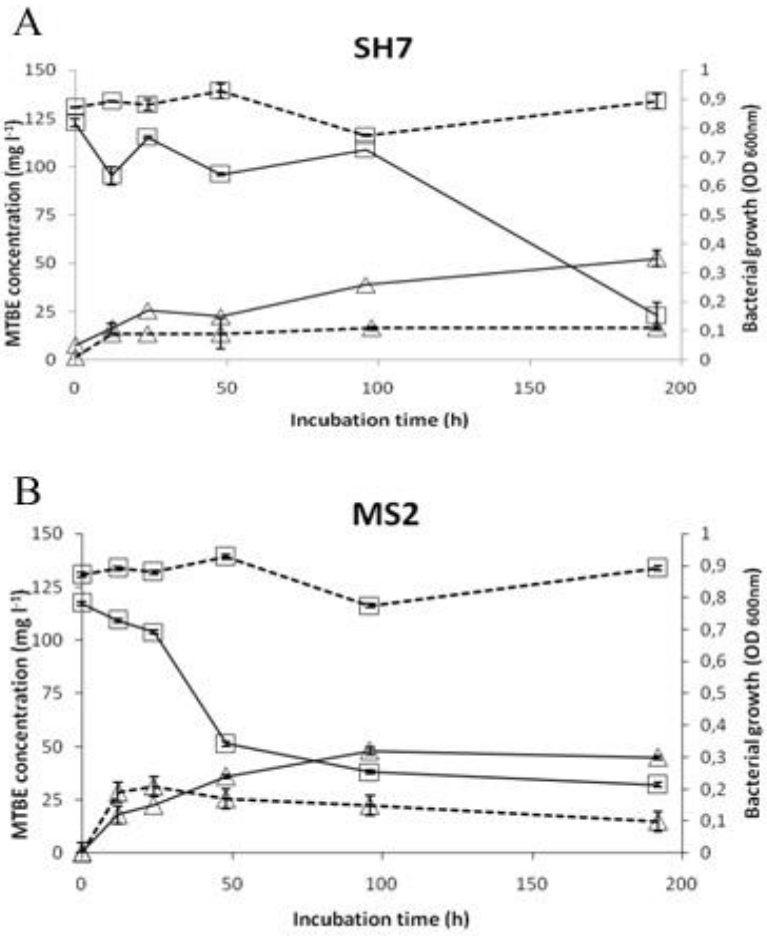

Fig. 5. Growth (as OD_{600nm})and MTBE degradation (amount, mg l^{-1}) of bacterial strains MS2 (A) and SH7 (B) in the presence of MTBE. Values represent means of triplicate samples, and error bars represent standard deviation of the mean. □– MTBE concentrations. △–Bacterial growth. Dotted lines represent the abiotic (□) control and the biotic control without MTBE (△).

Toxicity tests

The acute toxicity (as EC_{50}) of the culture medium inoculated by A5, EE1, EE6, MS2 and SH7 bacterial strains was evaluated using Microtox® bioassay. **Table 1** shows the acute toxicity values of the cultures after 96 and 192 h inoculation. A lower EC_{50} value represents a greater toxicity of the supernatant. Abiotic 150 mg l^{-1} medium was tested and resulted in an EC_{50} value of 3.3; all the biotic assays were higher than this value. The strain that produced the lowest toxicity in the assay was EE6 and that with the highest toxicity was MS2.

Table 1. Acute toxicity values (as EC_{50}) of the bacterial strain cultures amended with 150 mg l^{-1} MTBE after 5 minutes exposition.

Bacterial strain	A5	EE1	EE6	MS2	SH7
EC_{50} 96h after inoculation	15.0	9.0	44.0	5.0	12.0
EC_{50} 192h after inoculation	18.0	9.0	20.0	7.0	8.0

DISCUSSION

An important step in the design and use of a biological technology for the palliative treatment of contaminated water bodies and soils is the selection of bacterial strains with the ability to degrade or remove contaminants and their subsequent metabolites.

The objective of this study was the isolation and subsequent intensive selection processes of bacterial strains with gasoline oxygenate-degrading capacities by

physiological and genetic methods, for their future use in the bioremediation of contaminated groundwater and polluted soils. To date, there are few studies that describe pure bacterial cultures capable of using MTBE as a sole carbon and energy source (Hanson et al., 1999; Hatzinger et al., 2001; Piveteau et al., 2001; François et al., 2002; Nakatsu et al., 2006; Rohwerder et al., 2006).

The bacterial strains in this study were isolated from two hydrocarbon-contaminated sites according to two different methodologies; either directly from the original samples (soil and groundwater) or after an enrichment step process. In this sense, enrichment techniques have been used for years as a method to isolate bacterial strains with MTBE-degradation capacities (Mo et al., 1997; Hatzinger et al., 2001; Okeke and Frankenberger, 2003; Purswani et al., 2008). Besides, seven bacterial strains previously isolated from a continuously bioremediating MTBE biofilter were included, to increase the chance of obtaining a greater number of MTBE adapted strains.

The majority of bacterial strains isolated in this study were affiliated to the phyla *Actinobacteria* and *Firmicutes*, representing 60% of the total isolates. Genera belonging to these phyla have been reported to degrade aromatic hydrocarbon such as BTEX, or to be involved in MTBE aerobic degradation (Francois et al., 2002). The remaining bacterial strains were grouped within the phylum *Proteobacteria.* It is noteworthy that all of the microorganisms isolated after enrichment steps (MA1, MA2, MS1, MS2 and MS3) are included in this phylum. Microbial studies on the degradation of the xenobiotics vinyl chloride (Greń et al., 2011) and 1,3-dichloro-2-propanol (Bastos et al., 2002) found that Gram negative strains were more efficient than Gram positive strains, whereas Wagner-Döbler et al. (1998) describe that classical enrichments tend to isolate Gram-negative microorganisms due to their high growth rates.

Two strains that performed outstandingly well in the growth and degradation assays were *Agrobacterium* sp. MS2 and *Paenibacillus* sp. SH7. Strain SH7 achieved the highest

reduction in MTBE concentration (100 mg l^{-1}) in 8 days, and strain MS2 achieved a reduction of 85 mg l^{-1} within 4 days. Neither strain had a high growth rate or achieved a large number of generations, which can be explained by the lack of energy obtained in the process, which was mainly used in cell maintenance (Müller et al., 2007). In this sense, Hatzinger et al.,(2001) reported that the inefficiency of growth observed might be a result of a lack of enzymes for complete degradation or the presence of inhibitor compounds as intermediate products (i.e. TBA) in the biodegradation of these oxygenates. Especially in SH7, a 2 day growth lag phase was observed, accompanied by a 4 day lag phase before the active degradation period was observed, which might be due to the following factors: environmental strains grow slower than laboratory-adapted strains; transformation rates differ for non-identical enzymes; a lack of energy might exist due to the transformation and inhibition of other products such as those stated in the cited studies. According to Nadell et al., (2009), who studied social theory in biofilms, "bacteria slow their growth to use resources efficiently and secrete molecules that benefit other cells in their surroundings". *Agrobacterium* and *Paenibacillus* strains both isolated from contaminated soil sample, are accustomed to diversity of their natural environment, and thus even though their growth rates are slow, they might be more adaptable to biofilm-based bioreactors.

Another strain worth highlighting is *Rhodococcus ruber* EE6. This microorganism achieved the highest number of generations (6.3) under the same conditions as the other strains, but did not show the highest MTBE removal. The efficiency with which this strain grew might be due to efficient transformation rates. Toxicity values after growth on MTBE defined this strain as being the least toxic, thus, we infer that the most efficient removal of MTBE was probably achieved by EE6. Microtox® analyses revealed that the toxicity of the cultures after 96 and 192 h was always lower than that exhibited by the abiotic control supplemented with 150 mg l^{-1} MTBE (i.e., bacterial EC_{50} values were higher than the abiotic EC_{50}value of 3.3, data not shown), corresponding with the initial MTBE

concentration present in the cultures. The toxicity of the strains MS2 and SH7 after 192 h were high, despite the data for MTBE removal. An accumulation of a toxic metabolite in the culture medium might be inferred, nevertheless, the accumulation of TBA was never observed in any of the strains, thus the toxic metabolite is most likely an alternative compound (e.g., formaldehyde).

Numerous studies have suggested that the microbial oxidation of MTBE is initiated by monooxygenases (Lopes Ferreira et al., 2006; Hyman et al., 2013). In this study, the identified bacterial strains were subjected to genetic screening for the presence of monooxygenase ether-degrading genes (alkane or cytochrome P450 monooxygenases). However, the detection of these marker genes did not necessarily account for an effective biodegradation of MTBE, and thus, should not be used solely as an indicator of bioremediation in water/soil, especially since MTBE removal by strains MS2 and SH7 was the highest, and these strains did not test positive with the primers tested. This was also observed in other studies (Le Digabel et al., 2013; Purswani et al., 2014).The amplification of other known genes involved in the MTBE degradation pathway (MdpA, MdpB, MdpC) has also been tested, however, the primers were probably too specific, and amplification was not successful (Le Digabel et al., 2013). More general monooxygenase primers or multiplex-PCR using several monooxygenase primers might be a better approach to identifying a genetic MTBE bioremediation marker.

The main aromatic compounds of gasoline, namely BTEX, are an active part of the contaminants in aquifers after accidental spills or losses during the production, distribution, storage and use of gasoline. Happel et al., (1998) reported the co-occurrence of MTBE and BTEX compounds in a large number of contaminated groundwater sites with gasoline in the USA. Bacterial strains isolated after enrichment steps (MA1, MA2, MS1, MS2 and MS3) in this study were tested for their capacity to grow in medium supplemented with aromatic compounds (benzene, toluene, ethylbenzene and xylene) with or without MTBE

for 4 weeks. All strains exhibited a positive growth with all compounds, except *Cupriavidus basilensis* MA1, which did not grow in medium with toluene.

Based on growth results and on MTBE degradation in oxygenate/aromatic hydrocarbon pre-screening experiments of the isolated microorganisms, as well as on ether-degrading gene screening data, five bacterial strains (A5, EE1, EE6, MS2 and SH7) were selected for MTBE biodegradation studies in batch experiments. Although the strains MA2 (*Pseudomonas aeruginosa*) and A6 (*Nocardia nova*) were effective MTBE-degrading candidates, they were not included in further studies as they were potential human opportunistic pathogens. If the selection of microbial strains would have been based solely on MTBE degradation, A6 would have been selected as the best strain, however, this strain would not have been used in bioremediation processes due to its potentially pathogenic properties. Thus, the methods included in this pre-screening limited the number of suitable bioremediating strains at an early stage.

The results obtained in this work highlight the importance of complementary screening steps (i.e. physiological, genetic and ecotoxicological) to obtain a more representative selection of bacterial strains for bioremediation purposes.

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CHAPTER 2

Paenibacillus oxygenati sp. nov., able to growth on media supplemented with methyl tert-butyl ether (MTBE) isolated from hydrocarbon contaminated soil.

Adapted from: I.M. Guisado, J. Purswani, J. Gónzalez-López and C. Pozo. *Paenibacillus oxygenati* sp. nov., able to growth on media supplemented with methyl *tert*-butyl ether (MTBE) isolated from hydrocarbon contaminated soil.

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ABSTRACT

A bacterial strain designated as strain SH7^T was isolated from the hydrocarbon contaminated soil of a pilot plant (Granada, Spain). The strain was selected for its capacity to growth in media supplemented with methyl *tert*-butyl ether (MTBE) as sole energy and carbon source. Strain SH7^T was a Gram-stain-positive, facultatively anaerobic, spore-forming, rod-shaped bacterium. Phylogenetic analysis using 16S rRNA gene sequences showed that strain SH7^T belongs to a cluster comprising species of the genus *Paenibacillus* and was closely related to *Paenibacillus borealis* DSM 13188^T (97%) and *Paenibacillus odorifer* DSM 15391^T (98%). DNA-DNA hybridization tests showed low relatedness of the strain SH7^T with *Paenibacillus borealis* (16.9±1.5%) and *Paenibacillus odorifer* (16.6±2.1%) respectively. The cell wall contained meso-diaminopimelic acid. The predominant respiratory quinone was MK-7, anteiso-$C_{15:0}$ (32.9%) and $C_{16:0}$ (29.0%) were the predominant cellular fatty acids. Phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and three unknown aminophospholipids were the major phospholipids. The DNA G+C content was 44.3 mol%. The data obtained in this study indicate that the SH7^{T}strain represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus oxygenati* sp. nov. is proposed. The type strain is SH7^T (= CECT 8558^T =DSM 29760^T).

Keywords: *Paenibacillus oxygenati* sp. nov., grow MTBE, 16S rRNA, soil.

INTRODUCTION

Since in 1993, Ash and collaborators described the genus *Paenibacillus*, to date over 150 new species (including subspecies) have been described. Although are considered as saprophytic bacteria living on bare soil, members of the genus *Paenibacillus* can be isolated from numerous locations (environmental and/or human samples) like blood cultures, sputum, plant roots, seeds, food, agricultural soil, mineral soils….(Berge *et al*., 2002; Scheldeman *et al.*, 2004; Roux & Raoult., 2004). Members of this genus havebeen shown to produce numerous substances such as exopolysaccharides and extracellular enzymes (Rai*et al*., 2010), which can be used in industrial and biotechnological applications (Polizeli *et al.,* 2005; Goesaert *et al*., 2005; Roncero *et al*., 2005; Soriano *et al*., 2005; Margeot *et al*., 2009), and some of *Paenibacillus* species are involved in the degradation of environmental pollutants such as aromatic hydrocarbon, xylan and naphthalene, among others (Daane *et al.*, 2002; Sirota-Madi *et al.*, 2010; Khianngam *et al*., 2011).

Methyl *tert*-butyl ether (MTBE) has been the oxygenate compound most widely used in the last decades. It is added to reformulated gasoline (RFG) to enhance the octane number, so increasing its combustibility and reducing toxic emissions to the atmosphere. However this compound has been identified as an important contaminant substance of water bodies, mainly groundwater, so the development of technologies to treat them is of great importance. Among these, biological treatments are recognised as a cost-effective and environmentally friendly option (Kharoune *et al*., 2001). An important step in the design and use of a determinate biological technology for the treatment of contaminated water is the selection of bacterial strains with biotransforming/degrading xenobiotic compound capacity. In this sense, bacteria fromseveral genera such as *Rhodococcus, Gordonia,*

Nocardia, Corynebacterium, Micrococcus, Methylobacterium, Bacillus, Mycobacterium or Xanthobacter among others have been successively isolated from environmental samples based on their ability to growth and/or biotransforming/degrading MTBE (Liu *et al.*, 2001, François *et al.*, 2002; Guisado *et al.*, 2015).

In the present study, we show that strain SH7^T isolated from a hydrocarbon contaminated soil of a pilot plant (Granada, Spain) (Silva-Castro *et al.*, 2013) and with MTBE-growing capacities is a novel species of *Paenibacillus* genus based on the results from phylogenetic analysis of 16S rRNA gene sequences, DNA-DNA hybridization, physiological and chemotaxonomic analysis.

MATERIALS AND METHODS

Strain SH7^T was isolated from soil samples by serial dilution technique and plating on solid-modified mineral salts medium (FTW medium, Herman & Frankenberger, 1999), supplemented with 150 mg l^{-1} MTBE. The inoculated plates were incubated in a MTBE-saturated atmosphere at 28ºC for seven days. The plates were observed daily and the isolated colonies were streaked onto solid-modified FTW plates for further studies. The SH7^T strain isolation after enrichment steps from the contaminated soil samples was performed following the methodology previously described by Purswani *et al.*, (2008). Later, SH7^T strain was included in a procedure of pre-selection of MTBE-degrading bacterial strains, where was tested for growth (as$OD_{600\ nm}$) on modified FTW medium supplemented with MTBE and/or BTEX (benzene, toluene, ethylbenzene and xylene) as sole carbon and energy source, using sterile 96-well plates. SH7^T strain was pre-grown under controlled temperature (30ºC) and agitation (150 rpm) for 4 days, followed by centrifugation of 1 ml of culture (for 1min at 14,000rpm). 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 prepared as previously described. The plates were kept at 28ºC in a 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. MTBE biodegradation capacity of $SH7^T$ strain was evaluated in *batch*-experiments. For this, $SH7^T$ strain was inoculated into a sterile glass tube with 5 ml of modified FTW medium supplemented with 150 mg l^{-1} MTBE and incubated for 4 days at 28ºC under controlled agitation conditions. A volume of 500 µl of this bacterial pre-grown culture was inoculated in a glass vial (125 ml total capacity) with 25 ml of modified FTW medium plus 150 mg l^{-1} MTBE, sealed with polytetrafluoroethylene (PTFE) stopper and incubated for 8 days. Control glass vials non inoculated (abiotic controls) and others inoculated in mineral medium without MTBE (biotic controls) were included in this assay. After 12, 24, 48, 72, 96 and 192 h incubation, growth values (as OD_{600nm}) and MTBE-remaining concentrations were accomplished (Guisado *et al.*, 2015).

Cellular morphology and motility were observed by transmission electron microscopy (TEM Zeiss Libra 120 Plus). For intracellular endospores observation, strain $SH7^T$ was grown on tripticase soy agar (TSA, Difco, USA) medium during 2 days at 30ºC plus 3 days at 50ºC. Detection of intracellular endospores was achieved as follows: cells were pre-fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate at 4 ºC (24 h), washed, post-fixed in 1% OsO4 in a dark room at 4 Cº (1 h) and dehydrated in a graded ethanol series (50, 70, 90 and 100%). The sample was embedded in Epon resin and polymerized. Ultrathin sections were stained with uranyl acetate (1%) plus lead citrate (Reynolds, 1963) before being observed (**Fig S1, A**). For flagela observation, 25 µl of bacterial suspension were incubated in a grid support film for 5 min, after two washing step with ultrapure water. (**Fig. S1, B**).

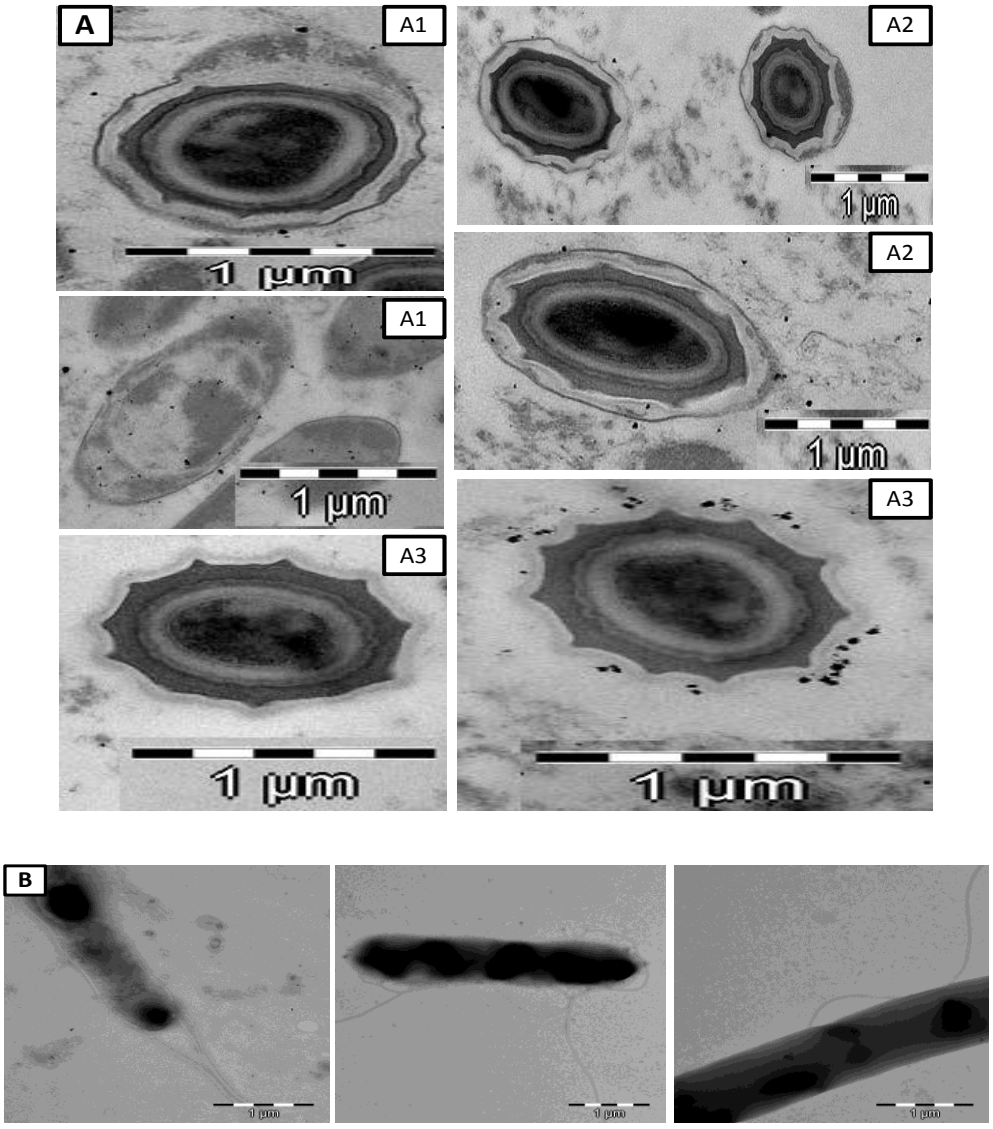

Fig. S1. A) Transmission electron micrographs of isolate SH7^T from a 15 d culture. **(A1)** Vegetative cells and spores at early stages of maturation. **(A2)** Spores at later stages of maturation. The exosporangium surrounding the maturing spore is visible.The developing 'spikes' have started to become visible (arrows). **(A3)** A free, mature spore with eleven apparent 'spikes' (arrow). **B)** Transmission electron micrographs of isolate SH7^T for the flagela observation.

Colony morphology was determined after 3 days growth at 30 ºC on TSA.Growth at several pH range values (1-13), different NaCl concentrations (0.1-8 % w/v) and several temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 ºC) was tested by using tripticase soy broth (TSB, Difco, USA). All tests were carried out by incubating the SH7^T strain and *Paenibacillus odorifer* DSM 15391^{T}at 30 ºC and *Paenibacillus borealis* DSM 13188^{T}at 28ºC, except for the investigation of the effect of temperature on growth and spore formation.

Gram staining, catalase and oxidase activities, hydrolysis of casein, DNA, starch, L-tyrosine, indole production, citrate utilization and H_2S production were performed as described by Barrow & Feltham (1993). For biochemical properties, utilization of carbohydrates and enzymatic activities of SH7^T strain, commercially available API test kits (API 20NE and API 50CH, combined with API 50CHB/E medium, BioMeriéux, France) were usedin accordance with the manufacturer's indications.

Genomic DNA extraction for amplification of the 16S rRNA gene of strain SH7^T was performed using the method described by Martín-Platero *et al.,*(2007).PrimersfD1andrD1(Weisburg *et al.,*1991) synthesized by Sigma Genosys (Dorset, UK), were used to amplify and sequence almost full-length 16S rRNA gene as described previously by Purswani *et al.*, 2008. The DNA sequences were processed, assembled, and edited for sequencing errors. Closely matching sequences were found in the GenBank database using the BLASTn algorithm (Altschul *et al*., 1997).The almost complete sequence of the 16S rRNA gene of SH7^T strain (approx. 1500 bp) was aligned with sequences from strains of closely related species of the genus *Paenibacillus* by using the ClustalX2 program (Larkin *et al*., 2007). The MEGA 5.0 package (Tamura *et al.*, 2011) was used to reconstruct phylogenetic trees by applying the neighbour-joining method (Saitou & Nei, 1987) (**Fig. 1 and Fig. S2)**. The stability of relationships was assessed by means of a bootstrap analysis of 1000 datasets. The phylogenetic tree constructed using the

neighbour-joining method suggested that strain SH7^T is a member of the genus *Paenibacillus* but represents a distinct species.

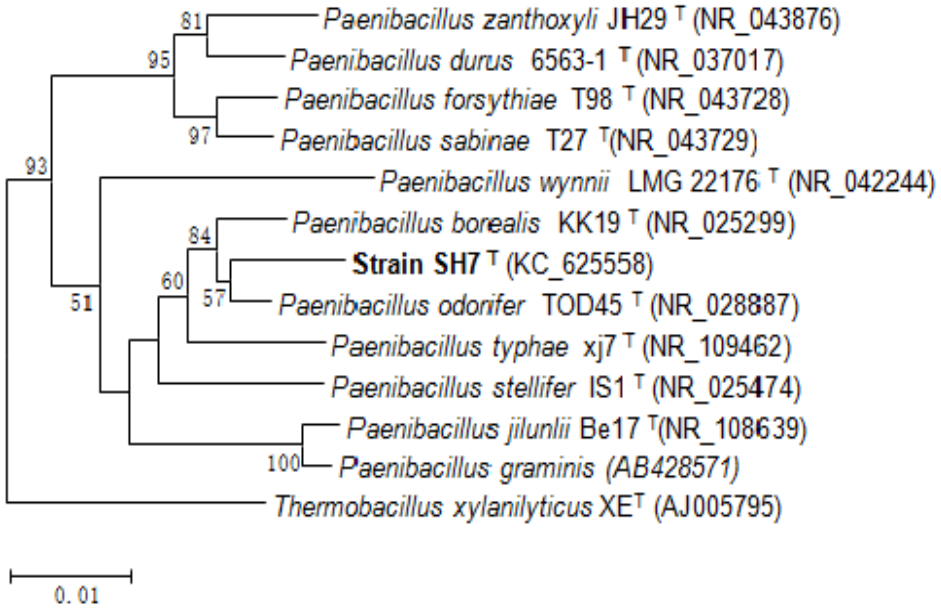

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain SH7^T andclosely related species within the genus *Paenibacillus*. *Thermobacillus xylanilyticus* was used as the outgroup. Based on 1000 resamplings, bootstrap percentages $\geq$ 51% are shown. Bar represents 0.01 substitutions per nucleotide position.

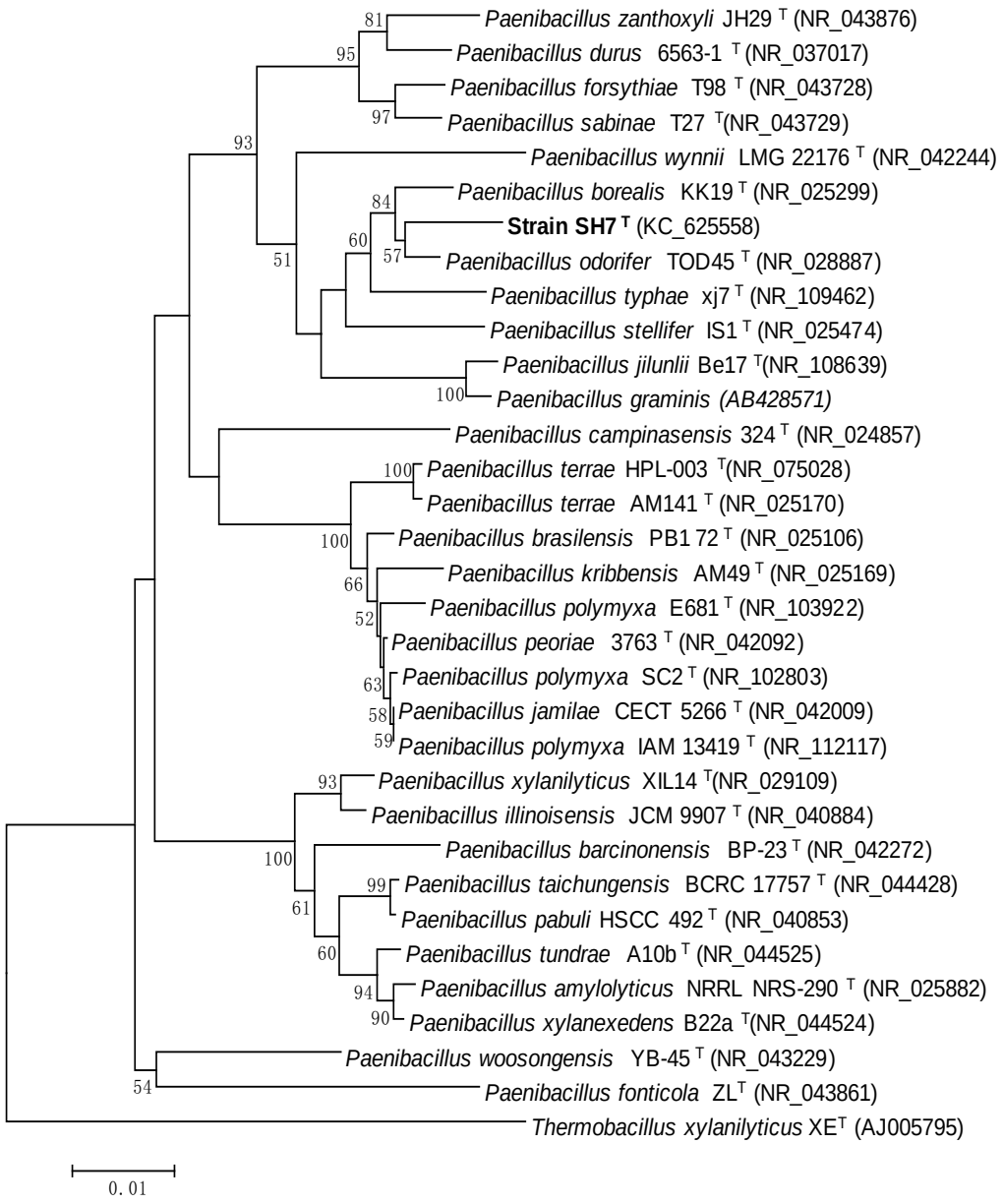

Fig. S2. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain SH7^T and representatives of all *Paenibacillus* species.

Strain SH7^T showed 16S rRNA gene sequence similarities with respect to the type strains of recognized species of *Paenibacillus* but it was most closely related to *Paenibacillus borealis* DSM 13188^T (97%) and *Paenibacillus odorifer* 15391^T (98%).

DNA-DNA hybridization among SH7^T and its closest neighbours,*P.borealis* DSM 13188^T and *P.odorifer* DSM 15391^T, G+C mol% content and chemotaxonomic analyses of strain SH7^T were carried out by the Identification Services, DSMZ (Braunschweig, Germany).

Cell disruption by using a Constant Systems TS 0.75 KW instrument (IUL Instrument, Germany) was used for DNA extraction and purified on hydroxyapatit according to the procedure of Cashion *et al.,* (1977). DNA-DNA hybridization was carried out as described by De Ley *et al.,* (1970) under consideration of the modifications described by Huss *et al.,* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The G + C (mol%) content of the genome of strain SH7^T was calculated from the ratio of deosyguanosine (dG) and thymidine (dT) according to the method of Mesbah *et al*., (1989). The cellular fatty acids were carried according to the instructions of the Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.). Analysis of respiratory quinones was carried out by the Identification Service and Dr. Susanne Verbag, DSMZ (Braunschweig, Germany) by thin-layer chromatography and by UV and mass spectroscopy.

RESULTS

Cells were Gram-positive, facultatively anaerobic, endospore forming, and rod-shaped bacterium (663.56 nm wide X 3240.67 nm long), peritrichous bacterium, motile with multi lateral flagella. Colonies were 1.5–5.0 mm in diameter,cream colored, ovoid

shape, and smooth with irregular entire margins when grown on TSA for 3 days. Growth was observed at pH 7–8, maximum NaCl tolerance 0.8%, temperature range 5-35ºC, being the optimum conditions for growth pH 7.5, 0% NaCl and 30 ºC. They were positive for catalase and negative for oxidase activities. **Table 1** shows phenotypic, biochemical, and physiological characteristics among strain SH7^{T}and type strains of phylogenetically related species.

Table 1. Differential characteristics of strain SH7^T and the type strains of closely related species of the genus *Paenibacillus.* Strains: **1**. SH7^T, **2**. P. *Paenibacillus borealis* DSM 13188^T, **3**. *Paenibacillus odorifer* DSM 15391^T

Characteristic	**1**	**2**	**3**
pH range	7.0-8.0	6.0-8.0	7.0-8.0
Temperature range (Cº)	5-50	5-35	5-35
Maximum NaCl tolerance (%)	0.8	0.4	0.5
Nitrate reduction	+	-	+
Hydrolysis of:			
Aesculin	-	W	+
Casein	-	+	-
Starch	+	-	+
Assimilation of potassium gluconate	W	-	-
Acid production from:			
Amygdalin	+	+	+
D-Arabinose	W	-	-
D-Arabitol	-	W	-
Cellobiose	+	+	+
Glycerol	+	+	+
Glycogen	+	+	+
Inositol	-	-	-
D-Lyxose	-	+	-
Melibiose	+	+	+
Raffinose	+	+	+
Xylitol	W	+	-
Ribose	+	-	+
D-Xylose	+	+	+
Methyl β-D-xyloside	+	+	+
Rhamnose	-	-	-
Methyl α-D-mannoside	-	-	+
Methyl α-D-glucoside	W	+	+
Lactose	+	+	+
N-Acetylglucosamine	+	+	+
Inulin	W	+	+
Assimilation of:			
L-Arabinose	+	+	+
D-Glucose	+	+	+
D-Mannose	-	+	W
D-Mannitol	-	+	-
Maltose	+	+	+
N-Acetylglucosamine	+	+	+
Adipic acid	-	-	-
Malic acid	-	-	-
Trisodium citrat	-	-	-
DNA G+C content (mol%)	44. 3	53.6*	44.0*

*Data no from this study. +, Positive; W, weakly positive; -, negative.

The DNA G+C content of strain SH7^{T}was 44.3 mol%, value within normal range for members of the genus *Paenibacillus* (Shida *et al*., 1997). DNA-DNA hybridization was performed with the two phylogenetically closest species *Paenibacillus borealis* DSM 13188^T and *Paenibacillus odorifer* 15391^T. Strain SH7^T displayed low levels of DNA similarity with *P. borealis* (16.9±1.5%) and *P. odorifer* (16.6±2.1%). According to the recommendation of a threshold value of 70% DNA–DNA relatedness for the definition of bacterial species (Wayne et al., 1987), strain SH7^T probably represent a novel species of *Paenibacillus* genus.

The predominant respiratory quinones for this strain was MK-7 (100 %).The major fatty acids were $C_{16:0}$ (29.0 %) and anteiso-$C_{15:0}$ (32.9 %) being this the major fatty acid in recognized members of the genus *Paenibacillus.* Moderate amounts of iso-$C_{14:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and $C_{16:1}$ w11c were also found. In addition, small amounts of $C_{10:0}$, $C_{12:0}$, $C_{15:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$ and $C_{16:1}$ w7c alcohol was detected. **Table 2** shows the fatty acid profiles of SH7^T strain and its closest relatives. Strain SH7^T, *Paenibacillus borealis* DSM 13188^T and *Paenibacillus odorifer* 15391^T showed similar profiles but differences were observed on $C_{16:0}$ or anteiso-$C_{15:0}$ amounts. The predominant polar lipids of strain SH7^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three unknown aminophospholipids (**Fig. S3**).

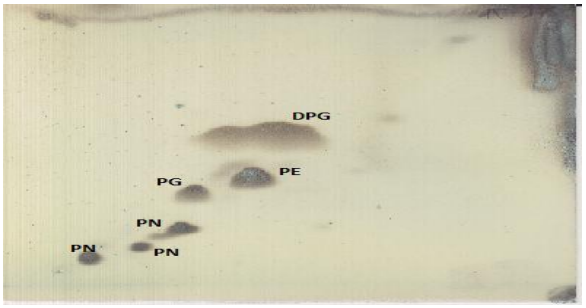

Fig. S3. Polar lipid profile of strain SH7^{T}after separation by two-dimensional TLC. **DPG**, diphosphatidylglycerol; **PE**, phosphatidylethanolamine; **PG**, phosphatidylglycerol; **PN**, aminophospholipid.

Table 2. Cellular fatty acid composition of the strain SH7^T and the type strains of closely related species of the genus *Paenibacillus.* Strains: **1**. SH7^T, **2**. P. *Paenibacillus borealis* DSM 13188^T, **3**. *Paenibacillus odorifer* DSM 15391^T

	1	**2**	**3**
Straight-chain saturated			
$C_{10:0}$	tr	nd	tr
$C_{12:0}$	tr	tr	tr
$C_{14:0}$	10.42	12.15	4.74
$C_{15:0}$	tr	tr	nd
$C_{16:0}$	29.00	14.00	14.45
Branched saturated			
iso-$C_{14:0}$	6.38	5.35	6.11
iso-$C_{15:0}$	6.59	6.66	12.65
iso-$C_{16:0}$	7.50	13.45	7.46
iso-$C_{17:0}$	tr	1.28	2.18
anteiso-$C_{15:0}$	32.98	43.59	48.86
anteiso-$C_{17:0}$	tr	2.72	1.91
Monounsaturated			
$C_{16:1}$ w7c alcohol	tr	nd	nd
$C_{16:1}$ w11c	4.73	nd	1.00
Summed features			
Summed feature 3	nd	0.23	Nd

Data were obtained in this study. Values are percentages of total fatty acids.
tr, trace (<1 %); nd, not detected.

The molecular, chemotaxonomic, physiological and biochemical data obtained in this study indicate that strain SH7^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus oxygenati* sp. nov. is proposed.

Description of *Paenibacillus oxygenati* sp. nov.

Paenibacillus oxygenati (o.xy.ge.na'ti. N.L. gen. n. of an oxygenate, e. g. MTBE, which was used as enrichment substrate of the type strain).

Cells are Gram-stain-positive, facultatively anaerobic, motile rods (663.56 nm wide X 3240.67 nm long). Subterminal ellipsoidal endospores are observed. Colonies are cream colored, ovoid shape, smooth with irregular entire margins and measure 1.5–5.0 mm in diameter after 3 d at 30°C on TSA medium. Growth is observed at pH 7–8 (optimally at pH 7.5), maximum NaCl tolerance 0.8%, temperature range 5-35ºC, being the optimum conditions for growth pH 7.5, 0 % NaCl and 30 ºC. They were positive for catalase and negative for oxidase activities. Nitrate is reduced to nitrite. With API systems, acid is produced from L-arabinose, ribose, D-xylose, methyl β-D-xyloside, galactose, glucose, fructose, methyl α-D-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, starch, glycogen and gentibiose.Strain SH7^{T} was able to produce acid from aesculin, D-fructose, metil-αD-glucopyranoside, D-galactose, D-glucose, glycerol, D-lyxose, maltose, D-ribose, D-xylose, N-acetylglucosamine, amygdalin, L-arabinose, arbutin, glycogen, inulin, melezitose, salicin, starch, b-xylopyranoside or xylitol; but not from D-mannitol, D-adonitol, L-arabitol, cellobiose, dulcitol, erythritol, D- or L-fucosegluconate, inositol, 2-ketogluconate, 5-ketogluconate, lactose, a-mannopyranoside, L-rhamnose, D-sorbitol, L-sorbose, D-tagatose, L-xyloseD-arabinose, D-arabitol. Gelatin was not liquefied.The following compounds are not assimilated: D-glucose, L-arabinose, D-mannose, D-mannitol, Tri-sodiumcitrate, malic acid, adipic acid, inositol, and phenylacetic acid. MK-7 is the predominant respiratory quinone. The predominant fatty acids are anteiso-$C_{15:0}$ and $C_{16:0}$. The cell wall contains meso-diaminopimelic acid. Major phospholipids are

phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and three unknown aminophospholipids. The DNA G+C content of the type strain is 44.3 mol%.

The type strain, strainSH7^T (= CECT 8558^T = DSM 29760^T), was isolated from an artificial hydrocarbon contaminated soil from a pilot plant (Granada, Spain).

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CHAPTER 3

Bacterial strains selection approach for methyl tert-butyl ether (MTBE) contaminated water bioremediation by extractive membrane biofilm reactor (EMBFR) technology.

Adapted from: I.M. Guisado, J. Purswani, L. Catón-Alcubierre, J. Gónzalez-López and C. Pozo. Bacterial strains selection approach for methyl *tert*-butyl ether (MTBE) contaminated water bioremediation by extractive membrane biofilm reactor (EMBFR) technology.
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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* sp. SH7 (CECT 8558), *Agrobacterium* sp. MS2 (CECT 8557) and *Rhodococcus ruber* strains A5 (CECT8556), EE6 (CECT 8612) and EE1 (CECT 8555), previously isolated from fuel-contaminated environments, were assayed for their adhering capacity to tubular semipermeable membranes assembled in systems at laboratory scale running for 10 days under aerobic conditions designed for the treatment of methyl *tert*-butyl ether (MTBE) contaminated influents. The biofilm established by the bacterial strains on the tubular semipermeable surface was observed by field-emission scanning electron microscopy (FESEM) at the end of each experiment, and the acute toxicity (as EC_{50}) of the bacterial growth media was assessed. EPS production by each bacterial strain was evaluated under different MTBE concentrations. *R. ruber* A5 and *Agrobacterium* sp. MS2 were the bacterial strains that showed the greatest ability to adhere to tubular semipermeable membranes and establish as a biofilm. No biofilm was recognized by *Paenibacillus sp.* strain SH7. The growth media inoculated with *R. ruber* strains EE6, A5 and *Agrobacterium* sp. MS2 showed the lowest levels of toxicity (EC_{50} 85.8 %, 43.0 % and 75.0 %, respectively). The results obtained from FESEM and toxicity analysis demonstrate that these bacterial strains could be excellent candidates to be used as selective inocula in EMBFR technology for MTBE bioremediation.

Keywords: MTBE, EMBFR, Biofilm, EPS, Microtox assay.

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is an oxygenated compound present in reformulated gasoline. As result of leakage from various locations and owing to its physicochemical characteristics, this substance has been detected in many aquifers, not only in America, but also in several European countries (Johnson et al., 2000; Rosell et al., 2007).

For the removal of pollutants, several remediation policies for contaminated groundwater have been developed, among which "bioremediation" has gained special importance. Among the strategies developed, those based on the use of bacteria adhered to inert supports and established as a biofilm have attracted interest. In this sense, biofilm-mediated bioremediation has an advantage over processes using planktonic cells due to the protection conferred by the biofilm matrix, allowing better cell adaptation and survival (Decho 2000; Singh et al., 2006), and also by the presence of a greater amount of enzymes involved in contaminant degradation as a consequence of higher bacterial density (Eweis et al., 1997; Fortin et al., 1999).

Extractive membrane biofilm reactor (EMBFR) technology (Katsivela et al., 1999; Ferreira and Livingston, 2000) can be successfully used for the treatment of MTBE-contaminated groundwater. The basis of this technology is using tubular semipermeable membranes through which the contaminant can move into another compartment (biological compartment) in which microorganisms with MTBE degradation/biotransformation capacities can grow and establish a biofilm on the tubing surface.

Effective biofilm formation on an inert support material is crucial for the application of biofilm-based technologies. Exopolysaccharide (EPS) production by the bacterial strains selected as inocula could indicate adequate biofilm formation. EPS are biopolymers located outside of microbial cells (in a layer around the cell) consisting mainly of carbohydrate

homo- or 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, forming channels through which the influent to be treated can flow, thereby increasing the growth and survival of the microorganisms (Decho and Herndl, 1995; Sutherland, 2001; Poli et al., 2007).

Although both the quantity and composition of EPS produced by a microorganism are genetically determined, under certain conditions (e.g. nutritional stress, presence of xenobiotic compounds or microbial growth inhibitors) they may be altered. In this sense, the composition of a waste could affect the composition and appearance of microbial EPS (Sponza, 2002).

Therefore, before a particular biofilm-based technology is applied (i.e. EMBFR technology), it is necessary to carry out preliminary experiments in which not only the ability of bacterial strains selected as future inocula to adhere to inert supports is tested, but also their ability to produce EPS. Moreover, in some cases, the acute toxicity exhibited by the bacterial cultures must be investigated.

In this study, we evaluated five previously isolated (Guisado et al., 2015) bacterial strains, i.e. *Rhodococcus ruber* strains EE1 (CECT 8555), A5 (CECT 8556) and EE6 (CECT 8612), *Agrobacterium sp.* strain MS2 (CECT 8557) and *Paenibacillus sp.* strain SH7 (CECT 8558), for their ability to attach to tubular semipermeable membranes assembled in systems at the laboratory scale as well as their EPS production capacity in media supplemented with MTBE as the sole carbon source. The acute toxicity (EC_{50} evaluated by the Microtox® test) exhibited by the bacterial cultures was also determined. The results obtained in this work provide valuable data for the potential use of these bacterial strains (solely or as consortia) as selective inocula in EMBFR technology for MTBE-contaminated groundwater remediation.

MATERIALS AND METHODS

Chemicals

All the chemical substances used in this study were of reagent grade or of the highest purity available. MTBE was supplied by Sigma-Aldrich and Fluka Chemical, (Milwaukee, WI, USA), with 99.9% purity.

Bacterial strains

The bacterial strains *Rhodococcus ruber* strains EE1 (CECT 8555), A5 (CECT 8556), EE6 (CECT 8612), *Agrobacterium sp.* strain MS2 (CECT 8557) and *Paenibacillus sp.* strain SH7 (CECT 8558) used in this study were previously isolated from different contaminated sites. They showed diverse MTBE biodegrading/biotransforming capacities (Guisado et al., 2015).

Growth media

The bacterial strains used in this work were pre-grown on modified mineral medium (FTW medium) amended with 150 mg/L MTBE as previously described by Purswani et al. (2008). The composition of this medium was (g/L): KH_2PO_4, 0.225; K_2HPO_4, 0.225; $(NH_4)_2SO_4$, 0.225; $MgSO_4$ $7H_2O$, 0.050; $CaCO_3$, 0.005; $FeCl_24H_2O$, 0.005 and 1mL of trace elements solution. The trace elements solution had the following composition (g/L): $ZnSO_27H_2O$, 0.1; $MnCl_24H_2O$, 0.03; H_3BO_3, 0.3; $CoCl_26H_2O$, 0.2; $CuCl_22H_2O$, 0.01; $NiCl_26H_2O$, 0.02 and $NaMoO_42H_2O$, 0.03. Moreover, the medium was supplemented with 1 mL of a 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 the cases, MTBE was added by filtration (Millipore, 0.22µm) to growth media previously sterilized by autoclaving. For the experiments on EPS production, modified FTW medium amended with 50 and 150 mg/L MTBE as the sole carbon source was used.

EPS production, extraction and characterization

EPS production by the selected bacteria was evaluated according to the methodology proposed by Quesada et al., (1993). For this, 5 mL of FTW medium amended with 150 mg/L of MTBE and inoculated with each bacterial strain was incubated for 4 days in a rotary shaker (100 rpm, 28ºC). After this time, each inoculum (5 mL) was added to an Erlenmeyer flask with 300 ml of FTW medium supplemented with 50 and 150 mg/L MTBE as the carbon source for biopolymer production. Erlenmeyer flasks with 300 mL of FTW medium without MTBE addition were inoculated as well and considered to be controls. After incubation for 4 days under the same conditions described above, the cells were harvested at 5ºC by centrifugation at 10,000 rpm for 45 min. The supernatants were mixed with a double volume of ice cold ethanol (96% v/v) and kept at 4°C for 24 hours to precipitate the EPS. Then, the mixtures were centrifuged at 5°C for 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. EPS (expressed as g/L) was estimated in each sample by dry weight. The total quantity of carbohydrates and proteins of the EPS produced by the bacterial strains were determined following the methodologies proposed by Dubois et al. (1956) and Bradford (1976), respectively.

Biofilm formation in tubular semipermeable membrane

To evaluate biofilm formation by the bacterial strains on the semipermeable tube surface, a system was constructed at laboratory scale **(Fig. 1)**.

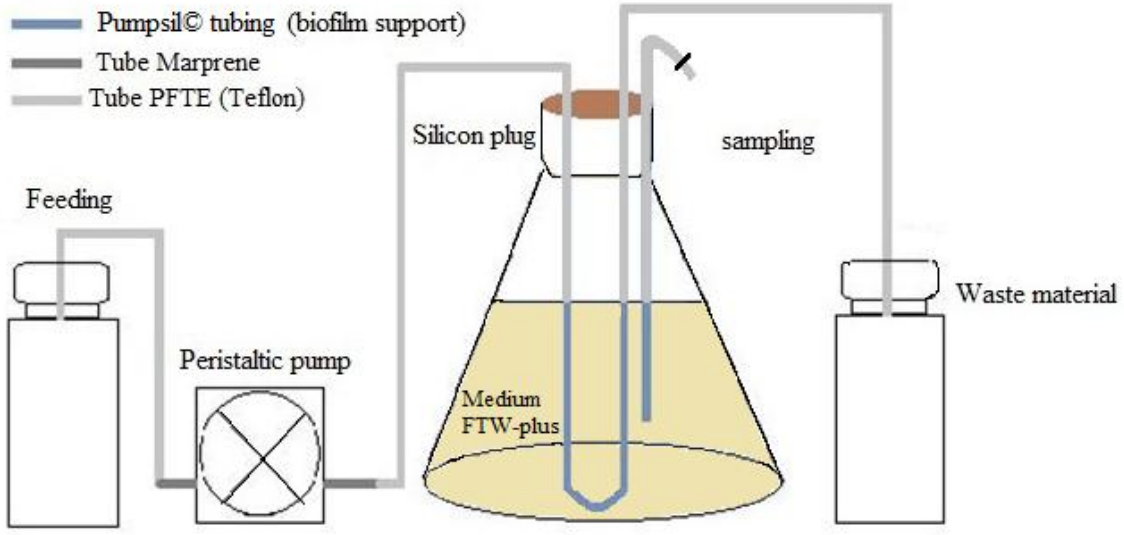

Fig. 1. Diagram of the pilot system at laboratory scale.

Erlenmeyer flasks (1 L total volume) were used, sealed with a silicon stopper and filled with 400 mL FTW medium. Semipermeable tubes (Pumpsil© tubing, 63.9 cm length and 3 mm internal diameter, Watson Marlow, Wilmington, MA, USA) were placed in the flasks through holes made in the plug, immersed in FTW medium and connected to a peristaltic pump. The FTW medium (400 mL) was inoculated with the bacterial strains (5 mL) previously pregrown on FTW medium amended with 1 g/L yeast extract for 4 days under agitation and at a controlled temperature (150 rpm, 30ºC).

The pump operated at a constant speed required to reach adequate influent (FTW medium amended or unamend with MTBE) flow (77.6 mL/day) for a hydraulic retention time (HRT) of 1.45 h. The fuel oxygenate could cross the semipermeable tubing and was available to bacterial strains growing in the FTW medium in the Erlenmeyer flasks (now called a "biomedium"). To prevent MTBE loss outside the biomedium, two different materials were used as connecting tubes: Marprene® and Teflon® (Watson Marlow, Wilmington, MA, USA). The inoculated flasks were incubated for 10 days under agitation and at controlled temperature (150 rpm, 30ºC).

To determine the differences in the adhesion behavior of the bacteria according to the presence or not of the fuel oxygenate as the carbon source, two experiments were run for each bacterial strain: one using FTW medium supplemented with MTBE (150 mg/L) as the influent and another without the fuel oxygenate, considered to be the biotic control. Furthermore, uninoculated Erlenmeyer flasks with FTW medium were included and considered to be abiotic controls.

Determination of MTBE diffusion through the tubular semipermeable membrane

MTBE diffusion through the tubular semipermeable membranes into the biomedium was assessed. Prior to bacterial inoculation, the system was operated for 24 h, after which 1.5 mL of the biomedium was collected up and the amount of MTBE was 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 for 90 min, followed by the injection of 50 µL of the gas phase into GC/MS equipment (Hewlett-Packard 6890 GC coupled to a MS Hewlett-Packard 5973 mass selective detector, Palo Alto, CA, U.S). The analysis was performed on a Quadrex

capillary column (007-1, dimethylpolysiloxane-PHAT Phase, 20 M x 0.18 mm x 6.0 μm). The temperature program was: 40ºC (3.5 min), 10ºC min^{-1} up to 85ºC, 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}. The quantification of MTBE was performed using an external standard calibration (R >0.99).

Examination of biofilm formation by Field-Emission Scanning Electron Microscope (*FESEM*) technique

For the examination of the biofilm established on the surface of the semipermeable tubing immersed in the biomedium, the methodology proposed by Purswani et al. (2011) was followed. After 10 days of incubation, the systems were uninstalled and several portions (three, 1 cm in length) of immersed tubing were collected from the lower part. The units were fixed in 2.5% glutaraldehyde in PBS pH 7.4 for 24 h at 4°C. The samples were then washed three times in PBS for 20 min. Post-fixation followed with the addition of 1% osmium tetroxide, in the dark for 1 h at room temperature; samples were then 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 to observation using a LEO 1530 field-emission scanning electron microscope available at *Centro de Instrumentación Científica* (CIC) from the University of Granada.

Microtox® test.

The Microtox® bioassay was used to measure acute toxicity (as EC_{50}) of each biomedium at the end of the experiment. The toxicity test is based on the bioluminescence reduction of *Vibrio fischeri* after 15 min of exposure to the medium using a Microtox® Model 500 toxicity analyzer (Instrumentación Analítica S.A. Madrid, Spain). The effective

concentration for 50% inhibition of luminescence (EC_{50}) after 5 min of incubation was calculated with data reduction software using the methodology proposed by the manufacturer.

RESULTS AND DISCUSSION

Bacterial EPS production

Fig. 2 shows the amount of EPS (as g/L) produced by each bacterial strain after 4 days of incubation under different culture conditions: EPS production in FTW medium without fuel oxygenate (0 mg/L) and medium supplemented with 50 or 150 mg/L of MTBE as the sole carbon and energy source.

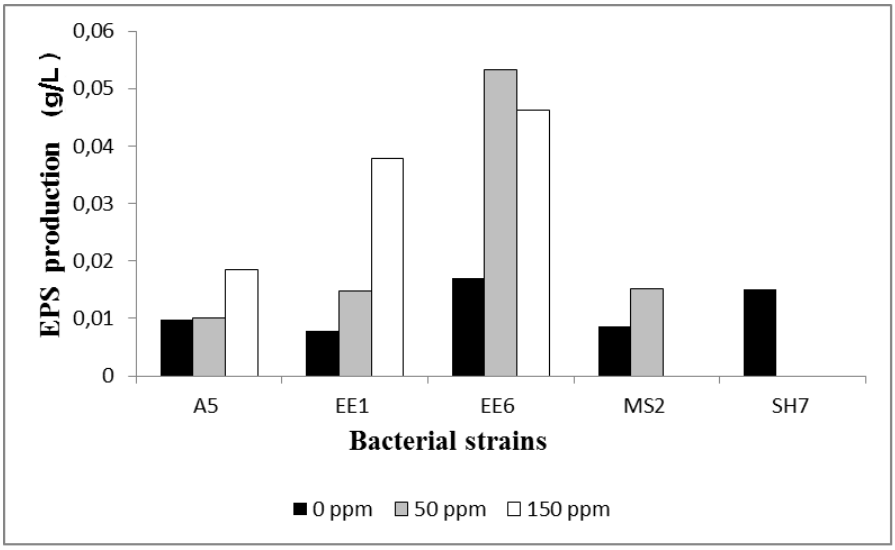

Fig. 2. EPS production (as g/L) on mineral medium without fuel oxygenate (0 mg/L) and amended with 50 and 150 mg/L of MTBE as sole source of carbon and energy.

When the growth medium was FTW medium supplemented with MTBE, the amount of EPS produced by the bacterial strains was lower than that produced in a rich carbon medium, i.e. MY medium (Moraine and Rogovin, 1966) (data not shown); *Paenibacillus* sp. strain SH7 was the exception, for which the amount of EPS produced was very low and similar in both media. Müller et al. (2007) observed low growth when MTBE was used as the sole carbon source by bacterial strains, and these authors concluded that the degradation of this substance provides slow energy release, which was mainly utilized for growth maintenance. In our study, the most EPS production was accomplished by *Rhodococcus ruber* strains A5, EE1 and EE6, but only in strains A5 and EE1 was a positive response to increasing MTBE dose recorded. Once the EPS was extracted, lyophilized and weighed, the total amount of carbohydrate and protein was determined.

Table 1 shows the percentage of carbohydrates **(A)** and proteins **(B)** in EPS produced by the bacterial strains in each growth medium.

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.

A

MTBE (mg/L)	*Bacterial Strains*				
	A5	***EE1***	***EE6***	***MS2***	***SH7***
0	16,0±1.59	16,2±0.23	12,3±2.89	8,9±0.69	4,0±0.23
50	17,7±1.93	15,1±0.51	15,3±1.41	6,1±1.20	-
150	9,6±0.70	14,9±0.49	13,9±1.30	-	-

B

MTBE (mg/L)	*Bacterial Strains*				
	A5	***EE1***	***EE6***	***MS2***	***SH7***
0	2,8±2.44	2,8±0.11	2,9±0.13	4,2±0.32	4,1±0.42
50	3,1±0.17	3,5±0.10	3,5±0.14	3,3±0.03	-
150	3,4±0.25	3,9±0.15	2,8±0.22	-	-

(-). Not detected

Several studies have reported that the yield and composition of EPS produced by bacteria can be influenced by changes in the culture conditions or the composition of the medium (Cerning et al., 1986; 1990). Onbasli and 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 the carbon source, variable monomer composition of the EPS can be detected. Thus, the culture conditions and the type of carbon source can affect the amount and composition of EPS produced. On the other hand, proteins play an important role in

aggregation processes, in structural functions and in enzymatic and biological functions, i.e., bacterial adhesion during the biofilm formation process (Higgins and Novak, 1997). Gao et al. (2013) showed that protein was the most important fraction in EPS, but this trend could change according to the environmental conditions, i.e. the temperature or carbon source.

In our study, the carbohydrate fraction was always larger than the protein fraction as a consequence of the C/N ratio in the media used for EPS production. As shown in **Table 1**, for similar amounts of EPS produced at the same MTBE concentration, the percentage of carbohydrates was different between bacterial strains (*R. ruber* A5, EE1 and *Agrobacterium* sp. MS2). No positive response in the percentage of carbohydrates with an increasing concentration of the carbon source (MTBE) was observed. The percentage of proteins in the EPS produced by the bacterial strains was similar independent of the composition of the growth media and bacterial strain.

Bacterial attachment observation by Field-Emission Scanning Electron Microscopy (*FESEM*)

FESEM analysis was carried out in order to determine the adhesion capacity of the bacterial strains to the immersed tubing surface after 10 days of incubation at a controlled temperature with agitation. For each bacterial strain, two microphotographs are shown: one from the tubing surface within which flowed the FTW medium supplemented with MTBE (150 mg/L) and another from the tubing surface within which flowed the FTW medium without fuel oxygenate in the influent. Microphotographs from the tubing surface of non-inoculated systems considered as abiotic controls are also included **(Fig. 3)**.

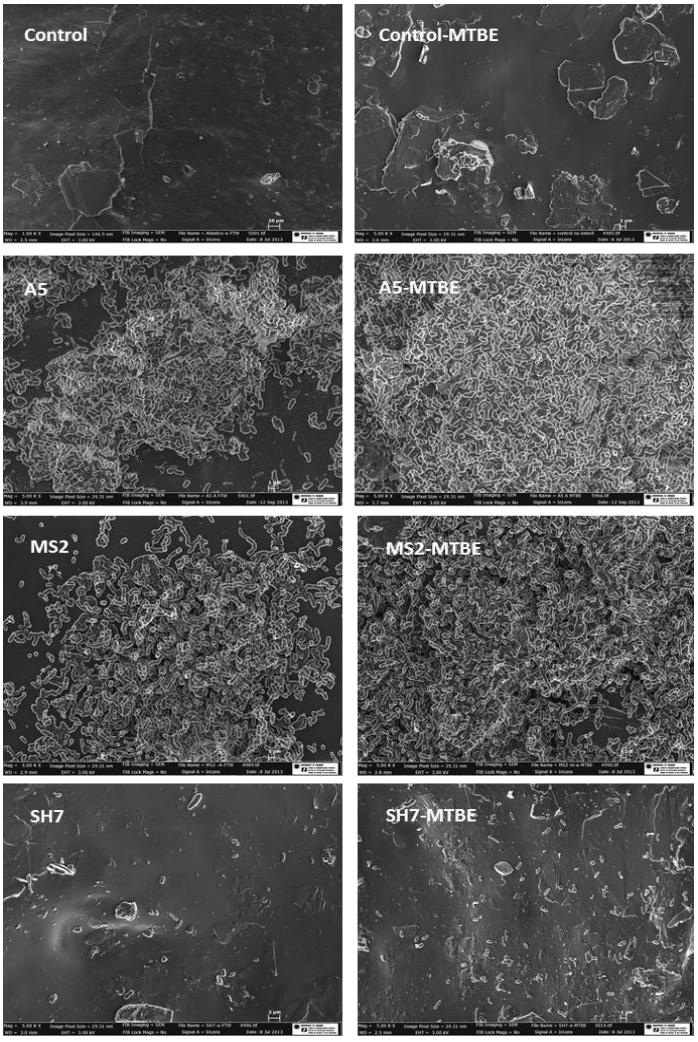

Fig. 3. Scanning electron microscope images of biofilm formation on tubular semipermeable membranes. Samples were taken from the bottom of immersed tubing after 10 days recirculation.

As **Fig. 3** shows, *R. ruber* A5 and *Agrobacterium sp.* MS2 were the bacterial strains that showed the greatest ability to adhere to the tubing surface despite being the bacterial strains that exhibited the lowest production of EPS under our experimental conditions. A higher cell density was observed when the fuel oxygenate was present in the influent.

However, no differences in cell density were observed in the biofilms established by *R. ruber* strains EE1 and EE6 on the tubing surface when the influent was amended with MTBE (data not shown).

The amount of MTBE detected (124 mg/L) by GC-MS in the biomedium used for the MTBE diffusion assay described in the Material and Methods section suggested that the fuel oxygenate could cross the tubing in response to a concentration gradient and hence be available for use as a carbon source by the growing microorganisms, supporting initial bacterial growth on the tubing surface and allowing biofilm formation. Even so, no biofilm was formed by *Paenibacillus sp.* strain SH7 under any condition.

Purswani et al. (2011) observed that the selective inoculation of an aerated submerged biofilter designed for treating contaminated MTBE groundwater with a strain showing a high capability for biofilm formation (*Acinetobacter calcoaceticus* M10) with another bacterial strain devoid of this capacity (*Rhodococcus ruber* E10), but highly effective in the degradation of MTBE, yielded a biofilm that was even more abundant and efficient than that developed only by *A. calcoaceticus* M10.

Previous studies (Guisado et al., 2015) showed the biotransforming/degrading MTBE capacity of *Paenibacillus sp.* strain SH7, so despite the small amount of EPS produced and the lack of attachment to the membrane as indicated by FESEM, this bacterial strain could be considered a good candidate for use as a member of a bacterial consortium in a bioremediation strategy.

Toxicity analysis

The acute toxicity (as EC_{50}) of the biomedium at the end of each experiment was tested by Microtox© assay. The **Fig. 4** illustrates the EC_{50} values after 10 days incubation under controlled conditions. The cultures showed varying levels of toxicity.

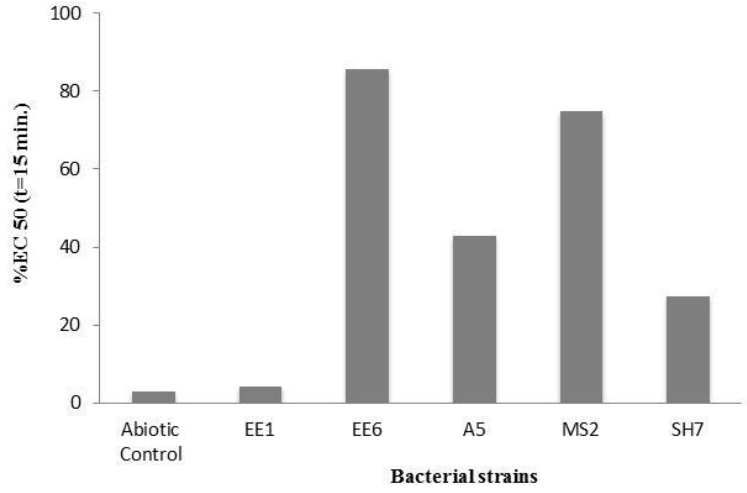

Fig. 4. Acute toxicity (as EC_{50} values) of the culture media after 10 days under controlled conditions.

The samples from the 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 %). On the contrary, *R. ruber* EE1 culture medium exhibited the highest toxicity data (EC_{50}= 4.2 %), which was similar to the value obtained for the abiotic control medium (EC_{50}= 3.0 %).

Guisado et al. (2015) showed the presence of genes encoding for MTBE-degrading enzymes (alkane or cytochrome P450 monooxygenases) in bacterial strains *R. ruber* EE1, EE6 and A5.These bacterial strains were able to use MTBE in batch experiments as a

carbon source in FTW medium amended with 150 mg/L MTBE. In their study, the acute toxicity analyses performed after 8 days of incubation indicated that growth media inoculated with strains *R. ruber* EE1 and EE6 were those that showed (as in this study) the highest and the lowest toxicity values, respectively. The accumulation of some toxic metabolites from MTBE degradation (i.e. TBA, *tert*-butyl-alcohol or formaldehyde) by *R. ruber* EE1 could be the reason for the acute toxicity values found with this microorganism. This result suggests that strain EE1 is not a good candidate as a selective inoculum in an MTBE bioremediation scenario.

Interestingly, the acute toxicity results for *Agrobacterium* sp. strain MS2 and *Paenibacillus* sp. strain SH7 founded are contradictory with those previously obtained by Guisado et al. (2015). In the current study, strain MS2 showed less toxicity. Although a gene screening study yielded negative results for alkane or cytochrome P450 monooxygenases, this strain not only grew in media amended with fuel oxygenate but also developed a patent biofilm on the tubing surface as shown by FESEM, so the growth of this strain as a biofilm seems to improve its MTBE degradative features in terms of toxic metabolite release. On the contrary, *Paenibacillus* sp. strain SH7 did not develop a biofilm, although it showed an important biotransforming/degrading MTBE capacity in batch, notwithstanding the absence of genes encoding for MTBE-degrading enzymes. Further research is needed to clarify which enzymes are involved in the degradation pathways of MTBE by these bacterial strains.

CONCLUSIONS

The results obtained in this study show that 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 ability to adhere to a

support and establish a biofilm as well as the acute toxicity level exhibited as a consequence of their biodegradation activities. The bacterial strains *Agrobacterium* sp. MS2, *Paenibacillus sp*. SH7 and *Rhodococcus ruber* strains EE6 and A5 showed appropriate characteristics for their use as selective inocula (solely or in a consortium) in an EMBFR designed for MTBE contaminated groundwater bioremediation.

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CHAPTER 4

An extractive membrane biofilm reactor (EMBFR) as alternative technology for the treatment of methyl tert-butyl ether (MTBE) contaminated water

Adapted from: I.M. Guisado, J. Purswani, J. Gonzalez-Lopez and C. Pozo. Extractive Membrane Biofilm Reactors (EMBFR) as alternative technology for the treatment of MTBE contaminated water.

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ABSTRACT

Among the strategies developed for contaminated groundwater bioremediation, those based on the use of bacteria adhering to inert supports and establishing biofilms have gained great importance in this field. Extractive membrane biofilm reactor (EMBFR) technology offers productive solutions for the removal of volatile and semi-volatile compounds. EMBFR technology is based on the use of extractive semipermeable membranes through which contaminants migrate to the biological compartment in which microorganisms with pollutant biotransformation and/or mineralization capacities can grow, forming an active biofilm on the membrane surface. The objective of this study was to assess the use of three bacterial strains (*Paenibacillus* sp. SH7 CECT 8558, *Agrobacterium* sp. MS2 CECT 8557, and *Rhodococcus ruber* EE6 CECT 8612), as inoculum in a lab-scale EMBFR running for 28 days under aerobic conditions to eliminate methyl *tert*-butyl ether (MTBE) from water samples. Three different hydraulic retention times (1 h, 6 h, and 12 h) were employed. MTBE degradation values were determined daily by a gas GC-MS technique, as well as suspended bacterial growth. The biofilm established by the bacterial strains on the semipermeable membrane was detected by FESEM at the end of each experiment. The acute toxicity of the treated effluents and biomedium was determined by Microtox© assay (EC_{50}). The results achieved from the MTBE degradation, biofilm formation and toxicity analysis indicated that bacterial strains MS2 and EE6 were the best options as selective inoculum, although further research is needed, particularly with regard to their possible use as a mixed culture. EMBFR technology is shown to be a real option for MTBE contaminated water remediation.

Keywords: Bioremediation, EMBFR, MTBE, Microtox assay

INTRODUCTION

Fuel oxygenates, such as methyl *tert*-butyl ether (MTBE), have been used to replace tetraethyl lead in gasoline formulations in order to reduce harmful emissions. However, their inclusion as a way to enhance air quality has been questioned due to the detection of these substances in both surface and groundwater (de Lacy Costello et al., 2005; Rosell et al., 2007).

Initially and due to its recalcitrant character (conferred by its chemical structure), MTBE was considered less biodegradable. However, in the last decade many studies have pointed out its degradation by several microorganisms under aerobic conditions (Fayolle et al., 2003; Schmidt et al., 2004; Lopes Ferreira et al., 2007; Müller et al., 2008). Bioremediation technologies are more environmentally friendly than physico-chemical ones since intrusion and environmental modification are reduced. Its effective and efficient and generally does not require complex mechanical or structural components. Basic and applied research on MTBE biodegradation has increased in recent years in order to develop new technologies to treat contaminated ecosystems. Among ex-*situ* practices for MTBE-contaminated groundwater bioremediation, those based on the use of fixed biofilms have gained great prominence (Eweis 1997; Fortin and Deshusses 1999;Acuna-Askar et al., 2000; Maciel et al., 2008; Purswani et al., 2014), since protection provided by the biofilm matrix allows for higher cell survival following adaptation to confer an increased production rate or enabling the acquisition of specific enzymes for toxic compound degradation (Decho 2000; Singh et al, 2006). However, the results offered by these studies revealed significant losses of oxygenate by air-*stripping* as well as slow growth and poor biomass yield of MTBE degraders, especially when a pure culture was used as the bioreactor inoculum. In this context, the use of extractive membrane biofilm reactor (EMBFR) technology could resolve these problems since this process employs a physical

barrier (semipermeable membrane) to remove different organic pollutants from contaminated water and transfer them to a biological reservoir ("biomedium") where the bacterial strains with degrading/biotransforming capacities would grow (Livingstone, 1993; Freitas dos Santos and Livingstone, 1994; Livingstone et al., 1998). The growing cells would be established as a biofilm on the biological zone of the membrane (Zhang et al., 1998; Katsivela et al., 1999; Ferreira and Livingstone, 2000), where there would be no contact between the contaminant and oxygen, thus preventing losses by air-*stripping*. The cell density could be higher since the degrading bacteria would develop under optimal conditions (pH, temperature, dissolved oxygen, etc.).

This paper describes the first start-up and operation of an EMBFR system designed at laboratory scale to treat MTBE contaminated water. The system was selectively inoculated with bacterial strains (as pure cultures) with oxygenate-degrading capacities previously isolated and identified (Guisado et al., 2015) and worked during 28 days under different hydraulic retention times. The technology's effectiveness was evaluated by measuring oxygenate values as well as by a toxicity assessment of treated effluent using the Microtox® assay. The biofilm-forming capacity of the bacterial strains on semipermeable membranes was evaluated by the FESEM technique.

MATERIALS AND METHODS

Chemicals

All the chemical substances used in this study were of reagent grade or the highest purity available. MTBE was supplied by Sigma-Aldrich and Fluka Chemical, (Milwaukee, WI, USA), showing 99.9% purity.

Bacterial strains and growth media

The bacterial strains *Rhodococcus ruber* EE6 (CECT 8612), *Agrobacterium sp.* MS2 (CECT 8557) and *Paenibacillus sp.* SH7 (CECT 8558) used in this study were isolated and identified in a previous study (Guisado et al., 2015). Partial 16S rRNA sequences of the isolates identified have been deposited in the GenBank nucleotide sequence database under accession numbers: *Rhodococcus ruber* EE6 (KJ792868), *Agrobacterium* sp. MS2 (KF021239.1), and *Paenibacillus* sp.SH7 (KC625558.1).

The bacterial strains were pre-grown on modified mineral salts medium (FTW medium, Herman and Frankenberger, 1999) amended with 150 mg l^{-1} MTBE as described by Purswani et al., (2008). When solid plates were used, agar-agar (16 g l^{-1}) was added. The fuel oxygenate was added by filtration (Millipore, 0,22 μm) to growth media previously sterilized by autoclaving. The FTW-plus medium was comprised of FTW medium supplemented with 1 g l^{-1} yeast extract.

Preparing pre-inoculums

Tubes of FTW liquid medium (5 ml) amended with 150 mg l^{-1} MTBE were inoculated with bacterial strains EE6, SH7 and MS2 and kept under agitation (150 rpm) and controlled temperature (30 ° C) for 4 days. After this time, 300 ml sterile FTW-plus liquid medium was inoculated with these pre-inocula and incubated again under the same conditions for 4 days.

Extractive membrane biofilm reactor (EMBFR) design and operation

The extractive membrane biofilm reactor (EMBFR) at laboratory scale (**Fig. 1**) consisted of a glass cylindrical vessel (3 l capacity) sealed by a glass lid with different holes for the insertion of several sensors (pH, temperature, dissolved oxygen) as well as influent and treated effluent tubes.

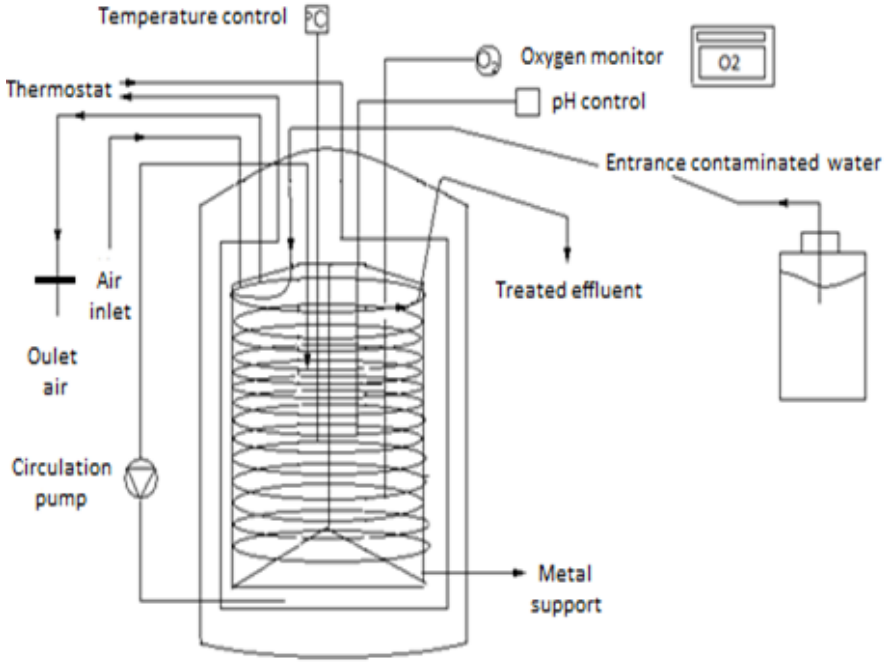

Figure. 1. Schematic diagram of Extractive Membrane Biofilm Reactor (EMBFR) at lab-scale used in the study.

Inside the glass vessel, tubular semipermeable membranes (3.8 mm internal diameter, 0.4 mm wall, 2.5 m length; Watson Marlow, Wilmington, MA, USA), were wound onto a stainless steel support with plastic washers to prevent the collapse of the

tubes. The water samples were amended with 150 mg l^{-1} MTBE and pumped into the system at different flow rates: 0.16 l h^{-1}, 0.026 l h^{-1} and 0.013 l h^{-1} i.e., HRTs (hydraulic retention times) of 1, 6 and 12 h, respectively, by a peristaltic pump (Watson Marlow 520S).

The bioreactors were filled with sterile FTW medium (2,700 ml) plus 300 ml of pre-grown FTW-plus medium inoculated with each bacterial strain (EE6, SH7 or MS2) prepared as previously described. To evaluate the non-biological degradation of MTBE, for each HRT condition, a non-inoculated bioreactor was included and considered as "abiotic control". The inoculated and abiotic bioreactors were kept under agitation during 28 days to ensure optimum contact between the microorganisms and mineral medium (i.e. growth medium, so-called "biomedium").

Oxygen was supplied by an air pump and available to the microorganisms by diffusion through another tubular semipermeable membrane, alternating both tubular membranes in order to provide sufficient oxygen for growing microorganisms and, most important, to avoid bubble formation and consequently the air-*stripping* phenomenon. The oxygen level (as dissolved oxygen, DO) of the biomedium was controlled by an oxygen probe (2-Channel DO-Transmitter Mettler Toledo M300) previously calibrated following the manufacturer's indications. Marprene® and Teflon® tubes were used to connect the influent bottles with the tubular semipermeable membranes immersed in the biomedium and from these to effluent tubes to prevent MTBE loss through the semipermeable membranes outside the bioreactor. The pH was maintained between 6.8 and 7.2 by a controller (Consort R3620 Controller pH, Medorex) connected to acid and base dosing pumps. The biomedium temperature was kept at 28ºC by a heating/cooling bath connected to a stainless steel cooling system (Huber Ministat 125 cc, Huber).

During the first eight days the systems were maintained under recirculation mode, being 150 mg l^{-1}, the amount of fuel oxygenate available at the experimental starting point.

After this time, the bioreactors were switched to continuous mode, fed with contaminated water (MTBE 150 mg l^{-1}) and so kept until the end of the study. Two weeks after beginning each experiment, 1/3 of the bioreactor volume (1 l) was drained and replaced by fresh sterile FTW medium both in inoculated bioreactors and abiotic controls.

Collection of samples

To evaluate the efficiency (as remaining MTBE concentrations) of the EMBFR at lab-scale during the running time, samples (1.5 ml) from the influent, biomedium and effluent were taken, stored in 2 ml glass vials, clamped and maintained at 4ºC until analysis. Samples were taken in triplicate. Microbial growth (as optical density, OD) in the biomedium was measured spectrophotometrically (λ = 600 nm). For this, samples (3 ml) were taken daily through a Teflon® tube located in the bioreactor cover and protected with a 0.22 µm filter to avoid contamination. Triplicate samples were measured.

At the end of the experimental period, samples (10 ml) from the influent, biomedium and treated effluents were analysed to evaluate their ecotoxicity (as EC_{50}) using the Microtox® test, followed by dismantling of the bioreactor and sampling from the immersed semipermeable membranes.

MTBE analysis

The gas chromatography mass spectrometry (GC/MS) headspace technique was used to quantify the remaining concentrations of MTBE in the samples following the methodology described by Purswani et al., (2011). Volumes of 1.5 ml of the unfiltered inoculated and control samples from the influent, biomedium and treated effluent were placed into 2 ml vials and clamped as previously described. The samples were heated at

90°C for 90 min, followed by the injection of 50 µl of the gas phase into the GC/MS equipment (Hewlett-Packard 6890 GC coupled to a MS Hewlett-Packard 5973 mass selective detector, Palo Alto, CA, U.S). 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: 40ºC (3.5 min), 10ºC min^{-1} up to 85ºC, 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).

Examination of biofilm formation by Field-Emission Scanning Electron Microscope (FESEM) technique

To examine the biofilms established on the tubular semipermeable membranes, the methodology proposed by Purswani et al., (2011) was followed. Briefly, after 28 days of operating time, the systems were uninstalled and several portions (three, 1 cm in length) of the lower part of the immersed tubing were cut. The units were fixed in 2.5 % glutaraldehyde in PBS pH 7.4 during 24 h at 4 °C. The samples were then washed three times in PBS 1 during 20 min. Post fixation was followed by the addition of 1 % osmium tetroxide, maintaining the samples in the dark during 1 h at room temperature and washing three times with distilled water. The samples were dehydrated in increasing ethanol concentrations, desiccated and covered by the evaporation of carbon with a HITACHI evaporator prior to 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 (Microtox Model 500 toxicity analyser, Instrumentación Analítica S.A. Madrid, Spain) was used to measure acute toxicity (as EC_{50}) of the influent, biomedium and treated effluent at the end of each experiment. Abiotic and biotic bioreactor samples (10 ml) were collected on the last operational day (day 28) for comparison.

RESULTS AND DISCUSSION

Suspended microbial growth at different retention times

Suspended microbial growth (as optical density, OD) in the biomedium of each bioreactor was evaluated by measuring their absorbance (ʎ=600 nm) as previously described. **Figure 2** shows the evolution of OD in the biomedium of each inoculated bioreactor at different hydraulic retention times (**Fig. 2**). Abiotic controls were included for comparison.

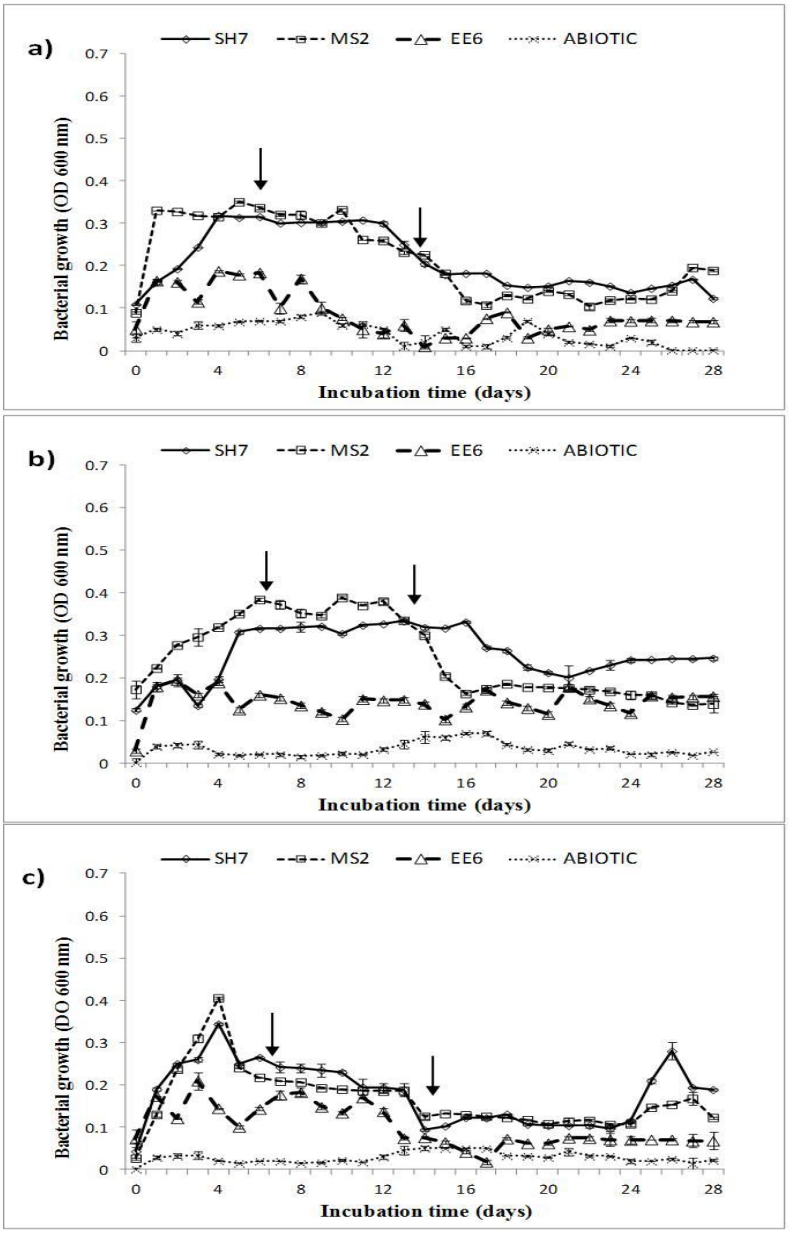

Figure. 2. Suspended growth (as optical density, $OD_{600\ nm}$) evolution in biomedium of each inoculated bioreactor under different HRTs. HRT of a) 1h, (b) 6 h and (c) 12 h. Abiotic controls were included for comparison. Two arrows indicating the time of operation mode change (8 d) and renewal of the biomedium (15 d) are shown.

The systems were maintained in recirculation mode for eight days, after which they were changed to continuous mode. Regardless of which HRT were run, the bioreactors inoculated with the SH7 and MS2 strains developed higher suspended bacterial growth. The OD increase was gradual until it reached a maximum that coincided in all cases with the recirculation period (OD= 0.3–0.4). However, growth values were maintained for additional days when the HRT was 1 h or 6 h (**Fig.2a** and **2b**). Moreover, the OD measured in bioreactors inoculated with *Rhodococcus ruber* EE6 was always the lowest regardless of HRT.

In order to renew the biomedium in the bioreactors and avoid possible negative effects of MTBE metabolites on bacterial growth and survival, we removed 1 l of biomedium and replaced it with fresh FTW medium after 15 days of operation. The OD decrease observed in the biomedium of the bioreactors after this change could be due to the dilution effect, although it should be noted that the growth of bacterial strains adhering to the surface of the tubular semipermeable membranes (and established as biofilm) in response to a MTBE diffusion gradient would also reduce the planktonic cell number in the biomedium. In this case, there should be a biofilm on the surface of these membranes. In this regard, Ferreira and Livingstone (2000) showed that the OD reductions detected in the biomedium of an extractive membrane bioreactor designed to treat wastewater containing organic compounds (monochlorobenzene and 1,2-dichloroethane) corresponded to an incipient biofilm on the surfaces of the semipermeable membranes tested by SEM analyses. In our study this fact was confirmed in bioreactors inoculated with the MS2 and EE6 bacterial strains by FESEM analyses as shown below.

Dissolved oxygen results

Laboratory MTBE biodegradation has been recorded under aerobic conditions by both pure and mixed cultures (Salanitro et al., 1994, Hyman et al., 1998; Liu et al., 2001; Francois et al., 2002, Zhong et al., 2007), although the use of mixed cultures seemed to offer better performance than pure ones when aerated biofilter technology was used. Monooxygenase enzymes (such as cytochrome P450) are responsible for the biotransformation of MTBE to *tert*-butyl alcohol (TBA), the main metabolite under aerobic degradation (Hardison et al., 1997; Steffan et al., 1997; Fayolle et al., 2001).

An important aspect to consider when aerated bioreactors are used for the removal of semi-volatile compounds such as oxygen ethers (i.e. MTBE) is the phenomenon of air-*stripping,* which reduces the performance of many conventional aerated biofilm reactors (Casey et al., 1999). To avoid this, in our study, the air was provided though a separate tubular semipermeable membrane positioned next to the one through which the MTBE amended water flowed. Thus, the oxygen could be released directly to the active microorganisms established in the biofilm or be dissolved in the biomedium and available to suspended microorganisms, preventing loss of MTBE to the gaseous phase.

Figure 3 shows the evolution of dissolved oxygen (DO, mg l^{-1}) in the biomedia of inoculated and non-inoculated ("abiotic control") bioreactors under different HRT (**Fig. 3**). The DO concentrations were recorded daily in the biomedia of all bioreactors by an oxygen probe.

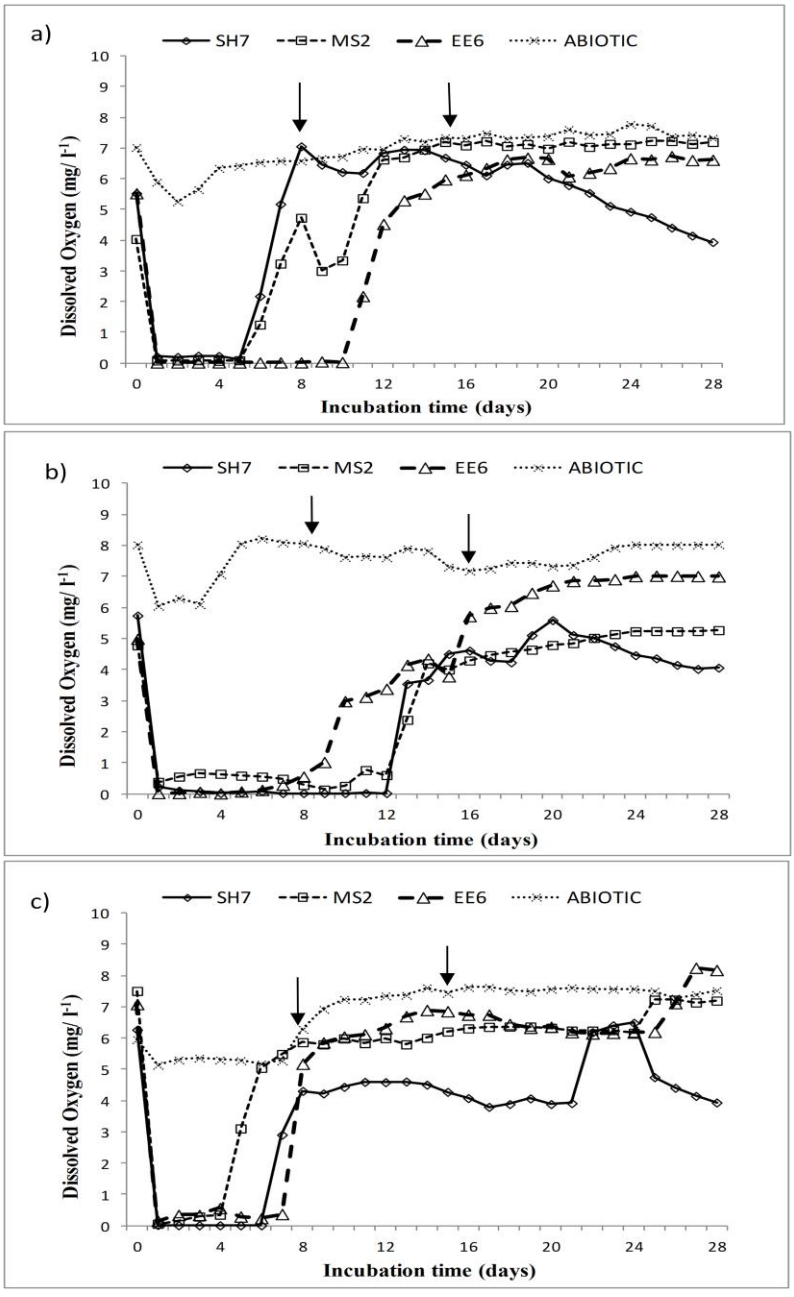

Figure. 3. Dissolved oxygen (DO, mg l^{-1}) evolution in the biomedium of inoculated bioreactors under different HRTs. HRT of a) 1h, (b) 6 h and (c) 12 h Abiotic controls were included for comparison. Two arrows indicating the time of operation mode change (8 d) and renewal of the biomedium (15 d) are shown.

During the first operating week the systems were under recirculation mode and the oxygen concentrations decreased in all the inoculated bioreactors whatever the operating conditions. In contrast, the level of oxygen in abiotic bioreactors remained more or less constant (between 7 and 8 mg l^{-1} O_2) during the study period.

The bacterial growth present in the inoculated bioreactors seemed to be the reason for the oxygen consumption observed during the recirculation period, though this consumption was extended longer in some cases, such as in bioreactors inoculated with bacterial strain EE6 at HRT 1 h and in all bioreactors inoculated at HRT 6 h (**Figure 3 a** and **3 b**, respectively). Zhong et al. (2007) showed in their study that there was a critical DO value for bacterial growth and, above this, MTBE degradation could be accomplished. They also pointed out that low DO values affect monooxygenase activity adversely and therefore MTBE degradation was carried out very slowly.

In our study, the biomedium was composed of 2.7 l of FTW medium plus 300 ml of FTW-plus medium (mineral medium with 1 g l^{-1} yeast extract), which was available to the microorganisms along with MTBE during this first phase as carbon and energy sources for growth. From the suspended growth data (**Figure 2**), it would seem that the bacterial growth observed was only due to the available carbon in the FTW-plus medium; however, the MTBE degradation data (**Figure 4**) showed that during the recirculation phase, MTBE concentrations decreased in the biomedium of all inoculated bioreactors. Acuna-Askar et al. (2000) studied the biodegradation of MTBE in biofilm reactors and concluded that the presence of other easily assimilated carbon sources in the culture medium favourably influenced MTBE biodegradation.

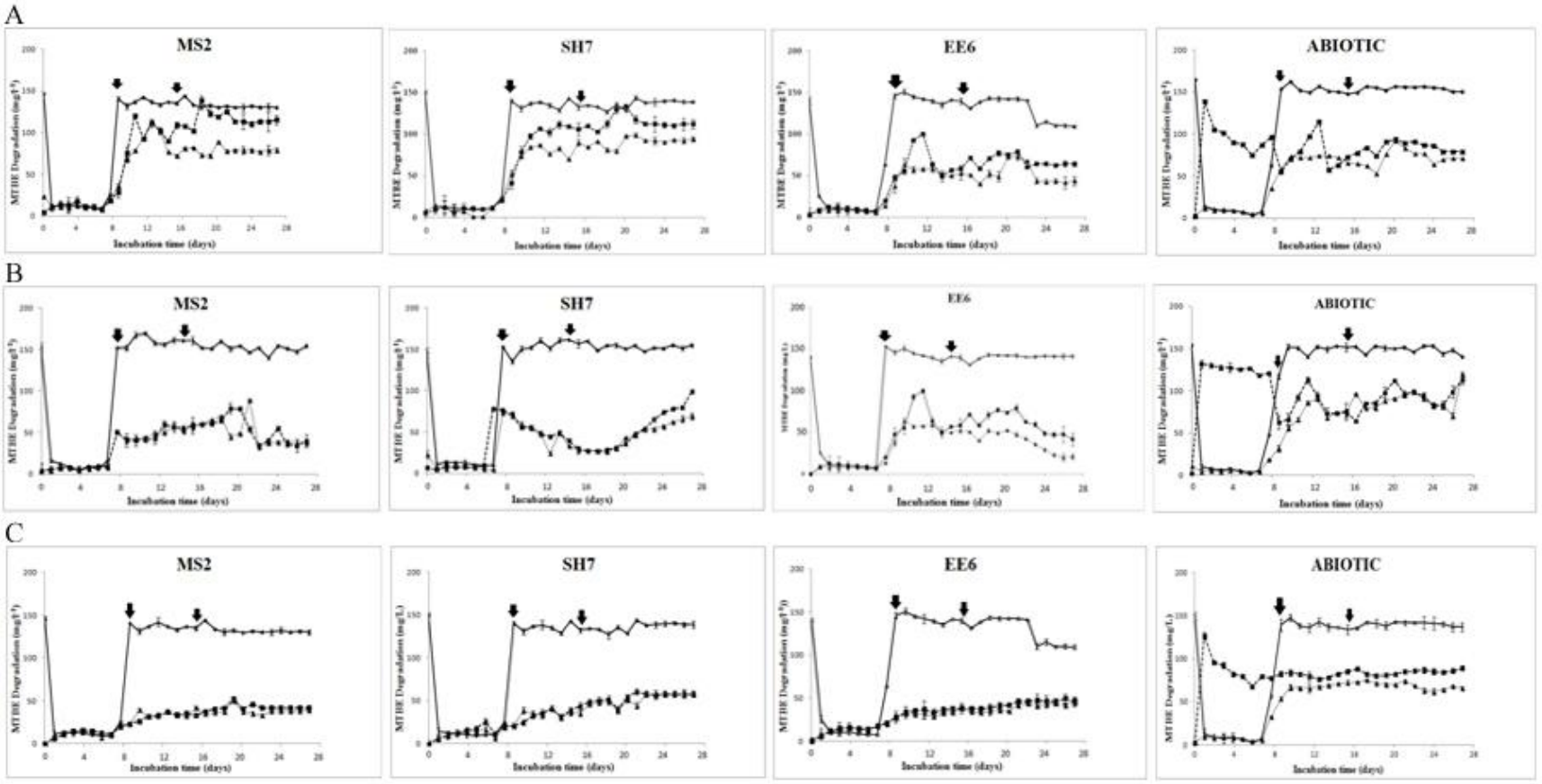

Figure. 4. Evolution of MTBE concentrations (mg l^{-1}) in each of the compartments (influent _._, biomedium --■--- and effluent ..▲..) of inoculated and non-inoculated bioreactors during 28 days of experiment under different HRTs. HRT of a) 1h, (b) 6 h and (c) 12 h. Two arrows indicating the time of operation mode change (8 d) and renewal of the biomedium (15 d) are shown.

MTBE degradation

Figure 4 shows the evolution of MTBE concentrations (as mg l^{-1}) in each of the compartments (influent, biomedium and effluent) in the tested systems (bioreactors inoculated with the strains EE6, MS2 and SH7) under different hydraulic retention times (**Fig. 4**). Data from non-inoculated bioreactors are included for comparison.

Influent MTBE concentrations were very similar in all bioreactors regardless of HRT, reaching their lowest values under recirculation mode. After switching to continuous mode (day 7), MTBE concentrations increased and then remained more or less steady until the end of the experiment. In order to maintain the same concentration of oxygenate available to the microorganisms, the feed bottles were supplemented daily during this phase with 1 l of sterile water amended with 150 (mg l^{-1}) of MTBE. However, certain variations due to environmental conditions were recorded despite having a chamber with controlled temperature.

The oxygenate was able to cross the semipermeable tubular membranes to the biomedium. In the abiotic bioreactors the amount of oxygenate in this compartment increased after the first running day and then remained steady during the whole study period. However, in the biomedium of inoculated bioreactors under recirculation mode, the fuel oxygenate concentrations were always very small regardless of the HRT, being HRT 12 h the operating condition that yielded the best oxygenate degradation results (i.e. 91.4% in the bioreactor inoculated with MS2, 95.63% in the case of SH7 and 94.22% in the bioreactor inoculated with EE6).

Purswani et al. (2014) highlighted losses of fuel oxygenates from aerated submerged biofilters by air-*stripping.* In our study, the decrease observed in MTBE concentrations could be attributed to this phenomenon but, to avoid this, air was supplied to the systems, as previously described, by semipermeable membranes, increasing system performance and

preventing loss of MTBE. Therefore consumption by the bacterial strains would be the reason for fuel oxygenate reduction.

The amounts of MTBE detected in the effluents of the abiotic bioreactors were similar to those from the inoculated ones (the bioreactor inoculated with EE6 being the exception) when the HRT was 1 h, but higher at hydraulic retention times of 6 h and 12 h. Under these latter operating conditions, the bacterial strains were able to use MTBE as a carbon source much more efficiently.

No differences in effluent MTBE concentrations were detected in bioreactors inoculated with MS2, SH7 or EE6 at HRT of 6 and 12 hours, although the latter operating condition yielded the best results in terms of degradation (with the MS2 strain degrading 56.81 % of MTBE, the SH7 strain 66.57 % and the EE6 strain 57.17%). Hu et al. (2004) observed that under their experimental conditions higher percentages of biodegradation removal were observed at longer HRT.

Biofilm formation on semipermeable tubing

The main goal of a study carried out by different authors (Ferreira and Livingstone, 2000) on extractive membrane bioreactors was to note if the development of a biofilm on semipermeable membranes could reduce mass transfer and thus limit system efficiency. They showed that the development of a biofilm on the membrane surface reduced mass transfer across it and revealed, under their experimental conditions, that the only real importance of the biofilm was to maintain an active microbiota capable of degrading various organic substances. In contrast, Katsivela et al. (1999) operating an EMBR for the treatment of industrial wastewater showed the excellent performance of the system and the pollutant-degrading efficiency of the biofilm.

In our case, biofilm formation on the membranes did not cause any damage (such as biofouling and/or corrosion) of the tubing material. In addition, the fuel oxygenate was able to cross the membrane and be available to the bacterial strains regardless of how they grew.

At the endpoint of each experiment (28 days) we proceeded to take samples from the tubular semipermeable membrane inside the biomedium to assess the biofilm established on it as described in the Material and Methods section. **Figure 5** shows FESEM micrographs from the tubular semipermeable membranes of each bioreactor under different HRT (**Fig.5**).

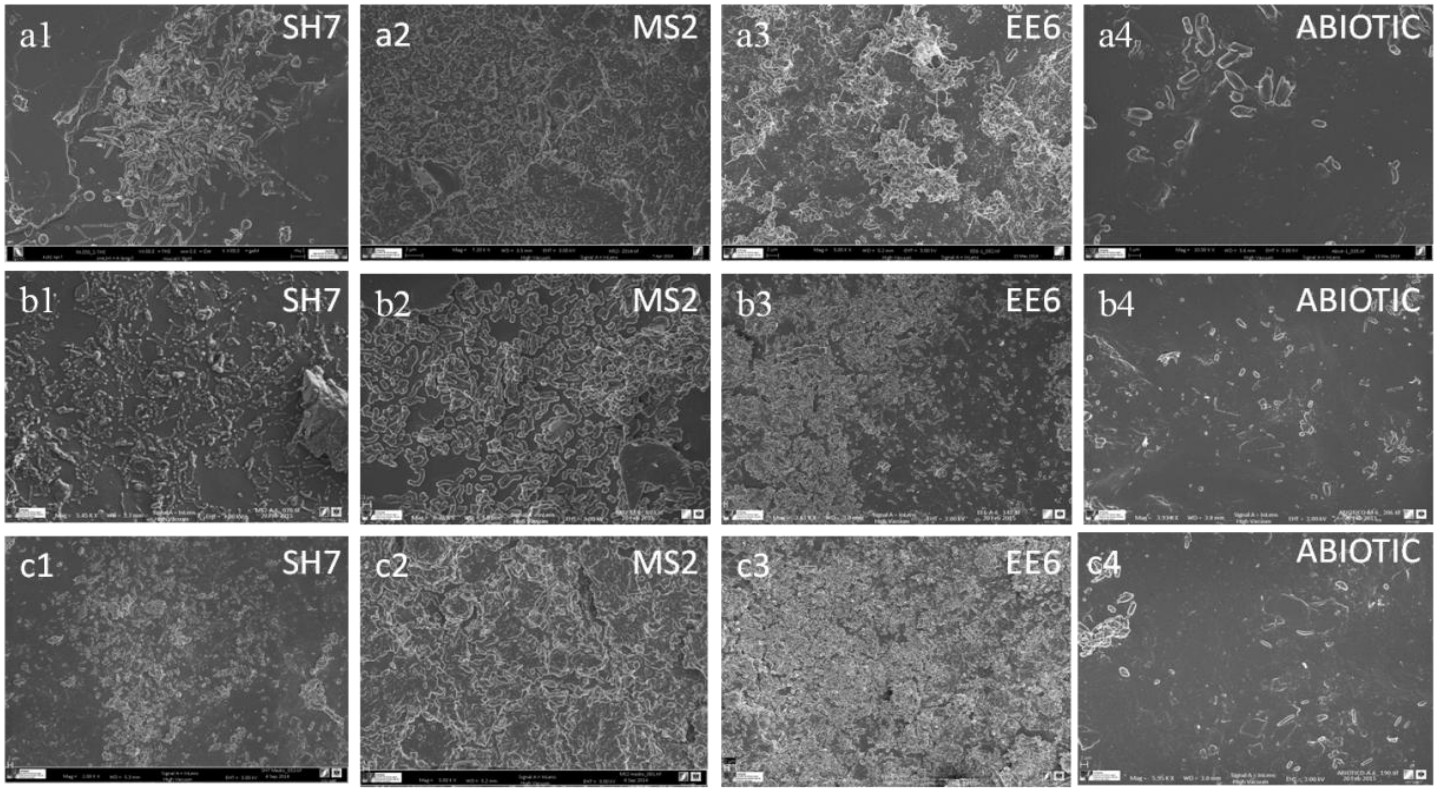

Figure. 5. Field-Emission Scanning Electron Microscope (FESEM) images of biofilm on tubular semipermeable membranes. Samples were taken from the bottom of immersed tubing of inoculated bioreactors at the end of each experiment (28 days) under different HRTs. HRT of a) 1h, (b) 6 h and (c) 12 h. Samples from abiotic controls were included for comparison.

The bacterial strains EE6 and MS2 were able to grow on the tubular semipermeable membranes and develop a more patent biofilm when the bioreactors were inoculated with *Rhodococcus ruber* (EE6) and maintained under a HRT of 12 hours. Suspended microbial growth data had already shown that in bioreactors inoculated with this strain, the biomedium's OD values were always the lowest of all, so the adhesion of the bacteria to the surface of the membranes would explain this fact. In contrast, the SH7 strain did not form any biofilm under either HRT tested, but this strain (together with *Agrobacterium* sp. MS2) exhibited the highest suspended growth measured as determined by OD. Previous studies (Guisado et al., 2015) have also shown the biotransforming/degrading MTBE capacity of *Paenibacillus* sp. SH7 in batch studies as well as the small amounts of EPS produced. Purswani et al. (2011) observed how selective inoculation of an aerated submerged biofilter designed to treat contaminated MTBE groundwater with a strain with a high capacity for biofilm formation (*Acinetobacter calcoaceticus* M10), together with another bacterial strain missing this capacity (*Rhodococcus ruber* E10) but highly effective in the degradation of MTBE, yielded a biofilm even more abundant and efficient at MTBE removal than that developed only with *A. calcoaceticus* M10. In this sense, the selective inoculation of an EMBFR with a bacterial consortium consisting of bacterial strains with MTBE-degrading capacities and different biofilm-forming behaviours (such as those used in this study) could offer a promising option for fuel oxygenate bioremediation.

Toxicity analysis of bioreactor biomedium/effluent

In order to determine whether the technology applied in this study was efficient in removing MTBE from contaminated water, together with determining the remaining MTBE concentrations, the Microtox® test was used to evaluate the ecotoxicity of the

treated effluent and the biomedium at the end of the experimental period. The ecotoxicity of the influent for each HRT was measured too. Blaschke et al. (2010) suggested in their study that the marine bacterium *Vibrio fisheri* was sensitive to MTBE and related metabolites, thus justifying the use of the above test.

Table 1 shows the ecotoxicity results (as EC_{50} after 15 minutes exposure) in both biomedium and treated effluent for each of the inoculated bioreactors under the HRTs assayed. The results from abiotic bioreactors are included for comparison.

Table 1. Acute toxicity (as EC_{50} values) of the culture media after 28 days under controlled conditions in each of the compartments under different hydraulic retention times. Values are media ± SD of three data.

		Bacterial strains (%EC_{50}, t=15min)			
	HRT	**Abiotic**	***EE6***	***MS2***	***SH7***
Biomedium	**1**	3.25±0.13	17,59±0.25	10,22±0.16	5,11±0.23
	6	3,82±0.18	24,96±0.12	20,81±0.15	9,03±0.22
	12	4,25±0.21	32,12±0.31	25,78±0.24	13,19±0.17
Treated effluent	**1**	3,06±0.11	24,31±0.29	18,65±0.24	7,70±0.19
	6	3,15±0.14	30,45±0.10	19,95±0.20	11,62±0.15
	12	3,26±0.26	38,21±0.21	29,24±0.16	21,95±0.23

In all cases, the ecotoxicity exhibited by the biomedium and treated effluent of the inoculated bioreactors was always less than those from abiotic bioreactors as well as from influents (average influent EC_{50}= 3.3 %, data not shown).

The bioreactors inoculated with the bacterial strain EE6 showed the lowest toxicity levels in both the biomedium and treated effluent, being HRT 12 h the operating condition

that yielded the best ecotoxicity results (biomedium EC_{50}= 32.12±0.31% and effluent EC_{50}= 38.21±0.21%). Nevertheless, the biomedium and effluent from bioreactors inoculated with the SH7 bacterial strain showed higher levels of toxicity with any HRT assayed. FESEM analyses revealed that this bacterial strain was unable adhere to the tubular semipermeable membrane and establish as a biofilm. The presence of secondary metabolites from MTBE degradation (TBA or formaldehyde) released by this bacterial strain in the biomedium would be sufficient to justify these ecotoxicity values. Roslev et al., (2015) pointed out in their study that TBA was far less toxic (as EC_{50} values) than MTBE, while formaldehyde was found to be the most toxic metabolite. However, the data from GC-MS analyses under our experimental conditions did not demonstrate the presence of either of these substances. It is noteworthy that similar results were obtained by Guisado et al. (2015) despite the high MTBE-degradation capacities in *batch* studies shown by this bacterial strain.

The results obtained in this study show that extractive membrane biofilm reactor (EMBFR) technology could be an effective option for the bioremediation of MTBE-contaminated water bodies under particular operating conditions (i.e. HRT), although further research is needed, particularly with regard to the use of bacterial strains with different oxygenate degrading capacities as bacterial consortia to improve system performance. In the other hand, the selection of bacterial strains as inocula for bioremediation purposes should not only be determined by their biodegradation capacities but also by other aspects like, for example, their contribution to the ecotoxicity level of treated effluents, so that the technology used can be an environmentally-friendly system. In this sense and based on our results, a consortium constituted by *Rhodococcus ruber* EE6, *Agrobacterium* sp. MS2, and *Paenibacillus* sp. SH7 would be an excellent selective inoculum for MTBE water bioremediation by EMBFR technology.

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IV. DISCUSIÓN GENERAL

OVERALL DISCUSSION

DISCUSIÓN GENERAL

El metil *tert*-butil éter (MTBE), es un compuesto semi-volátil, principalmente producido para la sustitución del plomo en la formulación de la gasolina. Es añadido a la gasolina reformulada (en distintas concentraciones, dependiendo del país) para mejorar el índice de octano y así su combustibilidad a fin de reducir las emisiones tóxicas a la atmósfera. Este compuesto oxigenante se puede detectar en numerosos acuíferos de Estados Unidos y Europa como consecuencia de derrames accidentales desde los contenedores de almacenamiento, fugas en los sistemas de conducción o fugas desde tanques de gasolineras, entre otras, favorecido por su tendencia a lixiviarse y su alta solubilidad en agua. Se han desarrollado numerosas tecnologías de remediación para paliar el grave problema de la contaminación producida por este compuesto en aguas subterráneas; entre otras, podemos citar a las tecnologías basadas en la adsorción, la oxidación química, la inyección de aire y las tecnologías biológicas. Los tratamientos que aplican sistemas biológicos para catalizar la transformación de compuestos químicos en otros menos tóxicos o llevar a cabo su mineralización mediante el uso de organismos vivos (fundamentalmente bacterias) para absorber, degradar o transformar los contaminantes presentes en el medio ambiente, retirándolos, inactivándolos o atenuando su efecto, son una óptima opción y son considerados el mejor método para eliminar los compuestos oxigenantes de las masas de agua, además de ser técnicas no invasivas y rentables (Lopes-Ferreira y colaboradores, 2006; Eixarch y Constantí, 2010). Dentro de este tipo de tratamientos, ha cobrado un especial interés en la remediación de aguas subterráneas contaminadas con diversos compuestos semi-volátiles, la aplicación de sistemas tales como biofiltros percoladores, biofiltros sumergidos, biorreactores de membrana y biorreactores de membrana extractiva (Livingston, 1991,1993; Freitas dos Santos y Livigstone, 1994; Eweis y colaboradores, 1997, 1998; Fortin y Deshusses, 1999 a,b; Casey y colaboradores, 1999; Nicolella y

colaboradores, 2000; Steffan y colaboradores, 2000; Hu y colaboradores, 2004; Purswani y colaboradores, 2011). En la mayoría de los casos se ha mostrado una mayor efectividad cuando se utiliza biomasa fija (Fortin y colaboradores, 1999 a, b; Acuna-Askar, 2000; Kharoune y colaboradores, 2001; Moreels y colaboradores, 2004; Hu y colaboradores 2004; Maciel y colaboradores, 2008).

La degradación aeróbica de MTBE es posible, y en algunos casos muy eficiente, mediante cultivos puros (Hatzinger y colaboradores, 2001; Francois y colaboradores, 2002) y mixtos (Salanitro y colaboradores, 1994). Estos microorganismos pueden utilizar el MTBE como única fuente de carbono y energía (Deeb y colaboradores, 2001; Mo y colaboradores, 1995) o ser efectiva esta degradación, gracias a procesos cometabólicos (Hyman y colaboradores, 1998, Liu y colaboradores, 2001). Sin embargo, y de forma general, se ha puesto de manifiesto los bajos rendimientos de biomasa así como las bajas velocidades de crecimiento por parte de los microorganismos con capacidad para biotransformar/degradar este compuesto xenobiótico (Müller y colaboradores, 2007).

El estudio técnico y biológico de un sistema de biorreactor de membrana extractiva con desarrollo de biopelícula para el tratamiento de aguas contaminadas con metil *tert*-butil éter (MTBE) ha sido el objetivo principal de esta Tesis Doctoral. Tal y como se ha descrito, un reactor de membrana extractiva con desarrollo de biopelícula (EMBFR, Extractive Membrane Biofilm Reactor) (Katsivela y colaboradores 1999; Ferreira y Livingston 2000) es una modificación de la tecnología EMBR (Extractive Membrane Bioreactor) (Livingston 1991,1993; Freitas dos Santos y Livigstone 1994) basada en el uso de membranas semipermeables, a través de las cuales los contaminantes migran al compartimento biológico en el que los microorganismos con capacidades para biotransformar y mineralizar un determinado contaminante crecen en un medio, formando una biopelícula en la superficie de la membrana, pudiéndose controlar las condiciones de crecimiento de los microorganismos en el compartimento biológico para así asegurar una

degradación eficiente de los compuestos contaminantes, independientemente de las condiciones en el efluente a tratar. En los sistemas aeróbicos, el oxígeno puede ser suministrado también a través de membranas semipermeables evitando así la pérdida del contaminante por volatilización. Anteriormente diversos autores (Purswani y colaboradores 2011, 2014) pusieron de manifiesto las limitaciones que determinadas tecnologías *ex situ* desarrolladas para la remediación de masas de agua contaminada con MTBE mostraban y entre las que podemos citar el fenómeno de *air-stripping* y la baja concentración de biomasa desarrollada por las cepas bacterianas utilizadas como inóculos selectivos y previamente seleccionadas por su capacidad para crecer en medios minerales suplementados con MTBE como única fuente de C y energía. Con la aplicación de la tecnología objeto de esta Tesis Doctoral se pretendió resolver estas limitaciones y así llevar a cabo una exitosa estrategia de biorremediación.

Un paso importante en el diseño y desarrollo de una determinada tecnología biológica para el tratamiento de aguas subterráneas contaminadas con oxigenantes de las gasolinas, como la evaluada en esta Tesis Doctoral, es el inicial aislamiento y selección de cepas bacterianas que presentan la capacidad de crecer ó biotransformar/degradar estos compuestos. Autores como Hanson y colaboradores (1999), Piveteau y colaboradores (2001), Nakatsu y colaboradores (2006), Rohwerder y colaboradores (2006) y Müller y colaboradores (2008), entre otros, describen cultivos bacterianos puros capaces de usar MTBE como única fuente de carbono y energía. Actualmente, los procesos de selección de bacterias tienden a ser bastante limitados, por lo que no se proporciona mucha información sobre las cepas bacterianas antes de ser utilizadas en ensayos posteriores de biorremediación. En la presente Tesis Doctoral, se ha descrito (Guisado y colaboradores 2015) una metodología precisa para el aislamiento y selección de cepas bacterianas capaces de usar MTBE como única fuente de carbono y energía basándose en los resultados de crecimiento en medios adicionados de MTBE y/o hidrocarburos aromáticos (BTEX),

ecotoxicidad exhibida por los cultivos bacterianos bajo estas condiciones, presencia/ausencia de genes involucrados en la degradación de MTBE (monooxigenasas), así como en la capacidad para degradar/biotransformar MTBE en medios líquidos.

Las cepas bacterianas se aislaron a partir de muestras contaminadas con hidrocarburos, siguiendo dos metodologías diferentes: directamente a partir de las muestras originales (suelo y agua subterránea) o después de un proceso de enriquecimiento. Diversos autores (Mo y colaboradores 1997; Hatzinger y colaboradores 2001; Okeke y Frankenberger 2003; Purswani y colaboradores 2008), utilizaron técnicas de enriquecimiento como un método para aislar, con cierto éxito, cepas bacterianas con capacidad de degradación.

El 60% del total de los aislamientos que se realizaron en el desarrollo de este estudio fueron afiliados a los filos *Firmicutes* y *Actinobacteria*. De hecho, géneros pertenecientes a estos filos, (Hernández-Pérez y colaboradores 2001; Youngster y colaboradores 2010; Beazley y colaboradores 2012) están involucrados en la degradación de hidrocarburos aromáticos tales como benceno, tolueno, etilbenceno y xileno (BTEX) o en la degradación aeróbica de MTBE (François y colaboradores 2002). El resto de las cepas aisladas, se agruparon dentro del filo *Proteobacteria*. Es destacable que todas las cepas bacterianas aisladas después de las etapas de enriquecimiento, estuvieron incluidas en este grupo. Wagner-Döbler y colaboradores (1998) describieron que las técnicas clásicas de enriquecimiento tienden a aislar microorganismos Gram negativos, debido a sus altas tasas de crecimiento.

Los compuestos aromáticos (BTEX) son parte activa de los contaminantes en los acuíferos después de derrames o pérdidas accidentales durante la producción, distribución, almacenamiento y uso de la gasolina, coexistiendo MTBE y compuestos BTEX en acuíferos contaminados (Happel y colaboradores 1998). En el desarrollo de esta Tesis Doctoral se evaluó la capacidad de las cepas aisladas para crecer en medios suplementados

con compuestos aromáticos BTEX (con o sin MTBE). En paralelo, también fueron sometidas a análisis génicos para determinar la presencia de genes que codifican para enzimas involucradas en la degradación de MTBE (alcano o citocromo P450 monooxigenasas). Sin embargo, los resultados obtenidos mostraron que la detección de estos genes marcadores no necesariamente estuvo ligada a una biodegradación efectiva de MTBE por parte de las cepas, y por lo tanto, este hecho no debería ser utilizado como indicador inequívoco de la capacidad de una cepa para degradar/biotransformar este xenobiótico. Por otra parte y aunque las cepas bacterianas *Agrobacterium* sp. MS2 y *Paenibacillus* sp. SH7 aisladas en este estudio exhibieron los niveles más altos de biotransformación de MTBE, la detección génica realizada no mostró resultados positivos con los cebadores ensayados. El uso de más cebadores monooxigenasa generales o multiplex-PCR utilizando varios cebadores podría ser una mejor estrategia para la identificación de un marcador génico como indicador de la existencia de biorremediación de MTBE.

En base a los resultados obtenidos de los experimentos de crecimiento y degradación de las cepas aisladas en medios adicionados de MTBE e hidrocarburos aromáticos, así como los datos de detección de genes, se seleccionaron un total de 5 cepas bacterianas (*Rhodococcus ruber* A5 CECT8556, cepa EE6 CECT 8612 y cepa EE1 CECT 8555, *Agrobacterium* sp. MS2 CECT 8557 y *Paenibacillus* sp. SH7 CECT 8558), para realizar estudios de biodegradación de MTBE en experimentos *in batch*. Aunque las cepas MA2 (*Pseudomonas aeruginosa*) y A6 (*Nocardia nova*) fueron capaces de crecer en medios con MTBE, no fueron incluidas en los demás estudios ya que son cepas patógenas oportunistas en humanos.

Paenibacillus sp. SH7 fue la cepa que logró la mayor reducción en la concentración de MTBE (100 mg/L^{-1}) en los ensayos de degradación en medio líquido tras 8 días de incubación; *Agrobacterium* sp. MS2 en cambio, logró una reducción de 85 mg/L^{-1} en 4

días. Sin embargo, ninguna de las dos cepas bacterianas presentó una alta tasa de crecimiento ni un gran número de generaciones, pudiendo ser explicado este hecho por la falta de energía procedente de la degradación de la fuente de carbono, MTBE en este caso. Tal y como apuntaron Hatzinger y colaboradores (2001), la falta de crecimiento observado podría ser el resultado de una deficiencia de enzimas para la degradación completa del MTBE o la presencia de compuestos inhibidores como productos intermediarios (TBA, *tert*-butil alcohol por ejemplo) en la biodegradación de estos compuestos oxigenados.

Otra cepa a destacar fue *Rhodococcus ruber* EE6. Este microorganismo desarrolló el mayor número de generaciones bajo las mismas condiciones que las otras cepas, pero este hecho no estuvo relacionado con la más alta tasa de eliminación de MTBE. Los valores de toxicidad (determinados mediante el bioensayo Microtox®) después del crecimiento en medio suplementado con MTBE, indicaron que los cultivos de esta cepa fueron los que mostraron la menor toxicidad, y por lo tanto se deduce que la eliminación más eficiente de MTBE fue llevada a cabo por ésta. Los análisis de ecotoxicidad revelaron que la toxicidad de los cultivos bacterianos después de 96 y 192 h fue siempre menor que la exhibida por el control abiótico suplementado con 150 mg /L MTBE, correspondiente con la concentración de MTBE inicial presente en los cultivos. La toxicidad de los cultivos de *Agrobacterium* sp. MS2 y *Paenibacillus* sp. SH7 después de 192 h fueron altas, a pesar de los datos reflejados para la eliminación de MTBE. Una acumulación de un metabolito tóxico en el medio de cultivo podría ser la razón de esta toxicidad; sin embargo, la acumulación de TBA nunca se observó en los medios de cultivo, por lo que el metabolito tóxico podría ser un compuesto alternativo (por ejemplo, formaldehído).

Los resultados obtenidos mediante esta metodología, resaltan la importancia de la aplicación de ensayos de detección complementarios (fisiológicos, genéticos y ecotoxicológicos) al objeto de obtener una selección más representativa de cepas bacterianas para fines de biorremediación.

Entre las cepas aisladas mediante la metodología descrita, *Paenibacillus* sp. SH7 fue objeto de un estudio taxonómico tal y como se refleja en el segundo capítulo del apartado de Resultados de esta Tesis Doctoral.

Tradicionalmente, las bacterias bacilares, Gram positivas, formadoras de esporas, se han incluido en el género *Bacillus*. Logan y Berkeley en 1984, desarrollaron un sistema de identificación de las especies de este género basándose en la utilización del sistema API y en ensayos enzimáticos y bioquímicos, pero la heterogeneidad de la fisiología, ecología y genética de sus representantes presentaba una gran complejidad para ordenar de una forma adecuada a bacterias provistas de una diversidad tan grande. Se incluyen así representantes fijadores de nitrógeno, desnitrificantes, nitrificantes, oxidadores y reductores de Mn, acidófilos, alcalófilos, psicrófilos y termófilos, etc. y además con un rango de G+C excesivamente amplio, entre 32-69 mol %.

El género *Paenibacillus* fue propuesto por Ash y colaboradores (1993) con la finalidad de acomodar a un grupo de bacilos anaerobios facultativos o aerobios estrictos, Gram positivos, Gram negativos o Gram variable, filogenéticamente relacionados, móviles, con esporas elipsoidales con esporangio hinchado. A fecha de hoy se han descrito 150 nuevas especies (incluyendo subespecies), con distintas características: especies patógenas para insectos (*P. lentimorbus, P. popilliae),* especies productoras de antibióticos *(P.polymyxa, P. thiaminolyticus),* secretoras de enzimas que degradan polímeros como la quitina y la condroitina (*P.illinoisensis*, *P. antarcticus*) ó participantes en la degradación de contaminantes ambientales (Daane y colaboradores 2002; Sirota-Madi y colaboradores 2010 y Khianngam y colaboradores 2011).

Entre algunos marcadores quimiotaxonómicos significativos de este género bacteriano, cabe considerar la presencia del ácido graso mayoritario antesio-$C_{15:0}$ y un contenido en composición de bases G+C (% mol) entre 44-54% (Shida y colaboradores, 1997).

El estudio filogenético de la secuencia del rRNA 16S de la cepa SH7^T presentó un nivel de similitud respecto a otras especies del género *Paenibacillus* igual o mayor al 97 %, límite para una posible relación a nivel de especie (Stackebrant y Goebel, 1994). Las especies más cercanas *Paenibacillus borealis* DSM 1388^T y *Paenibacillus odorifer* DSM 15391^T se sitúan a niveles de similitud del orden del 98% y 97% respectivamente. Los estudios de hibridación DNA-DNA entre *P. borealis* DSM 1388^T, *P. odorifer* DSM 15391^T y la cepa SH7^T, mostraron un nivel bajo de reasociación (16.9% y 16.6 % respectivamente), que junto con los resultados de composición de ácidos grasos celulares (anteiso-C $_{15:0}$ 32.98% y C $_{16:0}$ 29%), fosfolípidos así como composición de pared celular y quinonas respiratorias indicaron que podría ser una nueva especie del género *Paenibacillus,* para la cual la denominación de *Paenibacillus oxygenati* sp. nov. se ha propuesto, denominación que refleja su capacidad para crecer en medios adicionados del oxigenante MTBE.

Tal y como se ha descrito anteriormente, la aplicación de métodos fisiológicos, génicos y ecotoxicológicos nos permitió seleccionar 5 cepas bacterianas como potenciales inóculos selectivos para su uso en los bioreactores de membrana extractiva con desarrollo de biopelícula (EMBFR), sistemas diseñados y construidos a escala de laboratorio para el tratamiento de influentes contaminados con MTBE. Al ser la primera vez en la que estos sistemas eran ensayados para remediar muestras de agua contaminada con este xenobiótico, fue necesario diseñar y desarrollar ensayos previos en los cuales se evaluó no sólo la capacidad del contaminante para atravesar la membrana tubular semipermeable, sino también la capacidad de las cepas seleccionadas para establecerse como biopelícula sobre la superficie de las membranas tubulares, así como la ecotoxicidad exhibida por los cultivos bacterianos bajo estas condiciones, tal y como se recoge en el tercer capítulo de la sección Resultados de esta Tesis Doctoral.

Para ello, se construyeron sistemas muy básicos constituidos por matraces Erlenhmeyer con tapones de silicona con varios orificios para influente, efluente y tubo toma muestras, en los que se dispuso medio de cultivo ("biomedio") inoculado con las cepas anteriormente mencionadas. Las membranas tubulares semipermeables (Pumsil®) se introdujeron en estos sistemas, siempre inmersas en el medio y conectadas a tubos de Marprene® para evitar las pérdidas del oxigenante a la fase gaseosa. Las muestras de agua con MTBE (150 mg/L) fueron introducidas en los sistemas a través de bombas peristálticas y estos se mantuvieron en funcionamiento (en forma continua) durante 10 días tras los cuales se evaluó el desarrollo de biopelícula sobre la superficie de las membranas tubulares así como la ecotoxicidad de estos cultivos.

Las cantidades de MTBE detectados (124 mg/L) mediante la técnica GC-MS en el biomedio mediante el ensayo de difusión de MTBE, sugirieron que el compuesto oxigenado podría cruzar la membrana tubular en respuesta a un gradiente de concentración y por lo tanto estar disponible para usarse como fuente de carbono por parte de los microorganismos en crecimiento, apoyando el crecimiento bacteriano inicial en la superficie de tubo y permitiendo la formación de biopelícula en el mismo.

Los ensayos de producción de EPS por las cepas seleccionadas ante distintas concentraciones de MTBE y desarrollados en paralelo, pusieron de manifiesto que las cepas *Rhodococcus ruber* A5, EE1 y EE6 rindieron las mayores producciones de EPS pero sólo en las cepas cepas A5 y EE1, se observó una respuesta positiva en producción de EPS ante dosis crecientes de MTBE. Varios estudios han informado que el rendimiento y la composición de EPS bacteriano pueden estar influenciados por cambios en las condiciones de cultivo o en la composición del medio (Cerning y colaboradores 1986; 1990). De hecho, Onbasli y Aslim, (2009) observaron que la composición de los EPS producidos por algunas cepas de *Pseudomonas*, cambió en presencia de diferentes contaminantes orgánicos. En nuestro estudio, la fracción de carbohidratos fue siempre mayor que la de proteína; este

hecho fue debido a la relación C / N en los medios utilizados para el análisis de la producción de EPS.

El análisis mediante microscopía electrónica de barrido (FESEM) fue llevado a cabo con el fin de mostrar la capacidad de adherencia de las cepas bacterianas a la superficie de la membrana semipermeable tubular después de 10 días de ensayo bajo temperatura y agitación controlada. *R. ruber* A5 y *Agrobacterium* sp. MS2, fueron las cepas bacterianas que mostraron mayor capacidad para adherirse a la superficie de la membrana tubular semipermeable, a pesar de ser las cepas bacterianas que mostraron la menor producción de EPS bajo nuestras condiciones experimentales. Una mayor densidad celular fue observada cuando el compuesto oxigenado estaba presente en los influentes. Sin embargo, no se observaron diferencias en la densidad celular de las biopelículas establecidas por las cepas *R. ruber* EE1 y EE6 en la superficie de las membranas tubulares cuando los influentes se suplementaron con MTBE. Por otra parte, la cepa *Paenibacillus* sp. SH7 fue incapaz de adherirse a la superficie de las membranas bajo cualquier condición ensayada. En un estudio anterior, Purswani y colaboradores (2011) observaron cómo la inoculación selectiva de un biofiltro sumergido aireado diseñado para el tratamiento de agua subterránea contaminada con MTBE con una cepa con alta capacidad de formación de biopelículas (*Acinetobacter calcoaceticus* M10) y con otra cepa bacteriana exenta de esta capacidad (*Rhodococcus ruber* E10) , pero muy eficaz en la degradación de MTBE, rindió una biopelicula más abundante y mucho más eficiente en la eliminación del oxigenante que la desarrollada solamente con *A. calcoaceticus* M10.

Tal y como se muestra en el primer capítulo de Resultados de esta Tesis Doctoral, la cepa *Paenibacillus sp.* SH7 fue el microorganismo que más creció en medios suplementados con MTBE, por lo que a pesar de las pequeñas cantidades de EPS producidos y la incapacidad de fijación a las membranas como el análisis de FESEM

reveló, esta cepa bacteriana podría ser considerada como "buen candidato" para su uso como miembro de un consorcio bacteriano en una estrategia de biorremediación.

La toxicidad aguda (como EC_{50}) de los cultivos bacterianos al final de cada experimento fue evaluada mediante el ensayo Microtox®. Las muestras procedentes de los medios de cultivo inoculados con *R. ruber* EE6 y *Agrobacterium* sp. MS2 mostraron los niveles más bajos de toxicidad. Por el contrario, el medio de cultivo inoculado con *R. ruber* EE1 exhibió la toxicidad más alta (EC_{50} = 4,2) siendo estos valores similares a los del control abiótico, medio de cultivo sin inocular y adicionado de 150 mg/L de MTBE (EC_{50} = 3,0). Estos resultados son coincidentes con los obtenidos para esta misma cepa en el primer capítulo de la sección Resultados. La acumulación de algunos metabolitos tóxicos procedentes de la degradación de MTBE (TBA o formaldehído) por parte de *R. ruber* EE1 podría ser la razón para explicar los valores de toxicidad aguda exhibidos por este microorganismo. Este resultado determina por tanto que la cepa EE1 no sea un buen candidato como inóculo selectivo en la tecnología EMBFR para la biorremediación de MTBE.

Sin embargo, los resultados de toxicidad aguda que se detectaron en los medios de cultivo inoculados con *Agrobacterium* sp. MS2 fueron contradictorios con los obtenidos previamente. Aunque el estudio de detección de genes que codifican para alcano/citocromo P450 monooxigenasas no rindiera resultados positivos, esta cepa creció en medios suplementados con MTBE y también desarrolló una patente biopelicula en la superficie de las membranas tubulares, por lo que esta cepa parece que mejora la degradación de MTBE (en términos de consumo y/o reducción de metabolitos tóxicos) cuando es utilizada como inóculo selectivo en la tecnología EMBFR bajo nuestras condiciones de estudio. Por lo tanto, será necesario realizar más investigaciones para aclarar la vía de degradación de MTBE que lleva a cabo esta cepa bacteriana.

Tras el desarrollo de estos ensayos preliminares en los cuales se comprobó que el oxigenante MTBE era capaz de atravesar las membranas tubulares semipermeables, componentes de los EMBFR a escala de laboratorio, y que determinadas cepas bacterianas tenían capacidad para adherirse a la superficie de las membranas, sin exhibir un valor de ecotoxicidad (EC_{50}) excesivo, se procedió a evaluar la eficiencia de la tecnología EMBFR para biorremediar muestras de agua contaminada con MTBE utilizando las cepas *Paenibacillus sp*. SH7, *Agrobacterium* sp. MS2 y *Rhodococcus ruber* EE6 bajo distintos tiempos de retención hidráulica (TRH): 1 h, 6 h y 12 h. Para ello se construyeron biorreactores a escala de laboratorio tal y como se recoge en el cuarto capítulo de la sección Resultados de esta Tesis Doctoral. Dichos biorreactores fueron inoculados de manera individual por las cepas bacterianas anteriormente citadas y mantenidos en recirculación durante 8 días; trascurrido este tiempo los sistemas operaron en forma continua hasta 28 días. Los resultados obtenidos revelaron que independientemente del HRT, en el biomedio de los biorreactores inoculados con las cepas bacterianas *Paenibacillus* sp. SH7 y *Agrobacterium* sp. MS2, se detectaron los valores más altos de crecimiento bacteriano en suspensión. La presencia de una fuente de carbono alternativa (extracto de levadura) en el medio de cultivo al inicio del periodo de recirculación podría haber sido la razón del crecimiento bacteriano observado; sin embargo, los datos de degradación de MTBE durante este periodo mostraron que las concentraciones de MTBE disminuyeron en el biomedio de todos los biorreactores inoculados**.** Acuña-Askar y colaboradores (2000), estudiaron la biodegradación de MTBE en bioreactores de biopelícula fija y concluyeron que la presencia de otras fuentes de carbono fácilmente asimilables en el medio de cultivo influyó favorablemente en la biodegradación de MTBE.

Para evitar el posible efecto negativo de metabolitos del MTBE sobre el crecimiento y supervivencia bacteriana, parte del biomedio de los biorreactores fue renovado tras 15 días de operación, observándose una disminución en la densidad óptica del biomedio. Este

hecho pudo ser debido al mismo efecto de la dilución o a la adhesión de las células bacterianas en suspensión a la superficie de las membranas semipermeables tubulares en respuesta a un gradiente de difusión de MTBE a través de ellas. Autores como Ferreira y Livinston (2000), manifestaron que las reducciones de densidad óptica detectadas en el biomedio de un biorreactor de membrana extractiva para el tratamiento de agua contaminada con mono cloro benceno y 1,2-dicloroetano, correspondían a la formación de una biopelicula incipiente en las superficies de las membranas semipermeables. En nuestro estudio, este hecho se confirmó en los biorreactores inoculados con las cepas bacterianas *Agrobacterium* sp. MS2 y *Rhodococcus ruber* EE6 mediante el análisis por FESEM de las membranas semipermeables tubulares.

El fenómeno denominado "*air stripping*" (transferencia de compuestos volátiles desde la fase líquida a gaseosa), puede reducir el rendimiento de muchos reactores aireados convencionales (Casey y colaboradores 1999). En nuestro estudio, para evitar este hecho, el aire fue proporcionado a través de una membrana semipermeable tubular. De esta manera, se evitó la pérdida de MTBE a la fase gaseosa y el oxígeno fue liberado directamente a los microorganismos activos establecidos en la biopelícula o al biomedio, estando así disponible para los microorganismos creciendo en suspensión. Durante la primera semana de funcionamiento en la que los sistemas trabajaron bajo el modo de recirculación, las concentraciones de oxígeno disuelto (medido mediante sonda de oxígeno) disminuyeron en todos los biorreactores inoculados independientemente de las condiciones de operación. El crecimiento bacteriano que presentaron los biorreactores inoculados fue la razón del alto consumo de oxígeno observado durante el período de recirculación, aunque este consumo se extendió durante más tiempo en algunos casos, como en los biorreactores inoculados con la cepa bacteriana *Rhodococcus ruber* EE6 a un tiempo de retención hidráulica de 1 h y en todos los biorreactores inoculados en un HRT de 6 h. Zhong y colaboradores (2007)

demostraron la existencia de un valor crítico de oxígeno disuelto, por encima del cual la degradación de MTBE podría llevarse a cabo con éxito.

Para evaluar la eficiencia de la tecnología EMBFR en cada condición de TRH (1h, 6h y 12 h) establecida, se estudió la evolución de las concentraciones de MTBE en cada uno de los compartimentos de los biorreactores (influente, biomedio y efluente tratado) durante el periodo de funcionamiento**.** En los influentes, las concentraciones de MTBE fueron similares en todos los biorreactores independientemente del TRH, alcanzándose los valores más bajos en el modo de recirculación. Durante esta fase y en el biomedio de los biorreactores inoculados, las concentraciones de MTBE fueron muy pequeñas, independientemente del TRH ensayado. La disminución observada en las concentraciones de MTBE podría atribuirse a la pérdida del compuesto debido a la aireación del sistema, como ocurrió en estudios previos (Purswani y colaboradores 2014), pero para evitarlo, el aire fue suministrado mediante membranas semipermeables, aumentando así el rendimiento del sistema y previniendo la pérdida por volatilización del MTBE. Por lo tanto, el consumo por parte de las cepas bacterianas fue la razón de la reducción del MTBE detectada.

No se observaron diferencias en las concentraciones de MTBE en los efluentes de los bioreactores inoculados tanto a THR de 6 y 12 h, aunque bajo esta última condición pudieron detectarse valores levemente más bajos. Cuando el TRH fue 1 día, las concentraciones de MTBE fueron mucho más altas independientemente de la cepa inoculada. Hu y colaboradores (2004) observaron bajo sus condiciones experimentales que un porcentaje más alto de biodegradación se llevó a cabo cuando el HRT era más largo.

Katsivela y colaboradores (1999), mostraron el excelente rendimiento de un EMBFR y la eficiencia en la formación de biopelícula usado para el tratamiento de aguas residuales industriales. En nuestro caso, tal como FESEM demostró**,** *Agrobacterium* sp. MS2 y *Rhodococcus ruber* EE6 fueron capaces de adherirse a las membranas tubulares semipermeables y desarrollar una biopelícula más patente cuando los biorreactores se

mantuvieron a un TRH de 12 h. La cepa *Rhodococcus ruber* EE6, mostró los valores más bajos de crecimiento en suspensión, de manera que la adhesión de las bacterias a la superficie de las membranas tubulares explicaría este hecho. La cepa *Paenibacillus* sp. SH7, en cambio, no formó biopelicula bajo ningún TRH evaluado, pero esta cepa (junto con *Agrobacterium sp*. MS2) exhibió el mayor crecimiento en suspensión. En el primer capítulo de esta Tesis Doctoral, fue demostrada la capacidad de estas cepas para crecer ó biotransformar /degradar MTBE. Autores como Purswani y colaboradores (2011), concluyeron que la inoculación selectiva de un biofiltro sumergido aireado con un consorcio bacteriano constituido por cepas bacterianas con capacidad para utilizar MTBE y diferentes comportamientos en formación de biopelícula (como los utilizados en este estudio), podría ofrecer una opción prometedora para la biorremediación de MTBE.

Con el fin de determinar si la tecnología aplicada en este estudio fue eficaz en la eliminación de MTBE, junto con la determinación de las concentraciones de este xenobiótico en biomedio y efluentes, se utilizó el bioensayo Microtox® para evaluar la ecotoxicidad del influente, biomedio y efluente tratado. La ecotoxicidad exhibida por el biomedio y el efluente tratado en cada biorreactor inoculado fue siempre menor, en todos los casos, que en los biorreactores abióticos y los influentes. Cuando el inóculo selectivo fue la cepa *Paenibacillus* sp. SH7 la toxicidad exhibida por el biomedio y el efluente fue la mayor independientemente del TRH ensayado. Los análisis mediante FESEM mostraron que esta cepa bacteriana no pudo adherirse a la membrana tubular semipermeable y establecerse como biopelicula, por lo que la presencia de metabolitos secundarios procedentes de la degradación del MTBE (TBA o formaldehído) en el biomedio, sería la razón para justificar estos valores de ecotoxicidad. Roslev y colaboradores (2015) señalaron que el TBA es mucho menos tóxico (como valores EC_{50}) que el MTBE, siendo el formaldehído el metabolito secundario de la degradación del MTBE el que presentó la mayor toxicidad. Bajo nuestras condiciones experimentales, los datos proporcionados por

GC-MS no demostraron la presencia de ninguna de estas sustancias; la baja sensibilidad de la metodología utilizada podría ser la razón de ello. Por el contrario, el uso de la cepa *Rhodococcus ruber* EE6 como inóculo selectivo, demostró los niveles más bajos de toxicidad tanto en el biomedio como en los efluentes tratados bajo un TRH de 12 h.

Los resultados obtenidos en este capítulo mostraron que la tecnología EMBFR podría ser una opción efectiva para la biorremediación de aguas contaminadas con MTBE, bajo determinadas condiciones de funcionamiento. Además, el uso de cepas bacterianas con diferentes capacidades degradativas utilizadas como consorcios bacterianos, podría mejorar el rendimiento del sistema. En este sentido y en base a nuestros resultados, el consorcio constituido por *Paenibacillus* sp. SH7, *Rhodococcus ruber* EE6 y *Agrobacterium* sp. MS2, sería un excelente inóculo selectivo para la biorremediación de agua contaminada con MTBE por la tecnología EMBFR.

OVERALL DISCUSSION

Methyl *tert*-butyl ether (MTBE) is a semi-volatile compound that is mainly produced to replace lead in the manufacture of fuel. It is added to the reformulated fuel (in different concentrations, depending on the country) to enhance the octane rating and thus its combustibility, in order to reduce toxic emissions into the atmosphere. This oxygenating compound can be detected in many aquifers in the USA and Europe as a result of accidental spills from storage containers and leaks in pipe systems and from fuel station tanks, among other sources. Its entry into groundwater systems is favoured by its tendency to leach and its high solubility in water. Numerous remediation technologies have been developed to alleviate the severe problem of groundwater pollution caused by this compound, including methods based on adsorption, chemical oxidation, air injection and biological techniques. Treatments involving the application of biological systems to catalyse the transformation of chemical compounds into other, less toxic, ones or to achieve their mineralisation through the use of living organisms (fundamentally, bacteria) to absorb, transform or degrade contaminants in the environment, removing or inactivating them or mitigating their effect, are considered the best means of removing oxygenating compounds from water bodies. In addition, such approaches are non-invasive and cost-effective (Lopes-Ferreira et al., 2006; Eixarch and Constantí, 2010). Among such types of treatment, in the remediation of groundwater contaminated with various semi-volatile compounds, special interest has been taken in using systems such as trickling biofilters, submerged biofilters, membrane bioreactors and extractive membrane bioreactors (Livingston, 1991, 1993; Freitas dos Santos and Livingstone, 1994; Eweis et al., 1997, 1998; Fortin and Deshusses, 1999a, b; Casey et al., 1999; Nicolella et al., 2000; Steffan et al., 2000; Hu et al., 2004; Purswani et al., 2011). In most cases, greater effectiveness has been achieved when a fixed biomass is

used (Fortin et al., 1999a, b; Acuña-Askar, 2000; Kharoune et al., 2001; Moreels et al., 2004; Hu et al., 2004; Maciel et al., 2008).

The aerobic degradation of MTBE is possible, and in some cases it is very efficient, using either pure cultures (Hatzinger et al., 2001; Francois et al., 2002) or mixed ones (Salanitro et al., 1994). These microorganisms can use MTBE as the sole source of carbon and energy (Deeb et al., 2001; Mo et al., 1995) or achieve degradation through co-metabolic processes (Hyman et al., 1998; Liu et al., 2001). However, in general, low biomass yields and low growth rates are achieved by microorganisms capable of biotransforming/degrading this xenobiotic compound (Müller et al., 2007).

The main aim of this Thesis is to conduct a technical and biological study of an extractive membrane biofilm bioreactor (EMBFR) system for the treatment of water contaminated with MTBE. As reported elsewhere, an EMBFR is a modification of extractive membrane bioreactor technology (Katsivela et al., 1999; Ferreira and Livingston, 2000; Livingston, 1991, 1993; Freitas dos Santos and Livingstone, 1994), based on the use of semipermeable membranes through which contaminants migrate to the biological compartment in which microorganisms with the capacity to biotransform and mineralise a given pollutant grow within a medium, forming a biofilm on the surface of the membrane. Growth conditions of the microorganisms in the biological compartment can be controlled to obtain an efficient degradation of the pollutant compounds, regardless of the conditions prevailing in the effluent to be treated. In aerobic systems, oxygen can also be supplied via semipermeable membranes, thus avoiding loss of the contaminant by volatilisation. Previous studies (Purswani et al., 2011, 2014) have highlighted the limitations of certain *ex situ* technologies developed for the remediation of MTBE-contaminated water bodies, such as air-stripping and the low concentration of biomass produced by the bacterial strains used as selective inocula, and previously selected for their ability to grow on mineral media supplemented with MTBE as the sole source of carbon and energy. By means of the

technology described below, we seek to overcome these limitations and thus implement a successful bioremediation strategy.

An important step in the design and development of a biological technology to treat groundwater contaminated with fuel oxygenates, as examined in this Thesis, is the initial isolation and selection of bacterial strains that have the ability to grow or biotransform/degrade these compounds. Studies have described pure bacterial cultures capable of using MTBE as the sole source of carbon and energy (Hanson et al., 1999; Piveteau et al., 2001; Nakatsu et al., 2006; Rohwerder et al., 2006; Müller et al., 2008, among others). Most of the bacteria selection processes currently applied are quite limited, providing little information on the bacterial strains before they are used in subsequent bioremediation tests. However, we have described (Guisado et al., 2015) a precise methodology for the isolation and selection of bacterial strains that are capable of using MTBE as the sole source of carbon and energy, based on the results obtained for growth in media to which MTBE and/or aromatic hydrocarbons (benzene, toluene, ethylbenzene and xylene – BTEX) have been added, on the ecotoxicity exhibited by bacterial cultures under these conditions, on the presence/absence of genes involved in the degradation of MTBE (monooxygenases), and on the ability to degrade/biotransform MTBE in liquid media.

The bacterial strains were isolated from samples contaminated with hydrocarbons, using two different methods: either directly from the original samples (soil and groundwater) or after an enrichment process. Several authors (Mo et al., 1997; Hatzinger et al., 2001; Okeke and Frankenberger, 2003; Purswani et al., 2008) have quite successfully used enrichment techniques as a means of isolating bacterial strains with degradation capabilities.

60% of all the isolates obtained in the course of this study belonged to the phyla *Firmicutes* and *Actinobacteria*. Genera belonging to these phyla (Hernández-Pérez et al., 2001; Youngster et al., 2010; Beazley et al., 2012) are involved in the degradation of

aromatic hydrocarbons such as BTEX or in the aerobic degradation of MTBE (François et al., 2002). The remaining isolates belong to the phylum *Proteobacteria*. All the bacterial strains isolated after the enrichment steps formed part of this group. Wagner-Döbler et al. (1998) reported that classical enrichment techniques tend to isolate Gram negative microorganisms, due to their high growth rates.

Aromatic compounds (BTEX) are an active part of the contaminants in aquifers after spills or accidental loss during the production, distribution, storage and use of fuel, and MTBE and BTEX compounds coexist in contaminated aquifers (Happel et al., 1998). In the course of this Thesis, we have assessed the ability of the isolates to grow on media supplemented with BTEX aromatic compounds (with or without MTBE). In parallel, gene analysis was conducted to determine the presence of genes encoding for enzymes involved in the degradation of MTBE (alkane or cytochrome P450 monooxygenases). However, the results obtained showed that the detection of these marker genes was not necessarily linked to the effective biodegradation of MTBE by the strains, and therefore, this fact should not be taken as an unequivocal indicator of the ability of a strain to degrade/biotransform this xenobiotic. Furthermore, although the bacterial strains *Agrobacterium* sp. MS2 and *Paenibacillus* sp. SH7 isolated in this study exhibited the highest levels of MTBE biotransformation, the gene detection performed did not achieve positive results with the primers tested. The use of other general monooxygenase primers, or of multiplex-PCR with several primers, could be a better strategy to identify a marker gene indicative of the existence of MTBE bioremediation.

Taking into account the results of the experiments on the growth and degradation of isolates in media containing MTBE and aromatic hydrocarbons, together with the gene detection data results, five bacterial strains (*Rhodococcus ruber* A5 CECT8556, strain EE6 CECT 8612 and strain EE1 CECT 8555, *Agrobacterium* sp. MS2 CECT 8557 and *Paenibacillus* sp. SH7 CECT 8558) were selected to study the biodegradation of MTBE in

batch experiments. Although the strains MA2 (*Pseudomonas aeruginosa*) and A6 (*Nocardia nova*) were capable of growing in media with MTBE, they were not included in the subsequent assays because they are opportunistic pathogenic strains in humans.

In the degradation assays, *Paenibacillus* sp. SH7 was the strain that achieved the highest reduction in MTBE concentration (100 mg/L^{-1}) in a liquid medium after incubation for eight days; *Agrobacterium* sp. MS2, however, achieved a reduction of 85 mg/L^{-1} in four days. Nevertheless, neither of these bacterial strains presented a high growth rate or achieved a large number of generations, due to the lack of energy derived from the degradation of the carbon source, which in this case was MTBE. As pointed out by Hatzinger et al. (2001), the lack of growth observed could be because there were insufficient enzymes for the complete degradation of MTBE, or might be due to the presence of inhibitory compounds as intermediary products (for example, TBA, tert-butyl alcohol) in the biodegradation of these oxygenated compounds.

Another noteworthy strain was *Rhodococcus ruber* EE6. This microorganism developed the largest number of generations under the same conditions as the other strains, but this fact was not associated with the highest rate of elimination of MTBE. The toxicity values determined (by the Microtox® bioassay) after growth in the MTBE-supplemented medium indicated that the cultures of this strain presented less toxicity. It follows, therefore, that this strain achieved the most efficient removal of MTBE. Ecotoxicity analyses showed that the toxicity of the bacterial cultures after 96 and 192 h was always lower than that exhibited by the abiotic control supplemented with 150 mg/L MTBE, corresponding to the concentration of MTBE initially present in the cultures. The toxicity of the cultures of *Agrobacterium* sp. MS2 and *Paenibacillus* sp. SH7 after 192 h was high, despite the quantity of MTBE removed. This toxicity might be explained by an accumulation of a toxic metabolite in the culture medium; however, no accumulation of

TBA was ever observed in the culture media, and so the toxic metabolite might be an alternative compound (for example, formaldehyde).

The results obtained using this methodology highlight the importance of conducting complementary detection tests (physiological, genetic and ecotoxicological) to obtain a more representative selection of bacterial strains for bioremediation.

Among the strains isolated by the methodology described, *Paenibacillus* sp. SH7 was subjected to a taxonomic study, as described in the second chapter of the Results section.

Traditionally, spore-forming Gram positive bacillary bacteria have been included in the genus *Bacillus*. In 1984, Logan and Berkeley developed a system for identifying the species of this genus, based on the API system and on enzymatic and biochemical assays, but the heterogeneous physiology, ecology and genetics of their representative strains made it a highly complex task to properly order such diverse bacteria. Thus, representatives were included of strains corresponding to nitrogen fixers, denitrifiers, nitrifiers, oxidisers and reducers of Mn, together with acidophilic, alkalophilic, psychrophilic and thermophilic strains, and many others. Moreover, the guanine-cytosine (GC) range was excessively large, at 32-69 mol%.

The genus *Paenibacillus* was proposed by Ash et al. (1993) in order to accommodate a group of optional anaerobic or strict aerobic bacilli that were Gram positive, Gram negative or Gram variable, phylogenetically related, mobile and with ellipsoidal spores in a swollen sporangium. To date 150 new species (including subspecies) have been described, with diverse characteristics: some are pathogenic to insects (*P. lentimorbus*, *P. popilliae*), others produce antibiotics (*P. polymyxa*, *P. thiaminolyticus*), or secrete enzymes that degrade polymers such as chitin and chondroitin (*P. illinoisensis*, *P. antarcticus*) or participate in the degradation of environmental pollutants (Daane et al., 2002; Sirota-Madi et al., 2010; Khianngam et al., 2011).

Significant chemotaxonomic markers of this bacterial genus include the presence of the majority fatty acid antesio-$C_{15:0}$ and a mol%GC composition of 44-54% (Shida et al., 1997).

The phylogenetic study of the 16S rRNA sequence of strain SH7^T presented a level of similarity to other species of the genus *Paenibacillus* equal to or greater than 97%, which is the standard limit for a possible relation at the species level (Stackebrandt and Goebel, 1994). The nearest species, *Paenibacillus borealis* DSM 1388^T and *Paenibacillus odorifer* DSM 15391^T, are located at levels of similarity of around 98% and 97% respectively. Studies of DNA-DNA hybridisation between *P. borealis* DSM 1388^T, *P. odorifer* DSM 15391^T and the strain SH7^T revealed low levels of annealing (16.9% and 16.6% respectively), which, together with the results for the cellular fatty acid composition (anteiso-$C_{15:0}$ 32.98% and $C_{16:0}$ 29%), the phospholipids, the cell wall composition and the respiratory quinones, suggested this might be a new species of the genus *Paenibacillus*, for which the term *oxygenati Paenibacillus* sp. nov. has been proposed. This name reflects its ability to grow within a medium containing the oxygenate MTBE.

As described above, physiological, genic and ecotoxicological methods were used to select five bacterial strains as potential selective inocula for use in extractive membrane biofilm reactors (EMBFR), as laboratory-scale systems designed and built to treat MTBE-contaminated influents. As this was the first time that these systems had been tested to remediate water samples contaminated with this xenobiotic, it was necessary to design and develop preliminary experiments to evaluate not only the ability of the contaminant to pass through the semipermeable tubular membrane but also the ability of the selected strains to become established as a biofilm on the surface of the tubular membranes. It was also necessary to assess the ecotoxicity exhibited by the bacterial cultures under these conditions, as described in chapter three of the Results section.

For this purpose, very basic systems were constructed, consisting of Erlenhmeyer flasks with silicone stoppers and apertures for the influent, the effluent and the sampling tube. The culture medium (or biomedium), inoculated with the above-described strains, was introduced into this system, together with semipermeable tubular membranes (Pumsil®), which were immersed constantly in the medium and connected to Marprene® tubes to prevent leakage of the oxygenate into the gas phase. The water samples with MTBE (150 mg/L) were then introduced into the system via peristaltic pumps, which operated continuously for ten days. The development of biofilm on the surface of the tubular membranes was then evaluated, together with the ecotoxicity of these cultures.

The amounts of MTBE (124 mg/L) detected by the GC-MS technique in the biomedium, by MTBE diffusion assay, suggested that the oxygenated compound may have penetrated the tubular membrane in response to a concentration gradient, and therefore have become available for use as a carbon source by the growing microorganisms, thus supporting the initial bacterial growth on the surface of tube and enabling biofilm to form on it.

Tests of exopolysaccharide (EPS) production by the selected strains, at various concentrations of MTBE and performed in parallel, showed that the *Rhodococcus ruber* strains A5, EE1 and EE6 achieved the highest productions of EPS, but that only A5 and EE1 presented a positive response in EPS production to increasing doses of MTBE. Several studies have reported that the yield and composition of bacterial EPS may be influenced by changes in culture conditions or in the composition of the medium (Cerning et al., 1986, 1990). Indeed, Onbasli and Aslim (2009) noted that the composition of the EPS produced by some strains of *Pseudomonas* varied in the presence of different organic pollutants. In our study, the carbohydrate fraction was always larger than that of protein, due to the C/N ratio in the media used for the analysis of EPS production.

Field emission scanning electron microscopy analysis (FESEM) was conducted to determine the bacterial strains' capacity to adhere to the surface of the tubular semipermeable membrane after ten days of testing under controlled temperature and stirring conditions. According to this test, *R. ruber* A5 and *Agrobacterium* sp. MS2 presented the greatest adhesion capacity, although these bacterial strains achieved the lowest production of EPS under our experimental conditions. The cell density was found to be greater when the oxygenate was present in the influents. However, there were no differences in the cell density of the biofilms created by *R. ruber* EE6 and EE1 on the surface of the tubular membranes when the influents were supplemented with MTBE. Moreover, the strain *Paenibacillus* sp. SH7 was unable to adhere to the surface of the membranes tested, under any of our experimental conditions. In a previous study, Purswani et al. (2011) observed that following the selective inoculation of an aerated submerged biofilter designed for the treatment of MTBE-contaminated groundwater, using one strain with high biofilm formation capacity (*Acinetobacter calcoaceticus* M10) and another devoid of this ability (*Rhodococcus ruber* E10), but highly effective in the degradation of MTBE, the production of biofilm was more abundant and the removal of oxygenate more efficient than was the case when *A. calcoaceticus* M10 alone was used.

As reported in the first chapter of the Results section, the strain *Paenibacillus* sp. SH7 was the organism that grew most vigorously in media supplemented with MTB. Thus, despite the small amounts of EPS produced and the inability to adhere to membranes, as revealed by the FESEM analysis, this bacterial strain could be considered a "good candidate" to be used as a member of a bacterial consortium in a bioremediation strategy.

The acute toxicity (measured as EC_{50}) of the bacterial cultures at the end of each experiment was evaluated by the Microtox® test. The samples from the culture media, inoculated with *R. ruber* EE6 and *Agrobacterium* sp. MS2, presented the lowest levels of toxicity. By contrast, the culture medium inoculated with *R. ruber* EE1 exhibited the

highest toxicity (EC_{50}=4.2), with values similar to those of the abiotic control, which was an uninoculated culture medium to which 150 mg/L of MTBE had been added (EC_{50}=3.0). These results are consistent with those reported for the same strain in the first chapter of the Results section. The accumulation of toxic metabolites from the degradation of MTBE (TBA or formaldehyde) by *R. ruber* EE1 could account for the acute toxicity values exhibited by this microorganism. From this result, therefore, we conclude that the strain EE1 is not a good candidate as a selective inoculum in EMBFR technology for the bioremediation of MTBE.

However, the acute toxicity results that were detected in the culture media inoculated with *Agrobacterium* sp. MS2 were contradictory to those obtained previously. Although the detection study of genes encoding for alkane/cytochrome P450 monooxygenases did not produce positive results, this strain grew in media supplemented with MTBE and also developed an evident biofilm on the surface of the tubular membranes, and so this strain does appear to improve MTBE degradation (in terms of the consumption and/or reduction of toxic metabolites) when used as a selective inoculum in the EMBFR technology examined under our study conditions. Therefore, further research is needed to clarify the means by which MTBE is degraded by this bacterial strain.

Following these preliminary tests, which revealed that the oxygenate MTBE was capable of traversing the semipermeable tubular membranes, which were the laboratory-scale EMBFR components employed in this study, and that certain bacterial strains have the capacity to adhere to the surface of the membranes, without reaching an excessively high level of ecotoxicity (EC_{50}), we then evaluated the efficiency of the EMBFR technology to remediate samples of MTBE-contaminated water, using the strains *Paenibacillus* sp. SH7, *Agrobacterium* sp. MS2 and *Rhodococcus ruber* EE6, under hydraulic retention times (HRT) of one, six and twelve hours. For this purpose, laboratory-scale bioreactors were constructed, as described in the fourth chapter of the Results section.

These bioreactors were inoculated individually by the above-mentioned bacterial strains and maintained in recirculation for eight days, after which time the systems were operated continuously for a further 21 days. The results obtained show that regardless of HRT, the highest values of bacterial growth in suspension were detected in the biomedium of the bioreactors inoculated with the bacterial strains *Paenibacillus* sp. SH7 and *Agrobacterium* sp. MS2. The presence of an alternative source of carbon (yeast extract) within the culture medium at the beginning of the recirculation period might account for the bacterial growth observed; however, the degradation data for this period showed that MTBE concentrations declined in the biomedium of all the bioreactors that had been inoculated. Acuña-Askar et al. (2000) studied the biodegradation of MTBE in fixed biofilm bioreactors and concluded that the presence of other readily assimilable sources of carbon in the culture medium favourably influenced the biodegradation of MTBE.

To avoid the possible negative effect of MTBE metabolites on bacterial growth and survival, part of the biomedium in the bioreactors was renewed after 15 days' operation, after which a decrease in the optical density of the biomedium was observed. This outcome could be due to the dilution itself or to the adhesion of the bacterial cells in suspension to the surface of the tubular semipermeable membranes, in response to an MTBE diffusion gradient through them. Livingston and Ferreira (2000) reported that reductions in optical density in the biomedium of an extractive membrane bioreactor used for treating water contaminated with mono chlorobenzene and 1,2-dichloroethane were due to the formation of an incipient biofilm on the surfaces of the semipermeable membranes. In our study, this was confirmed in the bioreactors inoculated with the bacterial strains *Agrobacterium* sp. MS2 and *Rhodococcus ruber* EE6, by the FESEM analysis conducted of the tubular semipermeable membranes.

The phenomenon known as air stripping (the transfer of volatile compounds from the liquid to the gas phase) can worsen the performance of conventional aerated reactors

(Casey et al., 1999). In our study, this was prevented by supplying the air through a tubular semipermeable membrane. Thus, the loss of MTBE to the gas phase was avoided and the oxygen was released directly to the active microorganisms present in the biofilm or to the biomedium, and hence made available to the microorganisms growing in suspension. During the first week of operation with the system working in recirculation mode, the concentrations of dissolved oxygen (measured by an oxygen probe) decreased in all of the inoculated bioreactors regardless of the operating conditions. The high oxygen consumption observed during the recirculation period was caused by the bacterial growth within the inoculated bioreactors, although this consumption lasted longer in some cases, as in the bioreactors inoculated with the bacterial strain *Rhodococcus ruber* EE6 with an HRT of one hour, and in all of the bioreactors when the HRT was six hours. Zhong et al. (2007) reported the existence of a critical value for dissolved oxygen, above which MTBE degradation could be performed successfully.

To evaluate the efficiency of the EMBFR technology in each HRT condition (1, 6 and 12 h), we studied the evolution of MTBE concentrations in each of the bioreactor compartments (influent, biomedium and treated effluent) during the operating period. In the influents, the MTBE concentrations were similar in all of the bioreactors, regardless of HRT, with the lowest values corresponding to the recirculation mode. During this phase and in the biomedium of the inoculated bioreactors, the MTBE concentrations were very low, regardless of the HRT. The decrease in the concentrations of MTBE could be attributed to the loss of compound due to the aeration of the system, as observed in previous studies (Purswani et al., 2014). However, to prevent this effect, the air was supplied through semipermeable membranes, thus increasing system performance and preventing MTBE loss by volatilisation. Therefore, we conclude that the reduction in MTBE levels was due to their consumption by the bacterial strains.

No differences were observed in MTBE concentrations in the effluents from the inoculated bioreactors with HRT of 6 and 12 h, although under the latter condition slightly lower values were recorded. When the HRT was 24 h, MTBE concentrations were much higher, regardless of the strain used. In the experimental conditions used by Hu et al. (2004), a higher percentage of biodegradation was achieved when the HRT was longer.

Katsivela et al. (1999) obtained excellent performance from an EMBFR and the efficient formation of biofilm to treat industrial wastewater. In our case, FESEM analysis showed that *Agrobacterium* sp. MS2 and *Rhodococcus ruber* EE6 were capable of adhering to the semipermeable tubular membranes and of developing a more apparent biofilm when the bioreactors were maintained at an HRT of 12 h. The *Rhodococcus ruber* EE6 strain, in contrast, presented the lowest levels of growth in suspension, due to the adhesion of bacteria to the surface of the tubular membranes. The strain *Paenibacillus* sp. SH7, however, did not form biofilm at any of the HRT evaluated, although, together with *Agrobacterium* sp. MS2, it did exhibit the highest degree of growth in suspension. In the first chapter of this Thesis we discussed the ability of these strains to grow or biotransform/degrade MTBE. Authors such as Purswani et al. (2011) have concluded that the selective inoculation of a submerged biofilter aerated with a bacterial consortium composed of bacterial strains with the capacity to make use of MTBE and presenting different types of behaviour in biofilm formation (like the consortium used in the present study), could offer a promising option for the bioremediation of MTBE.

To determine whether the technology applied in this study was effective in removing MTBE, and to measure the concentrations of this xenobiotic in the biomedium and in the effluents, the Microtox® bioassay was used to evaluate the ecotoxicity of the influent, the biomedium and the treated effluent. The ecotoxicity presented by the biomedium and the treated effluent, in each inoculated bioreactor, was always lower than in the abiotic bioreactors and in the influents. When the selective inoculum was the strain

Paenibacillus sp. SH7, the toxicity of the biomedium and the effluent was greatest, regardless of the HRT tested. The FESEM analysis showed that this bacterial strain was unable to adhere to the semipermeable tubular membrane and attach itself as a biofilm, and so the presence of secondary metabolites derived from the degradation of the MTBE (TBA or formaldehyde) in the biomedium would be the reason underlying these ecotoxicity values. Roslev et al. (2015) noted that TBA is much less toxic (as EC_{50} values) than MTBE, with formaldehyde being the secondary metabolite of MTBE degradation that showed the highest level of toxicity. Under our experimental conditions, the GC-MS data did not reflect the presence of any of these substances, possibly due to the low sensitivity of the methods used. By contrast, the use of the strain *Rhodococcus ruber* EE6 as a selective inoculum achieved the lowest levels of toxicity, both in the biomedium and in the treated effluents, with an HRT of 12 h.

The results presented in this chapter show that the EMBFR technology could be an effective option for the bioremediation of water contaminated with MTBE, under certain operating conditions. Furthermore, the use as bacterial consortia of strains with different degradative capabilities could improve system performance. In this regard, and in view of the results obtained, the consortium of *Paenibacillus* sp. SH7, *Rhodococcus ruber* EE6 and *Agrobacterium* sp. MS2 would be an excellent selective inoculum for the bioremediation by EMBFR technology of water contaminated with MTBE.

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V. CONCLUSIONES

CONCLUSIONS

De acuerdo con los resultados obtenidos, así como con la revisión bibliográfica llevada a cabo, se presentan las siguientes conclusiones:

1. La evaluación conjunta de estudios fisiológicos, genéticos y ecotoxicológicos permite la mejor y más real selección de cepas bacterianas para su uso como inóculos selectivos en la biorremediación de aguas contaminadas con el oxigenante metil *ter*-butil éter (MTBE).

2. Como resultado del desarrollo de estos métodos de estudio se han seleccionado 5 cepas bacterianas como potenciales inóculos: *Rhodococcus ruber* cepa A5 (CECT 8556), cepa EE6 (CECT 8612) y cepa EE1 (CECT 8555), *Agrobacterium* sp. MS2 (CECT 8557) y *Paenibacillus* sp. SH7 (CECT 8558).

3. Los resultados generados por el análisis génico demuestra que la detección de genes que codifican para enzimas implicadas en la degradación de MTBE no necesariamente está ligada a una biodegradación efectiva de MTBE por parte de las cepas, y por lo tanto, este hecho no debería ser utilizado como indicador de la capacidad de una cepa para degradar/biotransformar este xenobiótico.

4. Basándose en los datos morfológicos, fisiológicos, quimiotaxonómicos y filogenéticos realizados, se ha propuesto a la cepa *Paenibacillus* sp. SH7 (CECT 8558) como nueva especie del género *Paenibacillus*, para la cual se ha sugerido la denominación de *Paenibacillus oxygenati* sp. nov.

5. Los estudios realizados con las cepas seleccionadas sobre la capacidad para adherirse a la superficie de membranas semipermeables tubulares y el nivel de toxicidad aguda exhibida por los cultivos bacterianos, concluyeron que sólo tres de ellas (*Rhodococcus ruber* EE6, *Agrobacterium* sp. MS2 y *Paenibacillus* sp. SH7) mostraron adecuadas características para ser usadas como inóculos selectivos en la tecnología EMBFR.

6. El estudio de los resultados de degradación de MTBE en los distintos compartimentos de los EMBFR inoculados con las cepas *Paenibacillus* sp. SH7, *Agrobacterium* sp. MS2 y *Rhodococcus ruber* EE6, junto con los valores de ecotoxicidad (EC_{50}) obtenidos mediante la aplicación del bioensayo Microtox®, pusieron de manifiesto que la inoculación de estos sistemas con la cepa *R. ruber* EE6 operando a un TRH de 12 h fueron las mejores condiciones en términos de eficiencia.

7. Aunque la cepa *Paenibacillus* sp. SH7 no mostró capacidad para adherirse a las membranas semipermeables tubulares y exhibió la mayor ecotoxicidad en los EMBFR, sí mostró el mayor crecimiento y degradación en medios líquidos suplementados con MTBE, a pesar de no presentar, al igual que ocurrió con la cepa *Agrobacterium* sp. MS2, genes que codifiquen para enzimas conocidas e involucradas en la degradación de MTBE. Por lo tanto, esta cepa bacteriana es de potencial interés biotecnológico en la biorremediacion de ambientes contaminados con MTBE.

8. La tecnología EMBFR es una opción efectiva para la biorremediación de aguas contaminadas con MTBE bajo determinadas condiciones de operación. Además, el uso de cepas bacterianas con diferentes capacidades utilizadas como consorcios bacterianos, puede mejorar el rendimiento del sistema. En este sentido el consorcio constituido por Paenibacillus sp. SH7, Rhodococcus ruber EE6 y Agrobacterium sp. MS2, es potencialmente un excelente inóculo selectivo para la biorremediación de agua contaminada con MTBE mediante la tecnología EMBFR.

The results obtained, together with the literature review performed, lead us to draw the following conclusions:

1. The joint evaluation of physiological, genetic and ecotoxicological studies makes it possible to obtain a better and more realistic selection of bacterial strains for use as selective inoculants in the bioremediation of water contaminated with the oxygenate methyl *tert*-butyl ether (MTBE).

2. Following the development of these study methods, the following bacterial strains were selected as potential inocula: *Rhodococcus ruber* strain A5 (CECT 8556), strain EE6 (CECT 8612) and strain EE1 (CECT 8555), *Agrobacterium* sp. MS2 (CECT 8557) and *Paenibacillus* sp. SH7 (CECT 8558).

3. The results generated by gene analysis show that the detection of genes that encode the enzymes involved in the degradation of MTBE is not necessarily linked to the effective biodegradation of MTBE by the strains, and therefore this fact should not be used as an indicator of the ability of a strain to degrade/biotransform this xenobiotic.

4. Taking into account the morphological, physiological, chemotaxonomic and phylogenetic data obtained, we propose the strain *Paenibacillus* sp. SH7 (CECT 8558) as a new species of the genus *Paenibacillus*, and suggest it be termed *Paenibacillus oxygenati* sp. nov.

5. The studies made of the selected strains, concerning their ability to adhere to the surface of tubular semipermeable membranes and the level of acute toxicity exhibited by the bacterial cultures, lead us to conclude that only three of them (*Rhodococcus ruber* EE6, *Agrobacterium* sp. MS2 and *Paenibacillus* sp. SH7) present characteristics making them suitable for use as selective inocula in EMBFR technology.

6. The study of MTBE degradation in the different compartments of EMBFR inoculated with the strains *Paenibacillus* sp. SH7, *Agrobacterium* sp. MS2 and *Rhodococcus ruber* EE6, together with the corresponding ecotoxicity values (EC_{50}) obtained by applying the Microtox® bioassay, show that the inoculation of these systems with the *R. ruber* EE6 strain, operating at a hydraulic retention time of 12 hours, constituted the best conditions in terms of efficiency.

7. Although the *Paenibacillus* sp. SH7 strain showed no ability to adhere to the tubular semipermeable membranes and exhibited the greatest ecotoxicity in EMBFR, it did present the strongest growth and degradation in liquid media supplemented with MTBE, although like the *Agrobacterium* sp. MS2 strain, it did not present genes that encoded enzymes known to be involved in the degradation of MTBE. Therefore, this bacterial strain is of potential biotechnological interest in the bioremediation of environments contaminated with MTBE.

8. EMBFR technology is an effective option for the bioremediation of water contaminated with MTBE, under certain operating conditions. Furthermore, the use of bacterial strains with different capabilities, used as bacterial consortia, can improve system performance. Thus, the consortium of *Paenibacillus* sp. SH7, *Rhodococcus ruber* EE6 and *Agrobacterium* sp. MS2 is potentially an excellent selective inoculum for the bioremediation of water contaminated with MTBE, through the application of EMBFR technology.

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III. CHAPTER 1

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