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"Biorremediación del alpeorujo: estudio de la microbiota asociada y producción del exopolisacárido *jamilano*"

Memoria que presenta **José Antonio Morillo Pérez**

para optar al Grado de Doctor por la Universidad de Granada

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LOS DIRECTORES DEL TRABAJO:

Dr. Alberto Ramos Cormenzana
Catedrático del Dpto. de Microbiología
Universidad de Granada

Dra. Mercedes Monteoliva Sánchez
Profesora Titular del Dpto. de Microbiología
Universidad de Granad

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Autor: José Antonio Morillo Pérez
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Abstract of the Thesis.

This thesis deals with the topic of bioremediation of alpeorujo (two-phase olive mill olive waste), a semi-solid effluent that is rich in contaminating polyphenols and is produced in large amounts by the industry of olive oil production. The first chapter of the Thesis is an up-to-date revision of the last and most important advences and treatments of oilve oil wastes. In the second chapter, laboratory-scale bioreactors were used to investigate the (bio)degradation of TPOMW by its indigenous microbiota. The effect of nutrient addition (inorganic N and P) and aeration of the bioreactors was studied. Microbial changes were investigated by PCR-temperature time gradient electrophoresis (TTGE) and following the dynamics of polar lipid fatty acids (PLFA). The greatest decrease in the polyphenolic and organic matter contents of bioreactors, was concomitant with an increase in the PLFA fungal/bacterial ratio. Amplicon sequences of nuclear ribosomal internal transcribed spacer region (ITS) and16S rDNA allowed identification of fungal and bacterial types respectively by comparative DNA sequence analyses. Predominant fungi identified included members of the genera *Penicillium*, *Candida*, *Geotrichum*, *Pichia*, *Cladosporium* and *Aschochyta.* A total of 14 bacterial genera were detected, with a dominance of organisms that have previously been associated with plant material. Most sequences were generally more than 97 % identical to those present in the databases.

The third chapter investigated the use of the two-phase olive mill waste (TPOMW) as substrate for the production of exopolysaccharide (EPS) by the endospore-forming bacilli *Paenibacillus jamilae*. This microorganism was able to grow and produce EPS in aqueous extracts of TPOMW as unique source of carbon. The effects of substrate concentration and the addition of inorganic nutrients were investigated. Maximal polymer yield in 100 ml batch-culture experiments (2 g l^{-1}) was obtained in cultures prepared with an aqueous extract of 20% TPOMW (w/v). An inhibitory effect was observed on growth and EPS production when TPOMW concentration was increased. Nutrient

supplementation (nitrate, phosphate and other inorganic nutrients) did not increase yield.

Finally, chapter 4 deals with the interaction of several heavy metals (Pb, Cd, Co, Ni, Zn and Cu) with the exopolysaccharide (EPS) produced by *Paenibacillus jamilae*. The biochemical composition of the EPS was determined. Glucose was the most abundant neutral sugar, followed by galactose, rhamnose, fucose and mannose. The polymer presented a high content in uronic acids (28.29%). The FTIR spectrum of the polymer evidenced the presence of carboxyl groups, which may serve as binding sites for divalent cations. Lead biosorption was tenfold higher (in terms of mg of metal adsorbed per g of EPS) than the adsoprtion of the rest of metals. The EPS precipitated Fe (III), but the EPS-metal precipitate did not form with Fe (II), Pb (II), Cd (II), Co (II), Ni (II), Cu (II) and Zn (II).

CONTENIDOS

Capítulo 1

Introducción General y Objetivos.

1.1. LOS RESIDUOS GENERADOS POR LA INDUSTRIA DEL ACEITE DE OLIVA.

1.1.1. El olivo

Existen aproximadamente 750 millones de olivos productivos en el mundo, los cuales ocupan una superficie de 7 millones de hectáreas. Un olivo produce de 15 a 20 kg de olivas por año. La producción mundial de aceitunas de mesa y aceite de oliva se estima en 8 y 1.743 millones de toneladas métricas respectivamente, repartidas en 25.000 explotaciones (International Olive Oil Council, Anónimo 2004). El área mediterránea proporciona por sí sola el 98% del total de superficie dedicada al cultivo del olivo y de olivos productivos, y el 97% de la producción mundial de aceite de oliva. Los mayores productores de aceite de oliva son España, seguida gran distancia por Italia y Grecia, y en menor medida Turquía, Túnez, Siria, Portugal, Marruecos y Argelia entre otros. España es el primer productor a nivel mundial en cuanto a total de superficie cultivada (2.121.181 ha) y número de árboles productivos (180 millones). Otros países que han incorporado el cultivo del olivo a sus actividades agrícolas son por ejemplo USA (California), Australia y Argentina (International Olive Oil Council, Anónimo 2004).

1.1.2. Compuestos fenólicos del olivo.

Compuestos fenólicos en las plantas superiores.

Los compuestos fenólicos (CFs) comprenden un gran número de moléculas con estructuras muy heterogéneas. La característica común de este importante grupo de moléculas biológicas es la presencia de al menos un anillo aromático con al menos un grupo hidroxilo como sustituyente.

Muchos de los compuestos fenólicos de plantas superiores (en ocasiones denominados polifenoles o biofenoles), son metabolitos secundarios. Los metabolitos secundarios son compuestos producidos por plantas y microorganismos, cuya función en el metabolismo es en muchos casos aún

desconocida, y suele estar relacionada con la interacción con otros organismos (función ecológica, no fisiológica). Se ha propuesto que estos compuestos le sirven a las plantas superiores como metabolitos de defensa frente al ataque de depredadores e insectos o son la respuesta a condiciones ambientales de estrés (Luckner, 1984).

La característica fundamental del grupo hidroxilo de los CFs es su acidez, que se debe a que el enlace que une el O y el H es propenso a romperse, para formar el correspondiente ión fenóxido con carga negativa (Waterman y Mole, 1994). El fenóxido exhibe una mayor solubilidad en agua, particularmente en presencia de cationes metálicos simples como el Na^+ o cationes como el NH_4^+. Si bien todos los compuestos fenólicos tienen esta propiedad, el grado en el cual ocurre la ionización puede ser modificado por otros sustituyentes del núcleo aromático. Por ejemplo la adición de dos grupos nitro (NO_2) en las posiciones C-2 y C-4 del fenol le confieren un carácter ácido mucho más fuerte que el del ácido acético.

Otras características químicas muy importantes de los CFs son (Waterman y Mole, 1994):

1. deslocalización de la carga negativa del ión fenóxido en el anillo aromático, lo que permite la atracción de grupos electrofílicos;
2. formación de radicales mediante la pérdida de un electrón, lo que permite que dos radicales formen puentes covalentes (C-C o C-O) mediante un proceso denominado oxidación acoplada, que conlleva la polimerización de los polifenoles;
3. la capacidad de formar puentes de hidrógeno con otras moléculas a través de la interacción del grupo ácido del fenol (carga positiva) y centros negativos en otras moléculas.

En la Tabla 1.1 se muestran los principales tipos de CFs en función de su estructura. La mayoría de los polifenoles corresponden al grupo de los flavonoides. La lignina, compuesto primario de la madera, es el ejemplo más común de flavonoide.

Tabla 1.1. Tipos de compuestos fenólicos mas importantes en plantas superiores (Harborne, 1980).

Número de átomos de C	Esqueleto básico	Tipo
6	C_6	fenoles simples, benzoquinonas
7	$C_6 - C_1$	ácidos fenólicos
8	$C_6 - C_2$	acetofenonas, ácido fenilacético
9	$C_6 - C_3$	ácido hidroxicinámico, coumarina, isocoumarina
10	$C_6 - C_4$	naftoquinonas
13	$C_6 - C_1 - C_6$	xantonas
14	$C_6 - C_2 - C_6$	estilbeno, anthrachinone
15	$C_6 - C_3 - C_6$	flavonoides, isoflavonoides
18	$(C_6 - C_3)_2$	lignanos, neolignanos
30	$(C_6 - C_3 - C_6)_2$	biflavonoides
n	$(C_6 - C_3)_n$ $(C_6)_n$ $(C_6 - C_3 - C_6)_n$	ligninas catecolmelaninas (taninas condensadas)

La variabilidad de los flavonoides depende especialmente del grado de hidroxilación y/o metilación del sistema de tres anillos. Es interesante mencionar que no todos los polifenoles son metabolitos secundarios. Por ejemplo las quinonas funcionan como co-factores. Los polifenoles abarcan un rango amplio de funciones biológicas, entre las que se pueden destacar: pigmentos de flores (flavonoides amarillos, flavonas, auronas) y frutas (antocianinas, isoflavonas), substancias alelopáticas (quinonas, fenoles simples, ácidos fenolcarboxílicos), protección frente a plagas (quinonas, taninas, flavonoles), fungicidas (isoflavonas, ácidos fenolcarboxílicos) y fitoalexinas (fenilpropanoides, isoflavonas, fucocumarinas) (Waterman y Mole, 1994).

Compuestos fenólicos de <u>Olea europaea</u>.

El aporte energético de la fruta del olivo se debe fundamentalmente a los lípidos polares y triglicéridos, que cuando ésta es aún un producto crudo, suman aprox. un 98% de su composición. El 2% restante se compone de biomoléculas tales como esteroles, tocoferoles, terpenos y compuestos fenólicos, los cuales se pueden encontrar distribuidos en el mesocarpo de la oliva, la pulpa y también en la semilla (Servili et al. 1999). Estos CFs, abarcan un amplio rango de metabolitos secundarios con una rica variedad estructural y

una gran diversidad de actividades biológicas. Pueden encontrarse en diferentes formas químicas: en forma soluble o simple, esterificados y ligados a la pared celular (Uccella, 2001).

Muchos de los CFs del olivo son también compuestos terpénicos, caracterizados por uno o más hidroxilos como substituyentes del anillo aromático lo que les confiere la funcionalidad característica. Según Uccella (2001) suelen denominarse incorrectamente polifenoles (nomenclatura que proviene de la terminología de los polifenoles del vino), ya que los CFs del olivo no son polímeros sino monómeros de peso molecular hasta 624 (Ryan et al. 1998). Los CFs engloban compuestos con estructuras que varían desde compuestos simples como el 2(4-hidroxifenil)etanol o tirosol, hasta compuestos muy polimerizados como las taninas (Saija et al. 2001). A lo largo de esta Memoria seguiremos utilizando el término CF de forma general para referirnos al conjunto de compuestos con funcionalidad fenólica presentes en el olivo, así como en los productos y sub-productos generados a partir de su biomasa, incluyendo por tanto a los monómeros de bajo peso molecular como a los compuestos polimerizados.

Ente las funciones de los CFs del olivo, cabe destacar la defensa natural frente a ataques de patógenos. Por otro lado, los CFs también juegan un papel importante en el mantenimiento del esqueleto de la pared celular, al servir de enlaces entre los polisacáridos, además de influenciar notablemente las características organolépticas del aceite de oliva y las aceitunas de mesa (Uccella, 2001.)

El mecanismo de defensa exhibido en el olivo por los CFs frente a algunos patógenos (bacterias, microhongos, insectos) (Sivinski et al. 1999), se debe a los complejos CFs del grupo de los secoiridoides (*seco*CFs), como la oleuropeína y el lingstrósido. Estos compuestos son los responsables de la resistencia a la infección, a través de la síntesis de fitoalexinas y su acumulación en los puntos donde se ha producido la infección, acompañado de otros mecanismos como modificaciones en la pared celular, protección frente a enzimas y muerte celular.

El ataque de patógenos a las células del olivo provoca la liberación enzimática de fitoalexinas, por ejemplo a partir de la oleuropeína (Mercier et al. 1977). La inducción de la síntesis de fitoalexinas se controla mediante la producción endógena de β-glucosidasas (Bianco et al. 1999). En el caso de la oleuropeína, la reacción enzimática produce la liberación de la oleuropeína aglicona (hidroxitirosileleonato), compuesto que proporcionan una defensa efectiva frente al patógeno (Fig. 1.1).

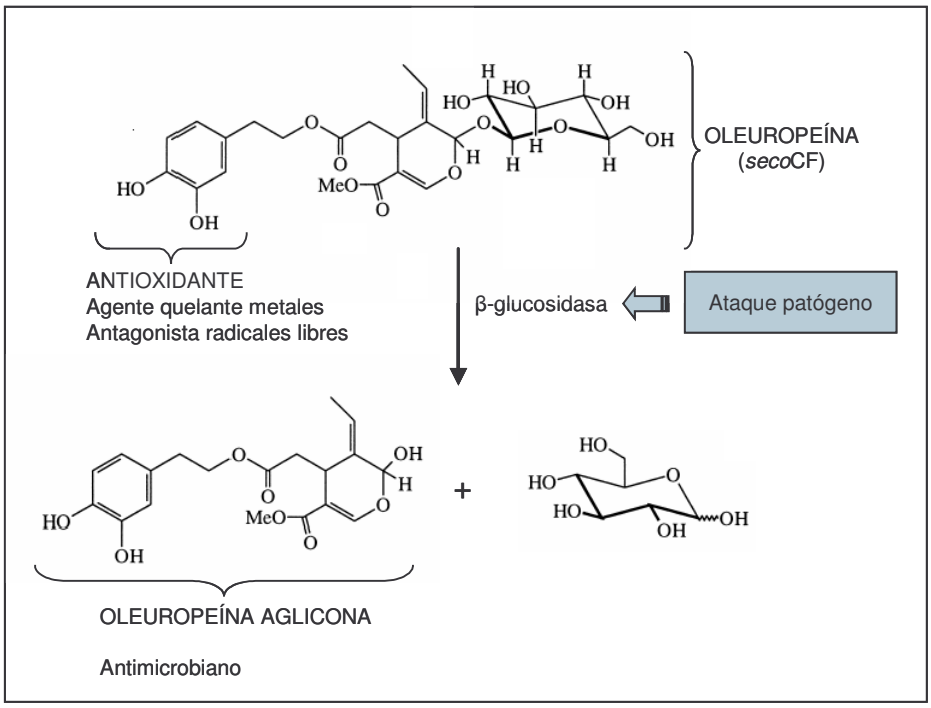

Fig. 1.1. Hidrólisis enzimática de la oleuropeína.

En general, los CFs ejercen una notable influencia sobre los productos que el ser humano produce gracias al cultivo de *Olea europaea*. Se ha demostrado que estos compuestos ejercen una acción antioxidante y antimicrobiana, habiéndose afirmado que la inclusión en la dieta de aceite de oliva y aceitunas de mesa (alimentos típicos de la cultura mediterránea), puede disminuir el riesgo de enfermedades degenerativas y de afecciones microbianas (Saija et al., 2001).

1.1.3. Procesos de obtención de aceite de oliva y residuos generados.

El aceite de oliva se extrae de la aceituna, fruto del olivo. La composición del fruto es muy variable, en función de la variedad de la aceituna, del suelo, del clima y del cultivo, pero en término medio está compuesta de: aceite (18-28%), agua de vegetación (40-50%), hueso y tejidos vegetales (30-35%) (Niaounakis and Halvadakis 2004).

El aceite de oliva se obtiene a partir del tratamiento de las aceitunas en las almazaras, a través de procedimientos mecánicos o medios físicos en condiciones térmicas controladas. Este aceite de oliva que es equivalente al zumo de la aceituna, es apto para el consumo, pero también se generan aceites no aptos para dicho consumo, a los que se denomina aceites de oliva vírgenes lampantes. Estos últimos serán sometidos a un proceso de refinado y una posterior mezcla con aceite de oliva virgen, obteniéndose aceite de oliva, que es el más consumido.

Tanto el cultivo del olivo como la extracción del aceite de oliva producen grandes cantidades de subproductos. Se ha estimado que tan solo la recolección de la aceituna produce 25 kg de residuos (ramitas y hojas) por árbol anualmente (las hojas representan el 5% del peso de las aceitunas que se destinan a la extracción del aceite).

De forma general, durante el proceso de extracción de aceite se producen tres “fases” o “efluentes”: aceite de oliva, residuo sólido y residuo líquido:

a) aceite de oliva

b) El residuo sólido, denominado en España **orujo** (*olive-cake* en inglés), está compuesto por una mezcla de la pulpa y los huesos de las aceitunas. Este material se suele reutilizar en plantas especializadas para la extracción de aceite residual o aceite de orujo, mediante su extracción con hexano tras secar el orujo con aire a 60 ºC.

c) El residuo líquido proviene del agua de vegetación y de los tejidos blandos de la fruta. La mezcla de este sub-producto acuoso con el agua que se utiliza en las diferentes etapas de la producción del aceite de oliva produce el denominado **alpechín** (en inglés, *olive-mill waste water*).

Esta biomasa residual que se obtiene durante el proceso de extracción de aceite se reparte entre las fases sólida y líquida en diferente proporción en función de la tecnología industrial de producción de aceite de oliva empleada. Estas tecnologías han experimentado diversos cambios evolutivos y actualmente se emplean diversos sistemas. Sin embargo, se mantienen una serie de operaciones preliminares comunes:

- **Recepción y selección:**

  Tras la recepción de las aceitunas en la almazara, se procede a una comprobación del estado de las mismas, siendo muy importante que las aceitunas sanas se encuentren separadas de aquellas defectuosas (enfermas, recogidas del suelo, rotas, etc).

- **Limpieza, lavado y pesada.**

  Es necesario eliminar las hojas y lavar las aceitunas antes de proceder a la extracción del aceite. Para ello se emplean generalmente máquinas automáticas provistas de un sistema de aspiración de las hojas y de una pila con circulación forzada de agua para el lavado de las aceitunas. El objetivo es eliminar de los frutos impurezas de origen vegetal como ramillas y hojas, y mineral, como polvo, tierra, piedras y otros cuerpos sólidos, así como restos de sustancias como pesticidas y contaminantes agrícolas. Durante esta operación se generan dos tipos de residuos: hojas de olivo u *hojín* y el *agua de lavado* de las aceitunas.

- **Molienda.**

  El objetivo de esta operación consiste en romper las células de la pulpa y provocar la salida del aceite de las vacuolas para su reunión en gotas más gruesas y permitir de este modo su separación. Para ello tradicionalmente se utilizaban molinos de rulos aunque hot en día se usan trituradores metálicos o molinos de martillos.

- **Batido.**

Esta operación tiene como finalidad romper la emulsión agua/aceite y facilitar la reunión de las minúsculas gotas de aceite en gotas de tamaño superior que permita la separación del aceite. Para ello la pasta de las aceitunas obtenida tras la molienda se somete a un removido lento y continuo que se efectúa en recipientes de acero inoxidable (batidoras) provistas de un sistema de calentamiento adecuado. Este proceso no es eficiente al 100% por lo cual parte del aceite se mantiene en gotas de pequeño tamaño formando una emulsión y es arrastrada a los subproductos.

La última etapa consiste en la separación de los líquidos contenidos en la pasta de la aceituna. Esta etapa fundamental se denomina **extracción**. Actualmente se emplean tres sistemas de extracción. El *sistema tradicional o discontinuo (por presión),* el *sistema de centrifugación o continuo de tres fases*, y el más recientemente introducido *sistema de dos fases*. En la Fig.1.2 se muestra un esquema de estos tres procesos. En el sistema de extracción por presión y en el sistema de tres fases se producen un residuo líquido (alpechín) y un residuo sólido (orujo). Sin embargo, en el sistema de dos fases se obtiene un solo residuo de textura fangosa o sólido-líquida, que se denomina **alpeorujo** u orujo de tres fases.

Sistema de extracción por presión.

Es el procedimiento más antiguo para extraer aceite de oliva. Este sistema utiliza un proceso de filtrado favorecido por presión para separar la parte líquida de la pasta (mezcla del aceite con el alpechín de las aceitunas) del orujo o parte sólida. El sistema discontinuo permite obtener aceites de gran calidad debido a las bajas temperaturas a lo largo del proceso. Los principales inconvenientes derivan de los elevados costes de mano de obra, la discontinuidad del proceso y los gastos inherentes al empleo de materiales filtrantes en condiciones óptimas.

Sistema de extracción por centrifugación de tres fases.

El sistema de tres fases se desarrolló en los años 70 con objeto de reducir los costes del laboreo necesarios e incrementar la calidad y rendimiento en la producción. Esta tecnología, ampliamente utilizada todavía en la actualidad a escala mundial, explota la diferencia de densidad entre al agua y el aceite. La pasta de las aceitunas se somete a un proceso de centrifugación a muy alta velocidad en centrifugadoras horizontales. Se requiere la adición de agua caliente y el resultado es que la pasta de aceitunas se separa en tres fases: aceite, alpechín (agua de vegetación más agua añadida) y orujo (mezcla de huesos y tejidos vegetales).

Ente las desventajas de este proceso, se incluye el problema ambiental generado por la gran cantidad de alpechín producido (1.25-1.75 más volumen que en el sistema tradicional, aprox. 1.7 kg de alpechín por kg de aceitunas), el elevado consumo de agua en las regiones generalmente secas donde se cultiva el olivo (80-100 litros de agua por cada 100 kg de aceituna prensada) y la pérdida de compuestos antioxidantes con el alpechín (compuestos fenólicos principalmente).

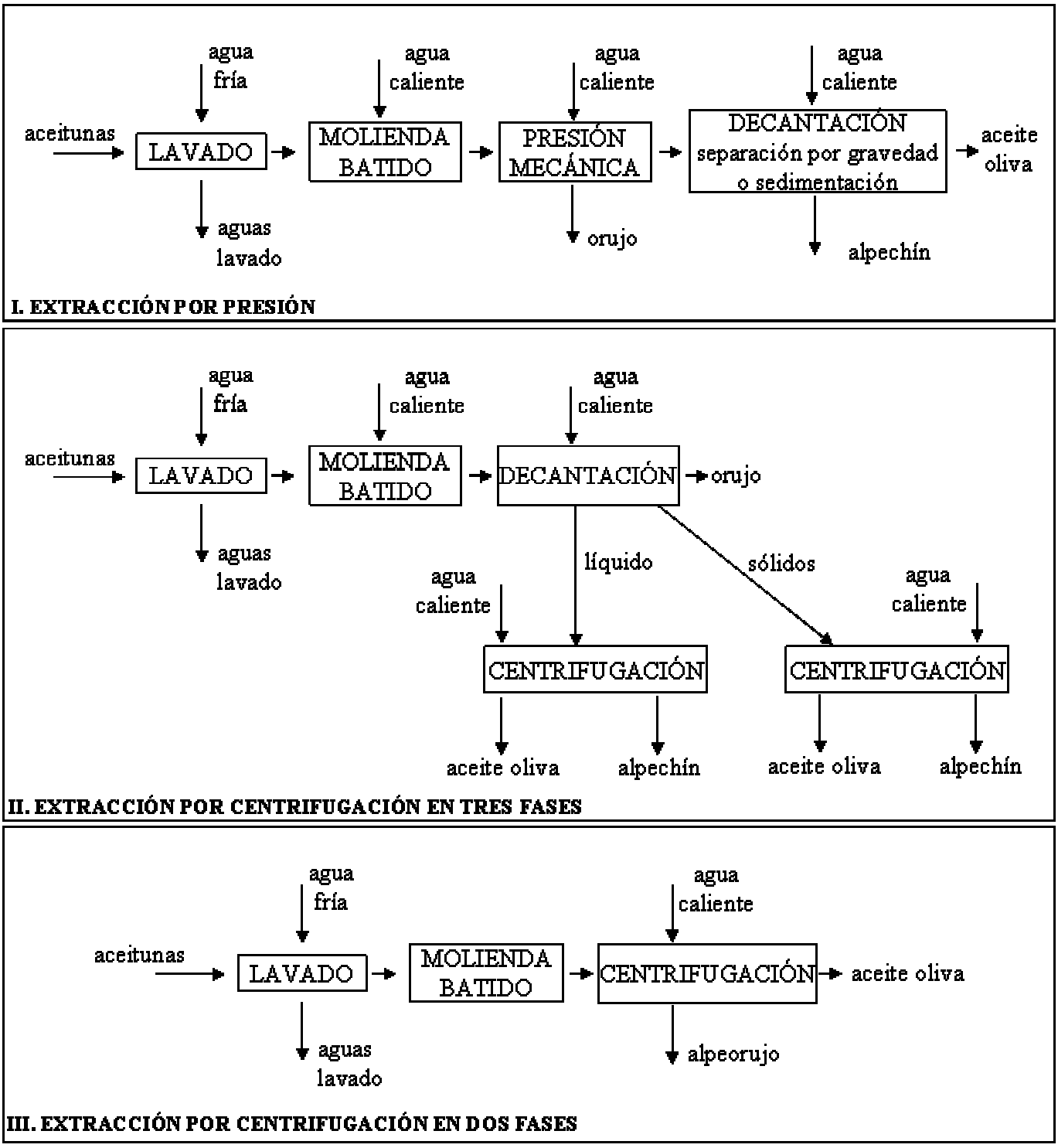

Fig.1.2. Sistemas actuales de producción de aceite de oliva (Vlyssides, 1998).

Esencialmente, la composición del alpechín es agua (80%), compuestos orgánicos (15-18%) e inorgánicos fundamentalmente sales de potasio y fosfatos (2%). La composición química puede variar ampliamente en función de la variedad de olivo, el tiempo de cosecha, las condiciones climáticas, el proceso de extracción etc (Niaounakis and Halvadakis 2004). Debido a la presencia de grandes cantidades de proteínas, polisacáridos, sales minerales y otras substancias útiles para la agricultura, como ácidos húmicos, el alpechín tiene un gran poder fertilizante. Por lo tanto, debería ser utilizado como abono natural de bajo precio y disponible en grandes cantidades. No obstante, desafortunadamente el alpechín contiene, además de estas substancias favorables para la agricultura, otros tipos de substancias que le confieren carácter fitotóxico y biotóxico, que impiden su uso directo como abono. Estos efectos fototóxicos y antibacterianos han sido atribuidos a su contenido fenólico. Se estima que la carga orgánica del alpechín en términos de compuestos fenólicos es más de 1000 veces mayor que la de las aguas residuales domésticas. De hecho, la presencia de tales substancias confiere una difícil biodegrabilidad al alpechín y consecuentemente limita considerablemente su uso y lo convierte en un problema ambiental. Además el alpechín es ácido (pH 4.5-5), tiene un color oscuro característico y desprende un fuerte olor. La producción total de alpechín asciende a 10-12 10^6 m^3 por año y se produce durante un pequeño concreto del año (Noviembre-Marzo).

Sistema de extracción por centrifugación de dos fases.

Como consecuencia de los problemas generados por el sistema de tres fases, más recientemente se desarrolló el sistema en dos fases, que utiliza mucho menos agua. El uso del sistema en dos fases se implementó por primera vez en España, durante la campaña 1991-1992, coincidiendo con una importante sequía, con lo que fue muy bien acogido al disminuir notablemente el consumo de agua (Alvarado 1998). El sistema en dos fases se basa en un tipo de centrifugación más efectivo y no requiere agua de procesado, produciendo tan solo dos efluentes: aceite de oliva y un nuevo tipo de subproducto de consistencia pastosa por su alto contenido en agua: el alpeorujo. Por lo tanto, con el sistema en dos fases, nació un nuevo problema de tipo ambiental, ya

que aunque no se produce un vertido líquido como en los anteriores sistemas, la naturaleza “semi-sólida” del alpeorujo, complica notablemente su transporte y tratamiento.

Diversos estudios han puesto de manifiesto la influencia del sistema de extracción sobre la calidad del aceite de oliva. Es interesante destacar uno de estos estudios realizado a nivel industrial (Ranalli y Angerosa, 1996), en el cual se procesaron lotes homogéneos de aceitunas mediante los sistemas de centrifugación de dos fases y tres fases. En general, los resultados indicaron que el sistema de dos fases generaba mayor producción de aceite, con la consabida disminución del residuo líquido. El sistema de extracción parece no afectar al perfil cualitativo de compuestos fenólicos presentes en el aceite, aunque los aceites vírgenes obtenidos por el sistema de dos fases contienen una concentración mayor de estos compuestos en relación al aceite homólogo obtenido por el sistema de tres fases.

Comparando los dos sistemas de extracción basados en la centrifugación, el sistema de dos fases presenta las siguientes ventajas e inconvenientes (Niaounakis y Halvadakis 2004):

a) Principales ventajas:

- La construcción de la centrifuga de dos fases es menos complicada y por lo tanto más barata y práctica que la centrifuga de tres fases.
- En el sistema de tres fases, se producen problemas técnicos asociados a la mezcla de agua y aceite debido a la precipitación de compuestos que forman una capa que se deposita en la centrífuga.
- El rendimiento del sistema de dos fases es mayor en relación a la cantidad de aceite producida, y el consumo global de energía es menor ya que al no añadirse agua, el volumen de material a procesar es menor.
- El aceite obtenido mediante el decantador de dos fases es de mejor calidad: especialmente se caracteriza por una mayor estabilidad frente a la oxidación y por sus más adecuadas características organolépticas.
- Los costos operacionales son menores. La cantidad de agua requerida es considerablemente menor.

b) En cuanto a los inconvenientes del sistema de dos fases, cabe resaltar:

- El sistema de dos fases no produce un vertido propiamente dicho. Sin embrago, al combinarse el residuo líquido con el sólido, se genera un solo efluente de consistencia pastosa. Este alpeorujo dobla la cantidad de residuo "sólido" generado en el sistema de tres fases, y requiere un tratamiento.
- El alpeorujo tiene un contenido de humedad del 55-70%, mientras que el orujo obtenido por el sistema tradicional tiene un contenido entre 20-25% y el orujo de tres fases entre 40-45%. Este gran contenido en agua, junto a las partículas finas y compuestos químicos que en el sistema de tres fases se concentraban en el alpechín, hacen que el alpeorujo tenga una consistencia "pastosa" que dificulta enormemente su transporte, almacenamiento y en general aumenta la dificultad de su manejo (no puede ser apilado y se ha de almacenar en grandes balsas).
- El alpeorujo es un residuo muy concentrado con una gran carga orgánica y rico en grasas y compuestos fenólicos de diversos tipos. Constituye un nuevo tipo de residuo y sus posibles tratamientos están aun en fase de investigación.
- En definitiva, el sistema de dos fases, aunque también se conoce con el apelativo de "sistema ecológico", transfiere el problema del depósito de los residuos de las almazaras a las estaciones extractoras de aceite de orujo. El alpeorujo, antes de ser extractado con hexano, debe ser secado y dado su alto contenido en humedad, este proceso es mas caro e ineficiente que en el caso del orujo del sistema tradicional y el de tres fases.

La exitosa introducción del sistema de dos fases en España (más del 90% de las almazaras han adoptado este sistema), no se ha producido en los otros productores importantes como Grecia o Italia (<5%). En Grecia por ejemplo, están censadas 2.786 almazaras de las cuales el 70% utiliza el sistema en tres fases y en el resto el sistema tradicional o diversas combinaciones (Niaounakis and Halvadakis 2004). En Portugal la extracción de aceite de oliva se basa

fundamentalmente en el sistema tradicional, aunque la industria se está modernizando en los últimos años. En Italia, están operando unas 10.000 almazaras y en la mayoría de ellas se sigue utilizando el sistema tradicional. Por lo tanto, aunque el sistema en dos fases se está introduciendo también en nuevos productores como Australia, se puede decir que por el momento el alpeorujo es fundamentalmente un problema español.

1.1.4. Efectos de los residuos sobre el medioambiente.

Dada la reciente aparición del alpeorujo en relación al alpechín, la mayor parte de los trabajos científicos publicados sobre los efectos ambientales de estos residuos tratan los problemas relacionados con el alpechín, que además sigue siendo en la actualidad un problema muy importante en la mayoría de los países productores de aceite de oliva, excluyendo principalmente a España. Por lo tanto la mayoría de los trabajos revisados en este apartado se refieren al alpechín aunque la información vertida por éstos es crucial para abordar el problema del alpeorujo ya que como sabemos, el origen de ambos residuos es idéntico y por tanto existen muchas similitudes en su composición, especialmente en la gran cantidad de carga orgánica que representan así como en la presencia de sustancias con posibles efectos tóxicos sobre plantas y microorganismos como polifenoles y ácidos grasos.

Actividad antimicrobiana.

La actividad antimicrobiana del alpechín es bien conocida, y ha sido asociada principalmente al contenido del residuo en compuestos fenólicos (Ramos-Cormenzana et al., 1996). La incidencia que esta actividad tiene sobre el contenido microbiano del suelo también ha sido descrita (Moreno et al., 1987, 1990).

Los compuestos fenólicos pueden afectar a los microorganismos de diferentes formas. Por ejemplo, el alpechín, aunque esté diluido, puede afectar a la esporulación de bacterias del suelo. Los extractos en n-propanol del alpechín presentan efecto bactericida frente a *Bacillus megaterium* ATCC 33085,

inhibiendo la esporulación y germinación a concentraciones de 5.6 mmol/l (Rodríguez et al. 1988). Sin embargo, aunque esta actividad se debe principalmente a los compuestos fenólicos, también ha sido relacionada con los ácidos grasos presentes en el alpechín, (González-López et al. 1994).

No todos los compuestos fenólicos presentes en los residuos originados en la extracción del aceite de oliva presentan actividad antimicrobiana, y los compuestos que sí presentan dicha actividad difieren tanto en la intensidad de la misma como en el grupo microbiano afectado. Por ejemplo, en un estudio publicado por Capasso et al. (1995), se demostró la actividad antibacteriana del alpechín frente a los organismos fitopatógenos *Pseudomonas syringiae* pv. *savastanoi* (Gram-negativa) y *Corynebacterium michiganense* (Gram-positiva). Entre los compuestos fenólicos más importantes encontrados en el alpechín, el metilcatecol fue el más activo frente a *P. syringiae* pv. *savastanoi* a 10^{-4} mol/l, mientras que tan solo fue ligeramente activo frente a *C. michiganense*. Catecol e hidroxitirosol presentaron algo de actividad frente a *P. syringiae* pv. *savastanoi*, pero fueron inactivos frente a la bacteria Gram-positiva. El tirosol y sus isómeros sintéticos 1,2- y 1,3-tirosol fueron completamente inactivos frente a las dos bacterias. Además, algunos de estos compuestos como el catecol presentaron actividad tóxica frente a las células humanas Hep2.

Fitotoxicidad.

El alpechín inhibe la germinación de diferentes tipos de semillas así como el desarrollo prematuro en diferentes especies vegetales (Capasso et al., 1992b; Della Greca et al., 2001). Así mismo, la aplicación directa del alpechín sobre plantas provoca la abscisión de hojas y raíces (Bartolini et al. 1994). La fitotoxicidad del alpechín se ha atribuido por diversos autores al contenido fenólico y a algunos ácidos orgánicos como el ácido acético o el fórmico que se producen durante el almacenamiento del alpechín debido a la actividad microbiana. Sin embargo no se dispone de mucha información específica sobre la actividad de la fracción aromática de estos residuos en la germinación de plantas. Wang et al. (1967) descubrieron que diversos compuestos fenólicos presentes en el alpechín tenían una considerable actividad fitotóxica. Pérez et

al. (1986) estudiaron los efectos del alpechín sobre la germinación de semillas y el crecimiento temprano de diversas especies vegetales. Demostraron el efecto fitotóxico del residuo en todas las variedades vegetales testadas siendo el tomate (*Lycopersicon esculentum*) la planta más afectada. Capasso et al. (1992) estudiaron el efecto de los principales compuestos fenólicos aislados del alpechín sobre el desarrollo del tomate (*L. esculentum*) y calabacín (*Cucurbita pepo*). Los compuestos fenólicos probados fueron selectivamente tóxicos frente a estas dos especies, excepto el 4-metilcatecol. También es interesante destacar que alpechín conservó en parte su actividad tóxica incluso después de extraerse los compuestos fenólicos, sugiriendo que otros productos químicos presentes en el residuo pudieran contribuir a la fitotoxicidad total.

Efecto sobre sistemas acuáticos.

En 1981 una ley española prohibió el vertido de alpechines a ríos y corrientes de agua (REAL DECRETO-LEY 18/1981, DE 4 DE DICIEMBRE, DISP. 28431). Esta ley surgió principalmente como consecuencia de los problemas de contaminación debido a loa vertidos incontrolados de alpechín en la cuenca del Guadalquivir. Posteriormente otros países mediterráneos adoptaron una legislación similar, aunque a pesar de que las políticas medioambientales de los principales países productores de aceite de oliva consideraron el peligro de los vertidos directos de alpechines a los ríos y otros cauces de agua, en la actualidad aún se producen ocasionalmente en España problemas de contaminación debido a vertidos incontrolados, fallos de las balsas de almacenamiento, etc. El alpeorujo, por su naturaleza sólida, no puede ser considerado un vertido líquido. Sin embargo, se han producido algunos casos de contaminación de ríos y acuíferos por alpeorujos debido a escorrentías e infiltraciones provenientes de balsas de almacenamiento.

Son varios los efectos que un vertido de un residuo de estas características puede provocar en los sistemas acuáticos (Niaounakis and Halvadakis 2004):

- Decoloración de las aguas.

Este efecto se relaciona con la oxidación y la consiguiente polimerización de las taninas produciendo compuestos que colorean el agua con tonos oscuros y que son difíciles de eliminar.

- Eutrofización.

El alpechín y el alpeorujo contienen diferentes nutrientes (azúcares, fósforo, nitrógeno) que pueden ser utilizados por microorganismos como bacterias y microalgas. Un aumento de la biomasa de los microorganismos de un ecosistema acuático, además del consiguiente desequilibrio ecológico, conlleva generalmente un aumento del requerimiento de oxígeno disuelto, circunstancia que puede desembocar en fenómenos de anoxia que pueden ser muy nocivos.

- Problemas en las redes de saneamiento de aguas domésticas.

La entrada de un vertido relativamente pequeño de alpechín en la red de saneamiento de aguas residuales, por ejemplo a través de las alcantarillas, puede provocar daños graves en las plantas de tratamiento ya que la carga contaminante de 1 l de alpechín equivale a 100-200 l de aguas residuales domésticas. Estas sobrecargas pueden ser dramáticas teniendo en cuenta que en algunas poblaciones, durante la temporada de producción de aceite de oliva, la carga contaminante de los residuos originados puede ser diez veces mayor que la carga orgánica de las aguas residuales. Por otro lado, la acidez propia de estos residuos puede provocar problemas de corrosión en tuberías y sistemas de conducción, siendo una de las razones por las que está generalmente prohibido verter alpechín en las redes de saneamiento.

- Efectos tóxicos.

La toxicidad del alpechín no solo afecta a especies vegetales y bacterianas. Se han descrito igualmente los efectos sobre otros componentes de la red trófica de ecosistemas acuáticos continentales. Una exposición a 40 mg/l de

alpechín durante 15 minutos es suficiente para intoxicar severamente al pez de río *Gambusia affinis* y el crustáceo *Daphnia magna* . Esta concentración ecológicamente dañina de alpechín se obtendría vertiendo un solo litro de alpechín en un cuerpo de agua de 1000.000 litros, con lo que considerando que una almazara típica puede producir 5000 litros de alpechín por hora, sería necesario verter el residuo sobre una corriente de al menos 100.000 litros por segundo para evitar el efecto tóxico (González et al. 1994). El alpechín al 10% tiene un efecto tóxico casi inmediato sobre el pez *Carasius auratus* y sobre poblaciones de fitoplancton del río Guadalquivir (Bellido, 1989). En un estudio mas reciente, se fraccionó una muestra de alpechín producido por una almazara italiana por medio de ultrafiltración y ósmosis inversa. Las fracciones obtenidas fueron sometidas a ensayos de toxicidad frente a organismos ubicados en diferentes niveles tróficos: el alga *Pseudokirchneriella sucapitata*, el rotífero *Brachionus calcyciflorus* y dos crustáceos: *Daphnia magna* y *Thamnocephalus platyrus*. La fracción más tóxica resultó la obtenida por ósmosis inversa y que consistía en los compuestos de bajo peso molecular (<350 Da), siendo catecol e hidroxitirosol los compuestos mas abundantes (Fiorentino *et al.* 2003).

Efectos sobre el suelo.

Una de las soluciones posibles para el problema de la gestión de la ingente cantidad de residuos generados por la industria del aceite de oliva podría ser su dispersión en suelo agrícola, con el objetivo de que en un primer paso la metabolización por parte de la microbiota natural reduzca la fitotoxicidad, y en un segundo, los nutrientes aportados pudieran servir como abono para su aprovechamiento agrícola. Sin embargo como veremos este enfoque presenta serias limitaciones.

Como se ha comentado anteriormente, es sabido que la aplicación directa de alpechín inhibe la germinación y el desarrollo de diferentes especies vegetales. En 1986 se realizó en nuestro Departamento de Microbiología un estudio sobre el efecto del alpechín en la comunidad microbiana del suelo, utilizando (Paredes et al. 1986). Se encontró que la contaminación por alpechín

aumentaba la cantidad total de microorganismos cultivables, y más específicamente se producía un aumento de bacterias coreniformes y una disminución del número de *Bacillus*.

El contenido microbiano no es el único componente del suelo que puede verse afectado por un vertido de alpechín. Como es sabido este líquido contiene ácidos orgánicos, sales minerales y otros compuestos que pueden alterar la capacidad de intercambio iónico de los suelos entre otras características. Paredes et al. (1987) demostraron que el efecto del alpechín sobre un suelo alcalino fue un aumento de la salinidad debido a la entrada de iones de sodio y potasio y un aumento del índice C/N, junto con una disminución de la concentración de bacterias productoras de esporas. Estos efectos, al cambiar las condiciones ambientales, afectando directamente a la microbiota y al balance de nutrientes, podrían disminuir la fertilidad del suelo como se ha demostrado posteriormente. También se ha comprobado que el alpechín afecta a las propiedades de agregación de las partículas del suelo (Colucci et al., 2002).

Sierra et al. (2001) investigaron las características de un suelo calcáreo que había sido afectado por una deposición incontrolada de alpechín durante 10 años. Para ello realizaron una completa caracterización (morfológica y analítica) de los diferentes horizontes del suelo comparándola con un área control con el mismo tipo de suelo pero que no había sido afectada por la contaminación. Los resultados mostraron que la infiltración del vertido provocó la disolución de carbonatos y la redistribución y modificación de los perfiles de pH, conductividad eléctrica, contenido en nutrientes, compuestos fenólicos y actividad biológica. Una vez que fue cerrado el punto de vertido, la infiltración natural y la actividad biológica redujeron la conductividad eléctrica y el contenido fenólico del suelo, aunque los niveles residuales de contaminación se mantuvieron altos por mas de dos años.

Los últimos estudios realizados sobre el efecto del alpechín, como posible abono orgánico sin ningún tratamiento previo, reflejan controversias. Probablemente esto sea debido a los efectos antagónicos de los diferentes

compuestos orgánicos e inorgánicos presentes en el residuo, cuyos efectos pueden ser beneficiosos o tóxicos (-Piotrowska et al. 2005-). Algunos trabajos insisten en la "línea clásica" de considerar a cualquier deposición de alpechín como un proceso destructivo del suelo, hecho que se demuestra mediante medidas de parámetros tales como cambios en el pH y perfil salino del suelo (Sierra et al. 2001), destacándose también el alto riesgo de contaminación de acuíferos cercanos (-S´Habout et al. 2005-), cambios en la estructura de la comunidad microbiana y problemas de toxicidad para microorganismos (Mekki et al. 2006a), e infiltración de compuestos fenólicos de los que una fracción fitotóxica permanece después de un año de producirse el vertido (Mekki et al. 2006b). Sin embargo otros trabajos defienden la descarga directa y controlada de alpechín a suelos agrícolas como una solución viable, especialmente si se trata de suelos calcáreos pobres en materia orgánica, ya que si bien se puede producir un aumento de la fitotoxicidad a corto plazo, a largo plazo el efecto es beneficioso pues desaparecen dicha toxicidad y se produce un enriquecimiento nutricional del suelo (Saadi et al. 2007).

Respecto a la aplicación directa de alpeorujo como abono, existe mucha menos información disponible. Si bien el alto contenido en materia orgánica (95% aproximadamente) de este residuo puede ser altamente beneficiosa para la agricultura, el aceite residual presente puede aumentar la hidrofobicidad del suelo y disminuir la retención de agua (Niaounakis y Halvadakis 2004). Aún así se ha encontrado que la aplicación de alpeorujo no tratado como abono puede aumentar el rendimiento en cosechas de trigo (-López-Piñeiro et al. 2006- y 2007).

Otros investigadores han abordado el efecto de la deposición de los residuos de las almazaras sobre el ciclo de nutrientes en el suelo y sobre el sistema planta-microorganismos del suelo. Así, se ha evaluado el efecto del alpechín sobre el ciclo de los siguientes nutrientes en suelos calcáreos: nitrógeno (-Pérez y Gallardo-Lara, 1987-), azufre (-Pérez y Gallardo-Lara, 1989-), calcio (-Gallardo-Lara et al. 1998-), y finalmente potasio, magnesio y manganeso (-Gallardo-Lara et al. 2000-).

Un problema añadido a la aplicación directa de alpechín es la solubilización de metales pesados previamente inmovilizados en el suelo, como por ejemplo Cu y Zn (-Madrid y Díaz-Barrientos, 1998-). En este sentido, parece que el efecto causado sobre el pH del suelo es el factor más importante. Además, a un pH determinado el alpechín puede provocar la movilización de metales pesados tales como Ni, Cd, Zn, Cu, Mn, Pb y Fe (-Bejarano y Madrid, 1996-). A su vez, un vertido de alpechín puede reducir la porosidad del suelo y aumentar notablemente el contenido en carbono orgánico, afectando a la movilización de otros compuestos tóxicos como pesticidas (-Cox et a. 1997-). Recientemente se ha demostrado que el efecto de depositar alpeorujo sobre un suelo contaminado por pesticidas consiste en una mayor retención del pesticida, especialmente de los compuestos más apolares (como por ejemplo herbicidas del grupo de la triazina), debido al contenido lipídico del residuo (-Delgado-Moreno et al. 2007-).

Efectos sobre la atmósfera.

Los residuos obtenidos durante la extracción del aceite de oliva producen un característico y desagradable olor que a veces afecta a los núcleos de población cercanos a almazaras y puntos de almacenamiento/tratamiento de estos residuos. Esto es debido procesos de fermentación que tienen como resultado la emisión de metano, sulfuro de hidrógeno y otros gases, que producen problemas de contaminación por olores (Balice et al. 1986). Se han propuesto algunas soluciones como el empleo de Ca $(OH)_2$ que produce una importante disminución del olor al aplicar una concentración de 10g/l en bidones de alpechín (Lagoudianaki et al. 2003).

1.2. CARACTERÍSTICAS QUÍMICAS Y MICROBIOLÓGICAS DEL ALPEORUJO.

1.2.1. Datos sobre la composición química del alpeorujo.

La mayoría de las publicaciones científicas en las que se recoge información sobre la composición química del alpeorujo tratan sobre un tratamiento realizado sobre una o varias muestras, incluyendo información específica sobre la composición de la(s) muestra(s) utilizadas (Cayuela et al., 2004; Cegarra et al. 2000; Madejón et al., 1998). En este apartado es conveniente resaltar por tanto el trabajo de Alburquerque et al. (2004), ya que el objetivo de este estudio fue precisamente la caracterización química del residuo, y para ello se analizaron 20 muestras provenientes de diferentes almazaras localizadas en diferentes provincias de España (Albacete, Córdoba, Cuenca, Granada, Jaén, Málaga, Murcia y Tarragona), durante sucesivas campañas incluidas en el periodo 1997-2000, aportando información estadísticamente significativa sobre la composición general del residuo.

Alburquerque et al. (2004) determinaron los siguientes parámetros: conductividad eléctrica y pH en extractos acuosos 1:10 (w/v), sólidos volátiles que reflejan el contenido en materia orgánica (OM) mediante pérdidas por ignición, contenido en nitrógeno total (NT) y carbono orgánico total (COT), contenido en P, K, Na, Ca, Mg, Fe, Cu, Mn, y Zn, y contenido total en grasas. En extractos acuosos 1:20 se analizó en contenido fenólico y en carbohidratos. Por último se determinó la concentración de lignina, celulosa y hemicelulosa. Los resultados obtenidos en estos análisis se muestran en las Tablas 1.2 y 1.3.

Tabla 1.2. Composición química del alpeorujo (n = 20). (Alburquerque et al. 2004)

Parámetro	Media	Rango	CV(%)
Humedad (% peso fresco)	64	55,6-74,5	7,6
pH	5,32	4,86-6,45	6,6
Conductividad (ds m^{-1})	3,42	0,88-4,76	33,9
Cenizas (g kg^{-1})	67,4	24,0-151,1	42,5
COT (g kg^{-1})	519,8	495,0-539,2	2,8
índice C/N	47,8	28,2-72,9	22,1
Nitrógeno Total (g kg-^{1})	11,4	7,0-18,4	24,5
P (g kg-^{1})	1,2	0,7-2,2	29,7
K (g kg-^{1})	19,8	7,7-29,7	34,2
Ca (g kg-^{1})	4,5	1,7-9,2	57,3
Mg (g kg-^{1})	1,7	0,7-3,8	58,7
Na (g kg-^{1})	0,8	0,5-1,6	36,6
Fe (mg kg-^{1})	614	78-1462	74,9
Cu (mg kg-^{1})	17	12,0-29,0	28,8
Mn (mg kg-^{1})	16	5,0-39,0	70,2
Zn (mg kg-^{1})	21	10,0-37,0	36,3

CV: coeficiente de variación

Tabla 1.3. Principales componentes de la fracción orgánica del alpeorujo (g kg^{-1} peso seco, n = 20), (Alburquerque et al. 2004).

Parámetro	Media	Rango	CV(%)
Materia orgánica total	932,6	848,9-976,0	3,1
Lignina	426,3 [45,8]	323,0-556,5	16
Hemicelulosa	350,8 [37,7]	273,0-415,8	12,7
Celulosa	193,6 [20,8]	140,2-249,0	14,8
Grasas	121,0 [13,0]	77,5-194,6	28,9
Proteínas	71,5 [7,7]	43,8-115,0	24,5
Carbohidratos (solubles)	95,8 [10,1]	12,9-164,0	50
Fenoles (solubles)	14,2 [1,5]	6,2-23,9	41

CV: coeficiente de variación

Los datos entre corchetes representan los porcentajes respecto al contenido orgánico total.

Como se aprecia en las dos tablas, el alpeorujo presenta un alto grado de humedad (mayor a 56%) que junto al pequeño tamaño de partícula lo hace un material poco poroso, plástico y con tendencia a compactarse, lo que supone un problema a la hora de transportarlo. Este material presenta también un pH ácido (media de 5.32). El alpeorujo es especialmente rico en K (19.8 g kg^{-1}),

como sucede con otros residuos procedentes de las almazaras. Sin embargo este residuo es pobre en P, Ca y Mg si se compara con residuos sólidos municipales (Alburquerque et al. 2004, -Cegarra et al. 1993-). El contenido en N (7.0-18.4 19.8 g kg^{-1}) es también bajo si se compara con otros residuos. Como micronutriente destaca el Fe (614 19.8 g kg^{-1}).

El alpeorujo tiene un alto contenido en materia orgánica (>93%) mayor que el del alpechín y el residuo seco de alpechín. Los mayores constituyentes de esta materia orgánica son lignina, hemicelulosa y celulosa que constituyen el 46%, 38% y 21% respectivamente del total de materia orgánica. Precisamente este alto contenido en lignina dificulta la degradación bacteriana del residuo (-Lynch et al. 1993-). Otros componentes del contenido orgánico son las grasas (13%), carbohidratos solubles (10%) y proteínas (cerca del 8%). El alto índice C/N (28.2-72.9) está por encima de los valores aconsejados para un eficiente compostaje de un residuo (25-35) (Alburquerque et al. 2004). El porcentaje en CFs solubles es de aproximadamente el 1.5% del contenido orgánico. No se detalla la composición de la fracción fenólica por métodos cromatográficos. Los autores de este estudio sobre la composición química del alpeorujo, consideran que las características químicas del residuo (alto contenido en CFs, en lípidos, elevado índice C/N, el pH ácido, etc) no son compatibles con su uso como abono orgánico.

Como se ha comentado, los residuos que se originan en la obtención del aceite de oliva son muy ricos en compuestos fenólicos, ya que durante el proceso de extracción de aceite, el 98% de los CFs presentes en el fruto pasan a formar parte de los residuos (Obied et al. 2005a). Servilli et al. (1999), estudiando los CFs durante un sistema de extracción en tres fases, compararon el perfil fenólico del la aceituna, el aceite obtenido, el alpechín y el orujo. Los resultados claramente mostraban divergencias importantes en la composición fenólica, ya que el perfil fenólico del fruto era muy simple, con una gran abundancia de secoiridoides y sus derivados. Sin embargo, en el alpechín aparecieron muchos más compuestos fenólicos y en concentraciones mayores que en el aceite de oliva. Este hecho se atribuye a el efecto de técnica de procesado y al hecho de que la mayoría de los CFs tienen un coeficiente de partición (aceite/agua) bajo,

del orden de 6 10^{-4} a 1.5 (Rodis et al. 2002), por lo cual tienden a concentrarse en los residuos (con elevado contenido en agua) y en menor medida en el aceite.

El contenido en CFs del alpeorujo es semejante en términos cualitativos al del alpechín. -Lesage-Meesen et al. (2001)- compararon el perfil fenólico de dos muestras de alpechín y alpeorujo mediante extracciones con acetato de etilo y su posterior análisis mediante HPLC. Hidroxitirosol y p-tirosol fueron los dos compuestos más abundantes, y también se detectaron otros CFs como los ácidos p-coumárico, cafeico, ferúlico y vainillico. El extracto fenólico del alpeorujo presentó mayor concentración en hidroxitirosol (1.16%) y una mayor actividad antioxidante que el extracto obtenido del alpechín. Es importante puntualizar que los resultados obtenidos en un análisis de los CFs pueden diferir notablemente según la metodología de extracción empleada. Obied et al. (2005b) dedujeron que las mezclas de metanol/agua más efectivas para extraer CFs del alpeorujo que otros disolventes como el acetato de etilo, etanol, propanol, acetona y acetonitrilo. Obtuvieron datos cuantitativos de los compuestos fenólicos más importantes del alpeorujo, entre los cuales seis de ello estaban presentes en más de 1g kg^{-1}: hidroxitirosol glucósido, hidroxitirosol, tirosol, verbascósido, y un derivado de la oleuropeína.

1.2.2. Contenido microbiano del residuo.

En bibliografía científica existe muy poca información sobre la microbiota característica del residuo alpeorujo, a pesar de la enorme importancia que esta microbiota puede tener en procesos de tratamiento tales como el compostaje, tratamientos anaerobios o la biorremediación natural en balsas. Además, el residuo podría servir de reservorio para especies potencialmente patógenas para el ser humano, plantas o animales que podrían verse afectados por una masiva acumulación de alpeorujo, problema que todavía no ha sido abordado. A continuación se comenta brevemente la información más relevante publicada hasta la fecha sobre la microbiota del alpeorujo. Es interesante también destacar que estos estudios se basan en técnicas dependientes de cultivo, a

pesar de la fuerte irrupción que se ha producido en los últimos años de las técnicas independientes de cultivo en el campo de la Ecología Microbiana.

La primera aproximación al estudio de la diversidad microbiana del alpeorujo se enfocó sobre la osmorregulación de bacterias aisladas del residuo (Jones et al. 2000). En este estudio se consiguió aislar seis grupos fenotípicamente distintos de bacterias Gram-positivas en muestras de alpeorujo procedentes de España y Grecia. Estos grupos presentaron diferentes tasas de crecimiento y respuestas osmoreguladoras distintas en condiciones de baja actividad del agua, estableciéndose una correlación entre la capacidad de crecer a baja actividad de agua (altas concentraciones salinas y de azúcares) y a crecer sobre el residuo. Se identificó uno de los aislados (1A) como cercano a *Bacillus amyloliquifaciens*, bacteria que presentó un atípico mecanismo de osmorregulación de adaptación a la alta concentración de materia orgánica del alpeorujo.

También se han estudiado cepas de levaduras aisladas de alpeorujo relacionadas con *Saccharomyces* sp., *Candida boidinii* y *Geotrichum candidum* (Giannoutsou et al. 2003).

Más recientemente se ha analizado mediante métodos moleculares (PCR-DGGE) la comunidad microbiana asociada a un proceso de tratamiento anaeróbico de alpeorujo en tanque de agitación continua (Rincón et al. 2006). Sin embargo en este caso el residuo fue inoculado con biomasa procedente de un proceso de digestión anaerobia de alpechín, no correspondiéndose por tanto con la "microbiota indígena" o "propia" del alpeorujo. Entre los grupos microbianos predominantes en el bioreactor destacó Firmicutes (principalmente representado por Clostridiales), seguido por Chloroflexi y Gamma-Proteobacteria (especialmente *Pseudomonas*). En cuanto al Dominio Archaea, el metanógeno *Methanosaeta concilii* fue la especie más representativa.

Por último, Ntougias et al. (2006) han publicado un artículo en el cual se describe de forma detallada aspectos relacionados con la ecofisiología y la filogenia de bacterias aisladas del alpeorujo "alcalino". Este residuo se obtiene

tras un tratamiento del alpeorujo convencional con $Ca(OH)_2$ para controlar la emisión de olores, con lo cual el residuo analizado fue un medio alcalino del que se aislaron una gran diversidad de bacterias alcalinotolerantes y alcalófilas, pertenecientes a géneros como *Bacillus*, *Idiomarina*, *Halomonas*, *Nesterenkonia*, *Corynebacterium*, *Serratia* y *Pseudomonas*.

1. 3. SOLUCIONES PROPUESTAS PARA LA GESTIÓN DE LOS RESIDUOS.

Desde hace más de cinco décadas se han realizado esfuerzos para buscar una solución a los residuos de la industria del aceite de oliva (Fiestas y Borja, 1992). Se han estudiado diferentes tipos de procesos, los cuales se pueden clasificar en dos categorías generales:

1. procesos de detoxificación
a) procesos físicos
b) procesos térmicos
c) procesos físico-químicos
d) procesos biológicos
e) combinación de procesos

2. reciclado y recuperación de componentes valiosos

Ninguna de las técnicas de detoxificación por sí solas permite resolver el problema de la deposición de estos residuos de una forma completa y efectiva, tanto desde un punto de vista económico como ecológico (Niaounakis y Halvadakis 2004). La industria todavía no se ha decantado por una solución global a gran escala. Esto puede ser debido a los grandes costos requeridos en investigación e inversión en las instalaciones, el corto periodo del año en el cual se producen los residuos (3-5 meses) y el pequeño tamaño de la mayoría de las almazaras, además de su gran dispersión regional (Boari et al. 1984).

La doble naturaleza de los residuos del aceite de oliva -vertido contaminante y recurso que debe ser reciclado-, provoca antagonismos entre grupos de investigación pertenecientes a ramas diferentes, debido a sus distintos puntos de vista sobre este tópico.

Las peculiaridades del alpechín que hacen que sea un problema de solución compleja pueden resumirse en:

a) alta carga orgánica (el alpechín está considerado como un residuo "fuerte" en este sentido, con valores de carbono orgánico disuelto (COD) de hasta 220 g/l);
b) naturaleza estacional de la producción, la cual requiere el almacenamiento del alpechín (muchas veces imposible en almazaras de pequeño tamaño);
c) alta dispersión regional de las almazaras;
d) pequeño tamaño de la mayoría de las almazaras, en las cuales los costes del tratamiento del alpechín no están debidamente integrados en la gestión económica;
e) presencia de compuestos orgánicos difíciles de degradar por microorganismos (como ácidos grasos de cadena larga y compuestos fenólicos);
f) alto porcentaje de sales minerales disueltas y sólidos en suspensión.

El alpeorujo presenta la mayoría de estas características a las que hay que sumar el hecho la consistencia pastosa y de difícil manipulación. A continuación se resaltan los aspectos más significativos de la investigación llevada a cabo en este tema.

1.3.1. Últimos avances en el tratamiento del alpechín.

En los últimos años, se han propuesto diferentes estrategias para el tratamiento y valorización del alpechín. La mayoría de estos métodos buscan la reducción de su fitotoxicidad con el objetivo de reutilizar el vertido para la agricultura, aunque como veremos también se han propuesto otros nuevos enfoques.

1.3.1.1. Evaporación.

En la práctica, el método de eliminación de alpechín mas empleado es almacenarlo en balsas de evaporación debido a la baja inversión requerida y a las condiciones climáticas favorables en los países mediterráneos. Si embargo este método precisa de grandes áreas y produce diversos problemas como mal olor, infiltraciones y proliferación de insectos (Roig et al. 2006).

La evaporación del alpechín produce fangos. La mayoría del fango producido en las balsas de evaporación se vierte en “landfill sites”, aunque puede ser utilizado en la agricultura o como biocombustible (Vitolo et al. 1999). La mayoría de los estudios sobre valorización de este residuo se centran en el compostaje. Por ejemplo, se ha conseguido obtener un buen abono mediante un compostaje combinado del residuo sólido de alpechín y residuos provenientes del cultivo del algodón (Paredes et. al 2002).

1.3.1.2. Aplicación directa al suelo.

Algunos autores han aplicado alpechín directamente al suelo y han comprobado sus efectos como fertilizante, que como hemos comentado en esta introducción, pueden ser positivos o negativos. Los efectos positivos están relacionados con la alta concentración de nutrientes, especialmente potasio, y con su potencial para movilizar iones del suelo; los efectos negativos, están asociados con el alto contenido en sales minerales, el bajo pH y la presencia de compuestos fitotóxicos, especialmente los polifenoles (Paredes et al. 1999). Dado que el alpechín puede reducir la movilidad de ciertos compuestos en el suelo, se ha sugerido su uso para atenuar la infiltración de de herbicidas en tierras cultivadas (-Cox et al. 1999-). Por su capacidad antimicrobiana, Kotsou et al. (2004) han experimentado con la aplicación de alpechín previa a las cosechas con objeto de inhibir al patógeno de plantas *Rhizoctonia solani*.

1.3.1.3. Tratamientos físico-químicos.

Este tipo de tratamientos consisten en la adición de sustancias químicas capaces de producir la precipitación, coagulación o destrucción de compuestos orgánicos disueltos. Por ejemplo se ha empleado hidróxido de calcio y sulfato de aluminio para reducir los valores de demanda química de oxígeno al 20-30% de los valores iniciales (Tsonis et al. 1989). Otra tecnología desarrollada consiste en la floculación de los compuestos orgánicos por medio del empleo de polielectrolitos, proceso que produce agua que puede ser utilizada para la irrigación de cultivos, y la fracción sólida del alpechín, que se puede compostar

con otros residuos agrícolas (García-Gómez et al. 2003). Otras técnicas novedosas como degradación fotoquímica de fenoles (-Cermola et al. 2004-) y electrocoagulación (-Adhoum y Monser, 2004) también han sido ensayadas.

1.3.1.4. Transformaciones biotecnológicas.

Tratamientos microbiológicos.

Algunos autores han desarrollado tratamientos microbiológicos para la producción de diversos polímeros microbianos a partir de alpechín, como xantano (López y Ramos-Cormenzana, 1996), pululano (Ramos-Cormenzana et al. 1995) y polihidroxialcanoatos (González-López et al., 1995).

También se han empleado tratamientos aeróbicos con microorganismos conducentes a disminuir la toxicidad del alpechín. Estos estudios se centran en la degradación de los polifenoles, los mayores responsables de la fitotoxicidad. Se han empleado diversos organismos tales como el hongo *Pleurotus ostreatus* (Tomati et al. 1991), la bacteria *Bacillus pumilus* (Ramos-Cormenzana 1996) y la levadura *Yarrowia lipolytica* (Scioli y Vollaro, 1997).

También se ha propuesto el uso del alpechín como substrato de crecimiento para la bacteria fijadora de nitrógeno *Azotobacter vinelandii* y la posterior aplicación del efluente obtenido como fertilizante (Piperidou et al. 2000).

Compostaje.

El compostaje es una de las principales tecnologías para el tratamiento del alpechín y su posterior tratamiento como fertilizante (Roig et al. 2006). Este proceso permite el retorno de nutrientes al área de cultivo. Además, el compostaje evita algunos efectos nocivos del alpechín derivados de su aplicación directa.

Para que se produzca un compostaje efectivo, el alpechín se debe absorber a substratos que permitan obtener una textura adecuada para el proceso, como

por ejemplo paja o residuos agrícolas. De esta forma se ha conseguido compostar el alpechín obteniendo un producto fertilizante con alto grado de humificación y sin fitotoxicidad (Tomati et al. 1995). El alpechín se puede compostar con éxito junto a diferentes residuos vegetales (Paredes et al. 2002).

Cegarra et al. (1996) han conseguido aplicar compost obtenido a partir de alpechín como fertilizante de cultivos de hortalizas, obteniendo rendimientos equiparables a los conseguidos con fertilizantes minerales y en algunos casos superiores.

Digestión anaerobia.

El principal interés de este tratamiento radica en la producción de energía (biogas) y en el potencial uso del efluente en irrigación (Marques, 2001). Una limitación importante es la inhibición de las bacterias metanógenas debido a los polifenoles y ácidos orgánicos (Hamdi, 1996). Filidei et al. (2003) han propuesto un pretratamiento de sedimentación-filtración como paso previo al tratamiento anaerobio del alpechín.

Extracción de compuestos valiosos.

Algunas sustancias presentes en el alpechín, como los compuestos antioxidantes, tienen interés en la industria cosmética y farmacéutica. Entre estos compuestos destacan polifenoles como el hidroxitirosol y oleuropeína. Se han propuesto diversos sistemas para la extracción de estos compuestos como el uso de enzimas hipertermófilas (Briante et al. 2004), o procesos de centrifugación-ultrafiltración (Turano et al. 2002) que permiten la reducción de la polución de forma simultánea a la extracción de compuestos útiles (lípidos, azúcares, polifenoles).

1.3.2. Tratamientos propuestos para el alpeorujo.

El alpeorujo, debido a su alto grado de humedad (ya que incluye las aguas de vegetación que en el sistema de tres fases constituían parte del alpechín),

presenta serios problemas para las almazaras ya que se requiere maquinaria específica para trasladarlo, almacenarlo, etc. El reducido beneficio de la extracción del aceite residual del alpeorujo en relación al orujo de tres fases, ha motivado que se investiguen más a fondo las posibles vías de valorización de este residuo.

1.3.2.1. Tratamientos físico-químicos.

Secado y segunda extracción de aceite.

En un primer momento se pensó que la mejor solución para revalorizar el alpeorujo era su secado para su posterior extracción de aceite residual mediante el uso de disolventes orgánicos (generalmente hexano). No obstante, el alto contenido en humedad causó problemas técnicos durante le proceso de secado en las tradicionales extractoras de orujo. Estos problemas motivaron el diseño de nuevos procesos ajustados a las características específicas del alpeorujo. Arjona et al. (1999) desarrollaron un procedimiento útil para determinar las condiciones operativas para el secado efectivo del alpeorujo. Krokida et al. (2002) propusieron el diseño de secador rotatorio a nivel industrial y discutieron su rentabilidad económica. Posteriormente, Arjona et al. (2005) desarrollaron un sistema de automatización que aumenta la automatización del proceso y mejora la eficiencia energética.

Valorización energética.

Tras la segunda extracción, el alpeorujo extractado normalmente se utiliza como combustible en las propias plantas extractoras para la producción de calor o electricidad (Caputo et al. 2003). Este método se utiliza en la mayoría de las extractoras debido a que el alpeorujo extractado tiene un elevado poder calorífico (400kcal/kg). Sin embargo, la mayoría de la energía obtenida se utiliza para el secado del alpeorujo fresco, por lo tanto el rendimiento final es bajo (Azbar et al., 2004). También se está exportando alpeorujo extractado al Reino Unido, donde es utilizado para alimentar calderas de los sistemas de calefacción (Dr. Juan Antonio Mata, *com. pers.*).

Un nuevo sistema propuesto para el alpeorujo seco consiste en la producción de "singas", una mezcla de CO y H_2 que se utiliza para la obtención de importantes compuestos químicos con NH_3 o CH_3OH y para la preparación de fuel (Jurado et al., 2003).

1.3.2.2. Aplicación directa al suelo.

Las ventajas del posible uso directo del alpeorujo como abono orgánico son su alto contenido en potasio, su bajo valor económico y que se produce cerca de zonas agrarias por lo cual no es necesario transportarlo a grandes distancias. Sin embargo, diferentes experiencias han demostrado que, si bien es menos fitotóxico que el alpechín, su aplicación directa provoca una pérdida del equilibrio nutricional del suelo debido al alto índice C/N (Thompson y Nogales, 1999), por lo que debe ser aplicado junto a una fuente de nitrógeno. Saviozzi et al. (2001) evaluaron la posibilidad de utilizar alpeorujo como aditivo de suelos junto a nitrógeno mineral, y concluyeron que la mineralización dependía del tipo de suelo utilizado, existiendo problemas en suelos ácidos.

1.3.2.3. Valorización biotecnológica.

Digestión anaerobia.

La digestión anaerobia de residuos orgánicos permite la obtención de biogás (una mezcla de CH_4 y CO_2) y materia orgánica parcialmente estabilizada. El biogás se puede utilizar para obtener energía y la materia orgánica se puede emplear como abono. Tekin y Dalgiç (2000) produjeron biogás a partir de orujo con un contenido del 75-80% de CH_4. -Borja et al.- Obtuvieron biogás a partir de alpeorujo pero destacaron el alto contenido en polifenoles como factor limitante.

Fermentación en estado sólido.

Una de las posibles salidas al alpeorujo podría ser utilizarlo para alimentar del ganado. Sin embargo el alto contenido en fibras no digeribles así como el bajo contenido proteico, especialmente en lisina, obligan a la suplementación con proteínas (Molina Alcaide et al., 2003).

La fermentación en estado sólido es un tratamiento factible para aumentar el valor nutricional de los residuos vegetales. Este proceso, desarrollado por microorganismos en estado sólido, ha sido explotado con éxito para la producción de piensos, combustibles y enzimas. Haddadin et al. (1999) consiguieron aumentar el contenido proteico del orujo de 5.9% a 40.3% mediante este proceso.

Compostaje.

El co-compostaje del alpeorujo con otros residuos agrícolas ha sido estudiado por diversos autores. Dado que se trata de un residuo con una textura pastosa que difícilmente deja pasar el aire, se debe mezclar con agentes como paja u otros residuos vegetales que permitan obtener una textura adecuada para el proceso de compostaje. Algunos materiales como paja (Madejón et al. 1998), residuos de algodón y virutas de madera (Filippi et al., 2002) han dado buenos resultados. En todos los casos el producto final muestra un buen grado de humificación, se eliminan los efectos fitotóxicos y se consigue un buen aporte de nutrientes minerales. Esta vía de valorización del residuo constituye una alternativa válida a la combustión.

Las características del alpeorujo dificultan su compostaje mediante aireación forzada, debido a la generación de vías preferenciales de flujo de aire que secan el material formando agregados (Cayuela, 2004); por lo tanto se ha aconsejado el volteado mecánico de las pilas de compost para airear la mezcla (Baeta-Hall et al., 2005).

Los residuos de la producción de aceite de oliva pueden tener un papel relevante en la agricultura ecológica (pie de página: sistema agrario cuyo objetivo fundamental es la obtención de alimentos de la máxima calidad

conservando la fertilidad de la tierra y respetando el medio ambiente, mediante la utilización óptima de los recursos naturales y sin emplear productos químicos de síntesis, ni organismos genéticamente modificados). Cayuela et al. (2004) propusieron el compostaje industrial con abono en el área cercana a la almazara y el cultivo como un buen método para revalorizar el alpeorujo.

El compost obtenido a partir de residuos del aceite de oliva se puede utilizar como abono en cultivos de hortalizas. No obstante, los altos valores de pH obtenidos durante el proceso de compostaje representan una importante limitación para su aplicación en suelos. Para resolver este problema se puede utilizar azufre elemental que mantiene el pH dentro de límites aceptables (Roig et al. 2004).

1.3.2.4. Extracción de compuestos valiosos.

El alpeorujo puede ser explotado como substrato de bajo coste para la obtención de diversos tipos de compuestos orgánicos valiosos. Entre estos destacan los compuestos fenólicos, que presentan un amplio rango de actividades biológicas (Obied et al. 2005). También se ha estudiado la extracción de pectinas a partir de alpeorujo. Estas substancias son muy abundantes en todo el reino vegetal, y pueden ser obtenidas en cantidades de las peladuras de cítricos y de restos de manzanas. No obstante existe una intensa búsqueda de nuevas fuentes de pectinas, especialmente de residuos. Cardoso et al. (2003) han obtenido resultados positivos en un estudio de viabilidad sobre la extracción de pectinas a partir de alpeorujo.

1.3.2.5. Otras propuestas de valorización del alpeorujo.

El alpeorujo podría ser utilizado como adsorbente de metales pesados con el objetivo de depurar efluentes contaminados (Pagnanelli et al., 2002). También se ha propuesto su aplicación al suelo con el objetivo de extender la adsorción de herbicidas (simazina) e insecticidas (imidacloprida), reducir su biodegradación y disminuir la infiltración, reduciendo el riesgo de contaminación de acuíferos subterráneos (Albarrán et al., 2004; Cox et al., 2004). Siracusa et

al. (2001) estudiaron la posibilidad de reciclar el alpeorujo con termoplásticos para producir nuevos materiales en la producción de contenedores.

1.4. OBJETIVOS.

Los objetivos de esta investigación son los siguientes:

1. Estudiar el efecto de la adición de nitrógeno y fósforo en el proceso bioremediación del alpeorujo en bioreactores a escala de laboratorio.

2. Caracterizar las comunidades microbianas presentes en el proceso de bioremediación experimental mediante técnicas independientes de cultivo (TTGE Y PLFA).

3. Optimizar la producción de exopolisacárido por *Paenibacillus jamilae* utilizando alpeorujo como única fuente de carbono y energía.

4. Estudiar la interacción del exopolisacárido por *Paenibacillus jamilae* con metales pesados (bioadsorción).

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Capítulo 2

Molecular microbial and chemical investigation of the bioremediation of two-phase olive-mill waste using laboratory-scale bioreactors

2.1. ABSTRACT.

Two-phase olive-mill waste (TPOMW) is a semi-solid effluent that is rich in contaminating polyphenols and is produced in large amounts by the industry of olive oil production. Laboratory-scale bioreactors were used to investigate the (bio)degradation of TPOMW by its indigenous microbiota. The effect of nutrient addition (inorganic N and P) and aeration of the bioreactors was studied. Microbial changes were investigated by PCR-temperature time gradient electrophoresis (TTGE) and following the dynamics of polar lipid fatty acids (PLFA). The greatest decrease in the polyphenolic and organic matter contents of bioreactors, was concomitant with an increase in the PLFA fungal/bacterial ratio. Amplicon sequences of nuclear ribosomal internal transcribed spacer region (ITS) and16S rDNA allowed identification of fungal and bacterial types respectively by comparative DNA sequence analyses. Predominant fungi identified included members of the genera *Penicillium*, *Candida*, *Geotrichum*, *Pichia*, *Cladosporium* and *Aschochyta.* A total of 14 bacterial genera were detected, with a dominance of organisms that have previously been associated with plant material. Most sequences were generally more than 97 % identical to those present in the databases. This is, to our knowledge, the first report of microbial community analysis of TPOMW bioremediation through the use of molecular biological techniques.

Overall, this work highlights that indigenous microbiota within the bioreactors through stimulation of the fungal fraction, is able to degrade the polyphenolic content without the inoculation of specific microorganisms.

2.2. INTRODUCTION.

The olive-oil industry generates large amounts of by-products that are harmful to the environment. Olive-mill wastes (OMWs) contain phytotoxic components capable of inhibiting microbial growth (Capasso et al. 1995, Ramos-Cormenzana et al. 1996) and the germination and vegetative growth of plants (Linares et al. 2003). Traditionally, the disposal of OMWs has become a great problem in Mediterranean countries, because of their polluting effects on soil and water (Sierra et al. 2001, Piotrowska et al. 2006). The production of olive oil is increasing worldwide, with a growth rate that is expected to be between 3.5 and 4% per year, according to the International Olive Oil Council. Moreover, this production is no longer restricted to the Mediterranean Basin, and new producers like Australia, USA and South American countries will have to face the environmental problems posed by OMWs.

At present, three systems are used worldwide for the industrial-scale extraction of oil from olives, *viz.* the traditional press-cake system, the three-phase decanter system and the modern two-phase centrifugation system. The present paper focuses on the two-phase centrifugation system, which was introduced in the 1990s as an ecological approach for olive oil production, since it drastically reduces the water consumption during the process. The waste-stream of this system consists of a semi-solid waste: the two-phase olive mill waste (TPOMW) or alpeorujo. The resulting waste comprises about 800 kg per 1000 kg of the processed olives, and its production may exceed 4 million tons annually in Spain alone (Aragón et al. 2001, Alburquerque et al. 2004). The TPOMW consists of a thick sludge that contains water and pieces of pit plus the pulp of the olive fruit. This semi-solid effluent has a water content of about 65% (Arjona et al. 2004), a slightly acidic pH, and a very high content of organic matter, mainly composed of lignin, hemicellulose and cellulose. It also has a considerable proportion of fats, proteins, water-soluble carbohydrates and a small but active fraction of hydrosoluble (poly)phenolic substances (Alburquerque et al. 2004).

The polyphenol content of olive residues consists of a complex mixture of compounds and it is considered to be the most problematic fraction of OMWs,

being associated with the well-known antimicrobial and phytotoxic effects of these residues. Bioremediation is a valuable tool for the detoxification of TPOMW by breaking down these phenolic compounds. Recent research on TPOMW bioremediation has included different approaches, such as anaerobic digestion (Borja et al. 2005, Rincón et a. 2006), natural biodegradation in evaporation ponds (Borja et al. 2006), removal of phenols by saprophytic fungi (Sampedro et al. 2004), biodegradation by co-composting with agricultural wastes (Paredes et al. 2002), vermicomposting (Benitez et al. 2005) and the production of metal-binding microbial exopolysaccharides (Morillo et al. 2006).

Despite the importance of bioremediation as an alternative to TPOMW detoxification and re-cycling, surprisingly little information is available on the indigenous microbiota of the residue and their potential for carrying out biodegradation of the waste. In a previous paper, we described six phenotipically distinct groups of bacteria that had been isolated from TPOMW. They were subsequently studied in order to characterize their growth on the waste and their osmoregulatory responses: with the exception of three isolates, the isolated bacteria were Gram-positive spore-forming organisms, belonging mainly to the genus *Bacillus* (Jones et al.2002). Ntougias et al. (2006) reported the isolation of a group of alkaliphilic and alkalitolerant bacteria from TPOMW treated with $Ca(OH)_2$. Cultivatable yeast diversity of TPOMW was studied in a fed-batch microcosm by Giannoutsou et al. (2004), who found three strains belonging to the genus *Saccharomyces*, *Candida* and *Geotrichum*, which could grow in TPOMW.

To our knowledge, the only detailed molecular characterization of the microbial communities of TPOMW is that of Rincón et al. (2006). They studied the anaerobic treatment of diluted TPOMW in a continuous-stirred tank bioreactor at laboratory-scale. The bioreactor was inoculated with methanogenically-active biomass from a different source. Molecular identification of the microbial species involved in the process was performed by PCR amplification of the 16S r RNA gene and denaturing gradient gel electrophoresis (DGGE). The Firmicutes (mainly represented by Clostridiales) were the most abundant

phylotype, followed by the Chloroflexi and the Gamma-Proteobacteria (*Pseudomonas* species) (Rincón et al. 2006).

The aim of the present investigation was twofold. First, to carry out an evaluation of the addition of inorganic nutrients and aeration on the bioremediation of TPOMW at the laboratory scale. Second, to perform a molecular analysis of the indigenous microbial communities of TPOMW (bacteria and fungi), and how they change in response to the different treatments applied of nutrient supplementation and aeration.

2.3. MATERIAL AND METHODS.

Two-phase olive-mill waste. The two-phase olive-mill waste (TPOMW) was obtained from the factory "Aceites Jimena S.A." (Granada, Spain), collected in sterile plastic containers and stored at -20ºC. The sample was dark brown in colour and had a smooth dough-like consistency, with a high content of water (56%) and organic matter (41.14%), slightly acidic pH (5.53) and a high C:N ratio (40.77 mol:mol).

Experimental design and sampling. Four experimental conditions were tested in triplicate using 63 in-vessel bioreactors over 55 days. The bioreactors were constructed from hardened glass jars with plastic lids modified with closeable inlet and outlet ports for aeration via stainless steel tubes when required. For sampling, the plastic lids could be unscrewed. Each glass bioreactor had a volume of approximately 200 ml, so that the waste mixture (see below) filled the bioreactor to about two-thirds of its capacity. The air inlet tube penetrated the full depth of the waste material to the bottom of the glass jar, whereas the air outlet tube ended above the surface of the waste. The conditions were: non-aerated (OA), non-aerated + nutrients (OAN), aerated (AA) and aerated + nutrients (AAN). For the aerated treatments (AA and AAN), the bioreactor units stood vertically with air flowing continuously to avoid oxygen content limitation and vented outdoors. Airflow to the mixture of TPOMW and straw via the stainless steel tube was provided by 100% oil-free diaphragm pumps (Model PXW-600-DIOV, VP1, 5 l min^{-1}, Fisher Scientific). The design of

the non-aerated bioreactors (OA and OAN) was exactly the same, except that they were not aerated. These laboratory-scale bioreactors each held 35 g of a mixture of TPOMW/water/straw (15:18:2.4, by wt). Temperature was kept constant at 25ºC and the initial moisture content was 65%. The water content was measured after 0, 7, 14, 21, 35 and 55 days. In the aerated bioreactors, the air inlet was bubbled through a water reservoir to avoid excessive water evaporation during aeration. The cylindrical bioreactor design permitted a better distribution of the air flow, preventing the creation of anaerobic pockets in the mixture. In the two treatments with nutrient addition, the mixture in each bioreactor was supplemented at the beginning of the experiment with 1g of nitrogen and 50 mg of phosphorus, using NH_4NO_3 and KH_2PO_4, respectively.

Destructive sampling, in triplicate, for each bioreactor mixture was done after 0, 7, 14, 21, 35 and 55 days. The contents of each bioreactor were thoroughly mixed in a 500-ml beaker, and three subsamples were collected (A, B and C). Subsample A was used to measure moisture content, ash content and pH. These three parameters were analysed on the same day of the sampling. Subsamples B and C were kept frozen (-20ºC) in sterile containers for subsequent polyphenol content determinations and microbial community analysis by PLFA and PCR-TTGE.

Determination of moisture content and total organic matter. Total organic matter (TOM) was determined by ashing using a loss-on-ignition procedure. Triplicate 2 g samples were dried for 24h at 110ºC and reweighed to give the moisture content. The dried samples were transferred to a muffle furnace held at 550ºC for 12h. Ash content was calculated from the ratio of pre-ignition and post-ignition sample weights. The TOM losses were calculated as described previously (Alburquerque et al.2006). According to the equation of Stentiford and Pereira Neto (1985), and taking into account the apparent increase in the ash content resulting from the loss of dry matter, the organic matter losses during bioremediation can be determined using the formula:

$$\text{OM-loss (\%)} = 100 \times [(TOM_i / Ash_i - TOM_t / Ash_t) / (TOM_i / Ash_i)],$$

where TOM_i and TOM_t are the organic matter contents of the initial material and at time t, respectively, and Ash_i and Ash_t are the ash contents initially and at

time *t*¸ respectively. The residual moisture of the samples was determined to produce results on a dry matter basis (110ºC).

Polyphenol analysis. Extraction of polyphenolic substances from the mixture was performed as follows. Approximately 1 g of sample was weighed and extracted with 20 ml of methanol:water (20:80, v/v) using an orbital shaker (150 rpm) for 2 hours at 25ºC. A modification of the Folin–Ciocalteu reagent assay (Maestro-Durán et al. 1991) was used to determine the total phenolic content. All the assays were performed in triplicate. An aliquot (1 ml) of each extract was centrifuged at 3000 rpm for 5 minutes, and 500 µl of the supernatant was mixed with 2.5 ml of Folin–Ciocalteu reagent (Sigma). The mixture was allowed to stand for 5 min at 25ºC before adding 2.5 ml of 20% (w/v) sodium carbonate solution and distilled water to a final volume of 25 ml. Absorbance at 725 nm was measured after 1 hour. The calibration curve was constructed using caffeic acid as standard solution (0-60 mg/l), and total amount of phenolic compounds was calculated and expressed as caffeic acid-equivalent, CAE (mg/g).

Polar lipid fatty acid (PLFA) analysis of the microbial community. Polar lipids were extracted from mixture samples (1 g) using a modified Bligh and Dyer method as described by Kates (1985) and their fatty acyl chains converted to fatty acid methyl esters (FAMEs) by transmethylation using 2.5% (v/v) sulphuric acid in dry methanol (Kates 1985).

The FAMEs were analyzed by GC-MS using a Hewlett Packard 6890 series gas chromatograph and a 7673 series auto-sampler and a 5973 series mass selective detector. The FAMES were separated on a HP-5MS capillary GC column (Supelco Inc, length 30 m, int. diam. 0.25 mm, film thickness 0.25 (m, using the following temperature program: 40ºC for 3 min, 10 centigrade degrees per minute to 150ºC, 3 centigrade degrees per minute to 230ºC, and 30 centigrade degrees per minute to 300ºC, which was maintained for 5 min to allow late-eluting peaks to exit the column. The MS was operated in the selective ion monitoring (SIM) mode, using m/z = 74 as the common fragment ion of FAME. To identify the fatty acids, their retention times were compared

with those obtained for standard bacterial acid methyl esters (Cat. No. 47080-U, Supelco, UK). The sum of the following fatty acids was used to represent total bacteria: i15:0, a15:0, i16:0, i17:0, cy17:0, 18:1ω7c and cy19:0 (19). Gram-positive bacteria were represented by i15:0, a15:0 and i17:0 (13) and Gram-negative bacteria by cy17:0, 18:1ω7c and cy19:0 (23). Thermophilic bacteria (largely thermotolerant bacilli) were represented by i15:0 and i17:0 (14). Fungi were represented by 18:2ω6,9 (19).

Molecular characterization of the microbial community. (i) DNA isolation and 16S rRNA gene (bacterial) and ITS2 (fungal) PCR amplification. Total DNA was isolated from the samples employing the PowerSoilTM DNA isolation kit (MoBio Laboratorioes, Inc.) following the manufacturer´s instructions. Following electrophoresis on a 1% agarose gel containing ethidium bromide, the integrity of bands was assessed visually and the concentration of nucleic acids measured spectrophotometrically. Primers U968-GC (5´-CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5´-GCG TGT GTA CAA GAC CC) were used to amplify the V6-V8 region of the bacterial 16S rRNA gene, giving a product of 450 bp. The ITS2 region of fungal genomic DNA was amplified with the primer pair ITS3-GC (5´-CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGC ATC GAT GAA GAA CGC AGC) and ITS4 (5´-TCC TCC GCT TAT TGA TAT GC), resulting in 370 bp product (Nikolcheva et al. 2005, White et al. 1990).

PCR was performed using Hot Star Taq DNA polymerase (QIAGEN, Courtaboeuf, France). PCR mixtures (25 µl) contained the following: 1 x PCR buffer, 1.5 mM MgCl2, 0.1 mM of each dNTP, 0.5 µM of primers, 2.5 U of Hot Star Taq polymerase, and approximately 1 ng of DNA. The DNA fragments were amplified using a GeneAmp PCR System 2700 (Applied Biosystem, Singapore). The following program was used with the primers U968-GC and L-1401: 95°C for 15 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min 30 s, and finally 72°C for 15 min. Amplification of genomic DNA with the primers ITS3-GC and ITS4 was performed with the following program: 94°C for 15 min; 35 cycles of 94°C for 30 s, 47°C for 1 min, and 72°C for 1 min, and

finally 72℃ for 10 min. Negative (without DNA) controls were used in every series of reaction. Amplicons were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide to check the correct size and concentration.

(ii) TTGE analysis of PCR amplicons. The Dcode Universal Mutation Detection System (Bio-Rad, Paris, France) was used for sequence-specific separation of PCR products. Electrophoresis was performed through a 1-mm-thick, 16 cm by 16 cm polyacrylamide gel (8% [w/v] acrylamide-bisacrylamide, 7 M urea, 1.25 x Tris-acetate-EDTA [TAE], 55 µl and 550 µl of Temed, and 10% ammonium persulfate) using 7 l of 1.25 x TAE as electrophoresis buffer. Electrophoresis was performed at a fixed voltage of 65 V for 969 min with an initial temperature of 66℃ and a ramp rate of 0.2 centigrade degrees per hour. For better resolution, the voltage was fixed at 20 V for 5 min at the beginning of electrophoresis. Each well was loaded with 100 to 200 ng of amplified DNA plus an equal volume of 2 x gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, and 70% glycerol). After completion of electrophoresis, the gel was stained in a SYBR Green I solution (Sigma-Aldrich, St. Quentin Fallavier, France), and destained in 1.25 x TAE. Gels were visualized under UV illumination using a gel image system (Kodak Gel Logic 100). Predominant bands were excised and treated for further analysis

(iii) Comparison of TTGE patterns. The TTGE gels were analyzed as previously described (Marie et al. 2006). Briefly, the digital images of the acrylamide gels were analyzed using the Cross-Checker software (available from from http://www.dpw.wau.nl/pv/pub/CrossCheck/). A matrix of the intersections between the lanes and the most intense bands was used to calculate distances using normalized Euclidian distances. Dendograms presenting the relationship within samples were computed (UPGMA) using the DDGEStat program (available from http://www.sb-roscoff.fr).

(iv) Sequence analysis. To perform sequence-based phylogenetic identification, specific bands were cut from the polyacrylamide gel. Gel fragments were washed once in 200 µl of PCR water and kept in 100 µl of PCR water overnight at 4℃ for diffusion. Both bacterial 16S rRNA and fungal ITS2

gene fragments were then amplified from the dialysate. The PCRs were performed in the same conditions as described above. The amplicons were purified using the GFX PCR DNA Purification Kit (Amershan Biosciences) and the size and concentration of the amplicons were evaluated on 1.5% agarose gels containing ethidium bromide. PCR products were sequenced with an Applied Biosystems 373A DNA sequencer by automated Sanger method. Similarity searches with sequences in the GenBank database were performed by using Blast algoritm.

(v) Nucleotide sequence accession numbers. The ITS (fungal) and rDNA (bacterial) amplicons sequences identified in each treatment have been included as supplementary material.

2.4. RESULTS

Chemical changes. The changes in concentration of polyphenols are shown in Figure1A. Generally, there was greater biodegradation of polyphenols during the first few days of the experiment (depending on the treatment) and thereafter their loss was slow. The greatest amount of biodegradation was in aerated bioreactors supplemented with nutrients, in which there was 36% and 54% losses after 7 and 55 d, respectively. There was no significant biodegradation in aerated bioreactors that were not supplemented with nutrients. The addition of nutrients stimulated the biodegradation of polyphenols during the first 7 d of treatment under both aerated and non-aerated conditions; however, after 55 d this stimulation was only apparent in the aerated bioreactors (Fig 1A). After 55 d of treatment in non-aerated bioreactors, there was 22% biodegradation of polyphenols, irrespective of whether or not they were supplemented with nutrients.

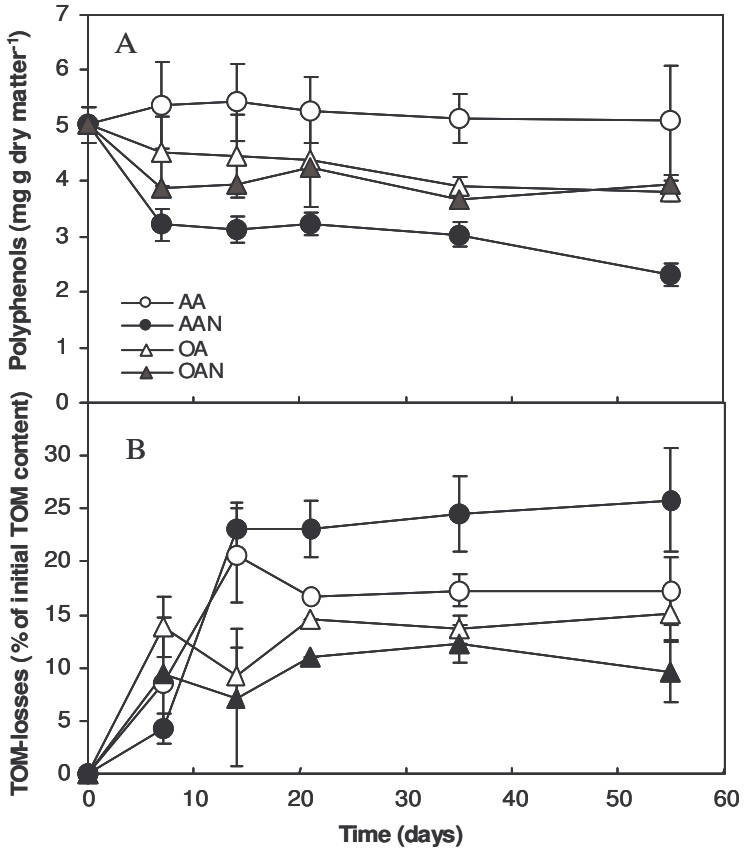

FIG.2.1. Polyphenol biodegradation (A) and organic matter losses (B). The data points are mean values of samplings from triplicate reactors (bars correspond to ± standard deviation) on the days shown. Symbols refer to the different treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN).

The bulk (about 95%) of the bioreactor material was organic matter, measured as total organic matter (TOM), and the losses over a 55 d incubation are shown in Figure 1B. Both aeration and nutrient addition influenced the rate of TOM losses. The addition of nutrient stimulated TOM loss in aerated bioreactors, but decreased TOM loss in non-aerated bioreactors. In all bioreactors the rate of loss of TOM was faster during the initial 14 d of incubation. Thereafter the rate of loss was slow. After 55 d of incubation, the loss of TOM was 10-25%, the greatest loss being in aerated bioreactors supplemented with nutrients (Fig 1B).

Microbial community structure.

(i) Phospholipid fatty acid (PLFA) analysis. The changes in relative amounts of fungal and bacterial biomasses in the bioreactors during a 55 d incubation are shown in Figure 2. The addition of nutrients increased the fungal/bacterial PLFA index (F/B index) in both aerated and non-aerated bioreactors, the greatest changes occurring during the initial 14 d of incubation, and generally being slow or static thereafter.

The increase in F/B index was greater in the supplemented aerated bioreactors, in which the presence of fungal biomass was sufficient to be seen as white mycelium by simple visual observation: this was not seen in any other bioreactor conditions, even in the nutrient-supplemented non-aerated bioreactors, which also displayed a steady rise in the F/B index throughout the 55 d incubation period (Fig 2). In contrast, in both aerated and non-aerated bioreactors without supplementation, there was a decrease in the F/B index. However, the kinetics of the decrease differed: in the aerated bioreactors the decrease occurred during the initial 7 d of incubation, in comparison with the non-aerated bioreactors in which it occurred during the latter half of the incubation period (Fig 2).

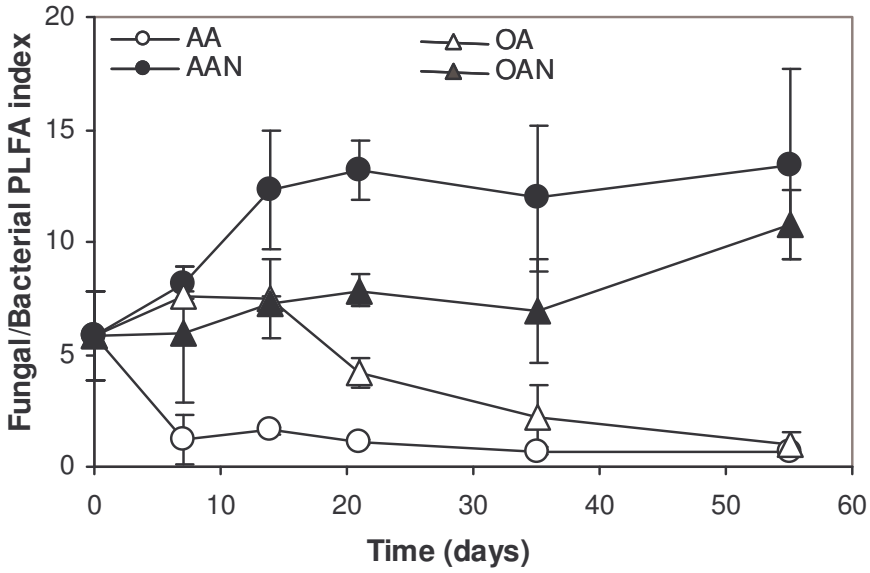

FIG.2.2. Time course of the change in fungal/bacterial PLFA index. The index was calculated as the ratio between fungal (18:2ω6,9) and bacterial (i15:0, a15:0, i16:0, i17:0, cy17:0, 18:1ω7c and cy19:0) fingerprints abundances. Each data point corresponds to the mean value for triplicate reactors (bars correspond to ± standard deviation). Symbols refer to the different treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN).

The relative proportions of Gram-negative and Gram-positive bacteria (G+/G- index) was also affected by aeration and nutrient supplementation, as shown in Figure 3. In general, the G+/G- index remained the same until after about 21 d of incubation, when its value increased. This effect was greater in bioreactors that were not supplemented with nutrients. The increase in G+/G- index was about 2-fold in aerated bioreactors, compared with up top 5-fold in non-aerated bioreactors. Although the increase in the aerated bioreactors was apparently less, it was maintained until 55 d, whereas in the non-aerated bioreactors it decreased back to the starting value.

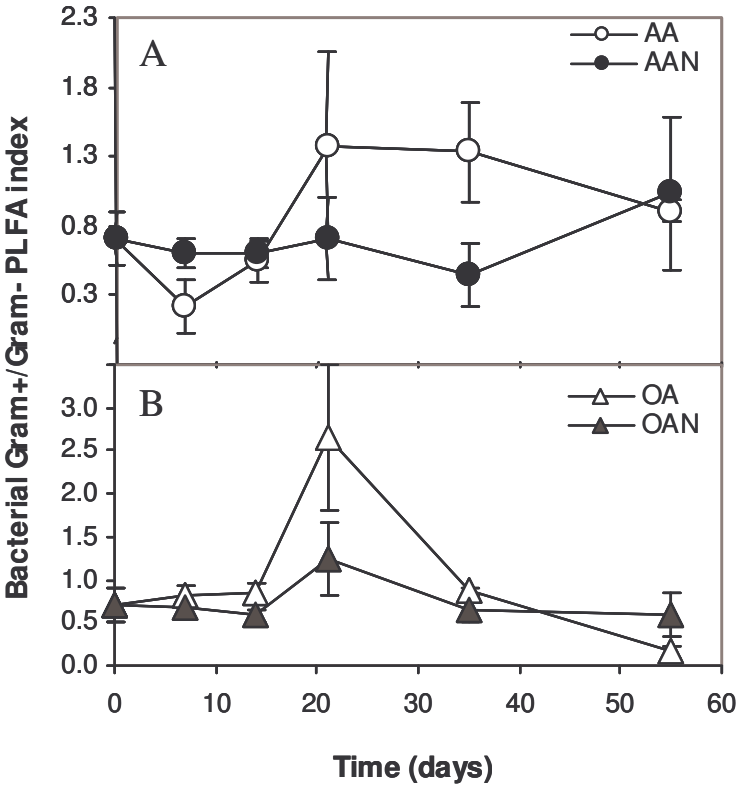

FIG.2.3. Time course of the change in the bacterial Gram+/Gram- PLFA index in aerated (A) and non-aerated (B) reactors. The index was calculated from the abundances of Gram+ bacterial PLFA (i15:0, a15:0 and i17:0) and Gram- (cy17:0, 18:1ω7c and cy19:0). Each data point corresponds to the mean value for triplicate reactors (bars correspond to ± standard deviation). Symbols refer to the different treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN).

(ii) TTGE analysis. Overall, the results of the comparative TTGE analyses revealed significant differences in bacterial and fungal communities when varying nutrient addition and aeration to the bioreactors (Figs. 4 and 5). These differences between the treatments were clear from the first week of the experiment (day 7), i.e. there was rapid establishment of the different microbial communities under all the conditions of nutrient status and aeration. Thereafter, the communities were stable, since their individual TTGE profiles did not change during the remaining 48 days of the experiment.

The greatest bacterial diversity, in terms of number of discrete bands, was detectable in OAN-bioreactors (14-17 bands per sample), followed by AA (6-15 bands), AAN (6-10 bands) and finally by OAN (4-5 bands). The TTGE profiles showed that the greatest fungal diversity was detected in OAN-bioreactors (14-17 bands), followed by AAN (10-13 bands) and AA (7-9 bands). Thus, the complexity of the microbial communities did not seem to be linked with the degradation of polyphenols. For instance, the maximal polyphenol degradation was detected in AAN-bioreactors, but the most complex communities, in terms of number of TTGE bands, was found in OAN-bioreactors. Therefore, these results show that only a few specific microorganisms, which were stimulated with nutrients and oxygen supply, were responsible for the greatest degradation of polyphenols.

As expected, in both bacterial and fungal TTGE analyses, samples from the same treatment clustered together (Fig. 6). No PCR product was obtained from any of the OA samples using fungi primers. In the cluster analysis of bacterial TTGE, two major groups could be observed, one corresponding to the AAN-bioreactors and the other to the rest of the samples. In contrast, in the fungal TTGE analyses, profiles from the OAN treatment clustered apart from profiles of aerated bioreactors. Moreover, TTGE profiles seemed to be independent of the incubation time. If we observe the cluster analysis at a finer level, TTGE samples within each treatment clustered without an apparent order based on the sequence of the sampling. It is important to note that, despite the high similarity of profiles that belonged to the same treatment (for bacteria and fungi analysis), each lane of the TTGE gels was obtained from DNA isolated from a different (independent) bioreactor.

To perform sequence-based phylogenetic identification, a sequence analysis of specific bands was performed. Twenty newly-determined sequences were compared directly with those in GenBank using BlastN. Database similarity is shown in Table 1. A total of 14 different genera of bacteria were detected, belonging to Gammaproteobacteria (5), Actinobacteria (4) and Firmicultes (5). Only two sequences were obtained from the samples of the initial mixture (day 0), both corresponding to the genus *Bacillus*.

In fungal TTGE gels, a total of 14 sequences were successfully obtained (Table 2). BLAST analysis confirmed the specificity of the primers used, as all sequences could be affiliated to fungal genera in more than 97% similarity. In total, 6 genera were identified, all of then belonging to *Ascomycota*, namely *Penicillium*, *Candida*, *Geotrichum*, *Pichia*, *Cladosporium* and *Aschochyta*.

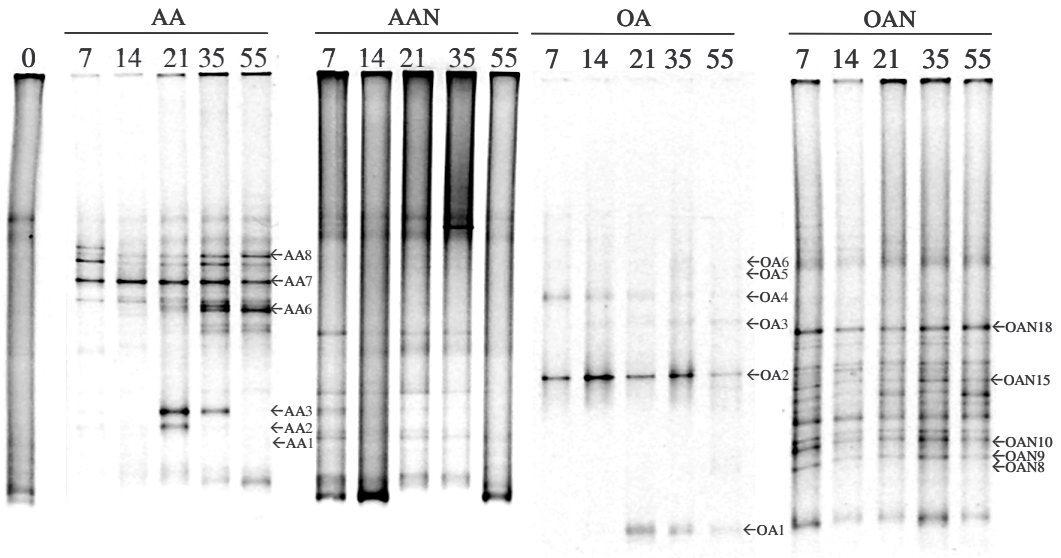

FIG.2.4. PCR-TTGE profiles of 16S rRNA fragments representing the bacterial diversity of species generated from the samples taken at days 0, 7, 14, 21, 35 and 55. The bands identified by sequencing are indicated by the arrowheads. Bioreactor treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN).

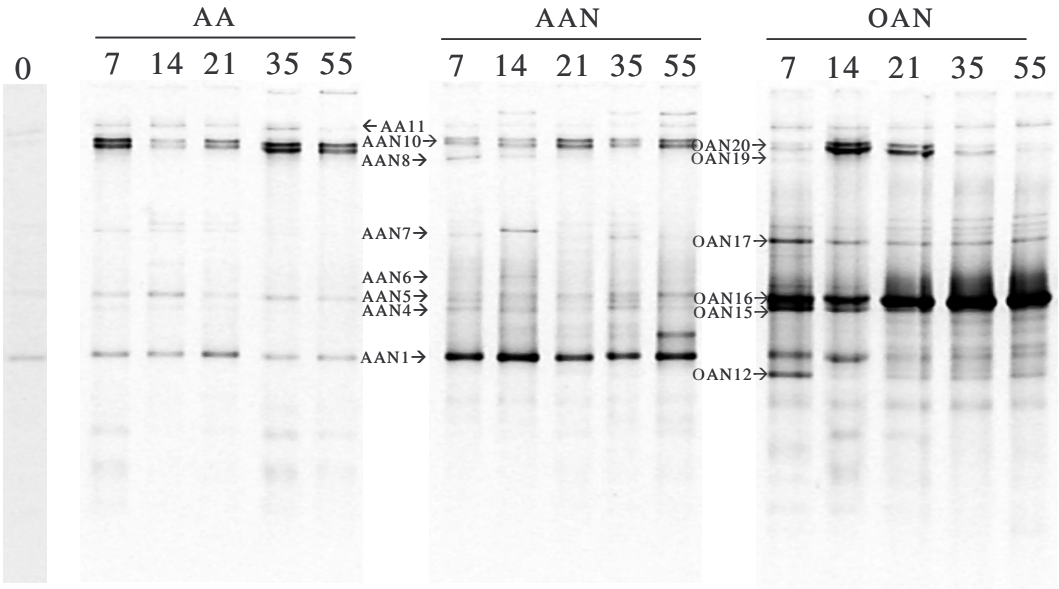

FIG.2.5. PCR-TTGE profiles of ITS region fragments representing the fungal diversity of species generated from samples taken at days 0, 7, 14, 21, 35, 55. The bands identified by sequencing are indicated by the arrowheads. Bioreactor treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN).

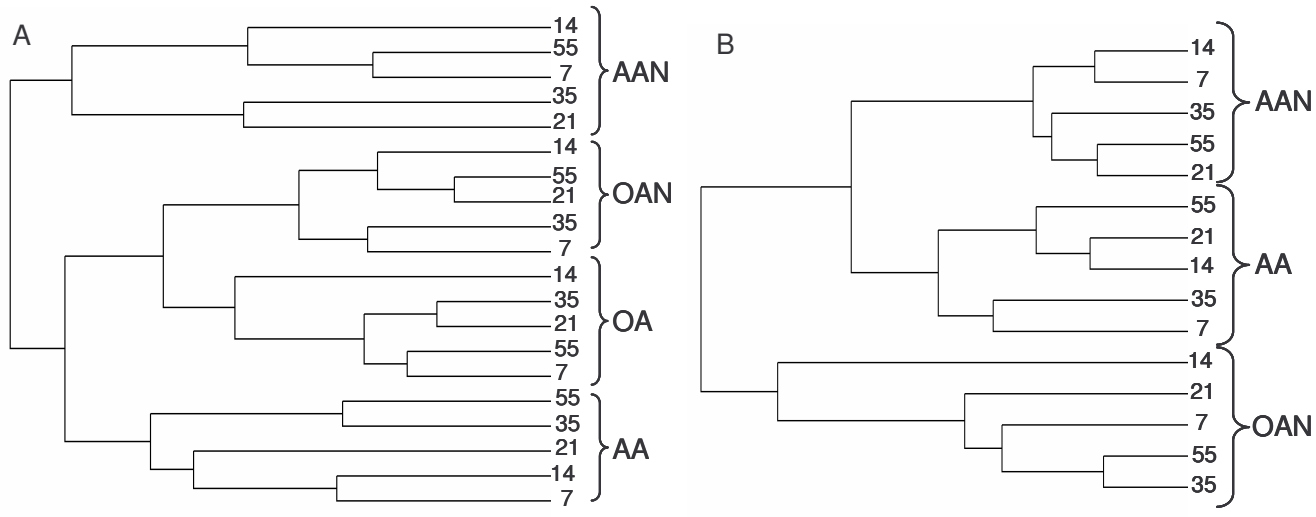

FIG.2.6. Cluster analysis of TTGE band patterns for A) bacteria, and B) fungi. The numbers indicate the day of sampling. Bioreactor treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN).

TABLE 2.1. Bacteria identified by PCR-TTGE analysis in TPOMW bioreactors according to their similarity to 16S rRNA gene sequences in the GenBank nucleotide sequence database.

Band no.	Treatment	Closest match	% similarity	Accession No.(*)
		Gamma proteobacteria		
OAN15	Non-aerated + nutrients	*Stenotrophomonas maltophilia*	94	AM261992
OAN18	Non-aerated + nutrients	*Erwinia persicina*	97	AJ937837
AA1	Areated	*Pantoea agglomerans*	98	DQ307453
AA2	Areated	*Luteibacter sp.*	99	DQ986460
AA3	Areated	*Stenotrophomonas maltophilia*	99	AM262002
		Actinobacteria		
OA7	Non-aerated	*Rhodococcus fascians*	98	AY730713
OAN8	Non-aerated + nutrients	*Clavibacter michiganensis*	97	L43096
OAN10	Non-aerated + nutrients	*Frigoribacteirum sp.*	97	AY439250
OAN9	Non-aerated + nutrients	*Curtobacterium albidum*	95	AM042692
		Firmicutes		
AA6	Areated	*Clostridium saccharolyticum*	98	AY604565
AA7	Areated	*Clostridium xylanolyticum*	98	X71855
AA8	Areated	*Clostridium sp.*	97	AB114227
OA3	Non-aerated	*Clostridium tyrobutyricum*	93	L08062
OA6	Non-aerated	Uncultured *Clostridium* sp.	98	DQ168846
OA2	Non-aerated	*Lactobacillus vaccinostercus*	99	AB218801
OA4	Non-aerated	*Leuconostoc mesenteroides*	98	EF068254
OA5	Non-aerated	*Leuconostoc mesenteroides*	99	EF068254
D10	Initial mixture	*Bacillus aquimaris*	97	DQ923229
OA1	Non-aerated	*Sporolactobacillus inulinus*	98	D16283

TABLE 2.2. Fungi identified by PCR-TTGE analysis in TPOMW bioreactors according to their similarity to ITS2 region sequences in the GenBank nucleotide sequence database.

Band no.	Treatment	Closest match	% similarity	Accession No.(*)
AAN1	Aerated + nutrients	Penicillium roquefortii	99	DQ148947
AAN4	Aerated + nutrients	*Penicillium roquefortii*	99	AY373929
AAN6	Aerated + nutrients	*Penicillium roquefortii*	97	AY373929
AAN7	Aerated + nutrients	Candida norvegica	92	DQ249194
AAN10	Aerated + nutrients	*Geotrichum* sp.	100	AY787702
OAN12	Non-aerated + nutrients	Pichia membranifaciens	99	DQ104713
OAN15	Non-aerated + nutrients	*Cladosporium herbarum*	100	AY251078
OAN17	Non-aerated + nutrients	*Ascochyta* sp.	100	AY305377
OAN19	Non-aerated + nutrients	*Geotrichum* sp.	99	AY787702
OAN20	Non-aerated + nutrients	*Geotrichum* sp.	99	AY787702

2.5. DISSCUSION.

The majority of previous studies dealing with the biodegradation of olive wastes have involved sterilization of the residues before inoculation of a specific microorganism or consortium. However, not only does autoclaving kill the indigenous microbiota, but also the high temperature causes significant physico-chemical modifications such as the oxidation of aromatic compounds present in the olive wastes (Aggelis et al. 2003). A simple, unmodified system was selected because a major aim of this work was to provide fundamental information that is relevant to the development of a robust biotechnological application for eventual scale-up to industrial scale. Therefore, in the work presented here, the TPOMW was not sterilized or manipulated in any other way before preparing the (bio)degradation mixture, adding only double distilled water and wheat straw to improve the porosity of the material and thus enable bioremediation to occur. The straw will have contributed some microbial activity, but given the organic richness of the TPOMW it is most likely that the bulk of the

microbiota were derived from the original olive waste. Therefore, we conclude that when appropriate conditions are achieved, the genetic potential of the indigenous microbiota of the waste (olive waste plus straw) is able to carry out an auto-biodegradation of the polyphenolic content without the inoculation of specific microorganisms. It is here suggested that the stimulation of this indigenous microbiota is a promising alternative bioremediation approach. Compared with the inoculation of a single strain (or consortium), the indigenous microorganisms have a range of different biodegrading activities that could act simultaneously or sequentially.

Similarly, a temperature of 25°C was selected, rather than a higher value, as being one that could be readily achieved in an industrial scale development without the necessity for expensive energy input.

Breakdown of polyphenols occurred mainly during the first 7 days of incubation, and the organic matter losses were mainly achieved during the first 14 days (Fig. 1). During the initial 1-2 weeks incubation period, in all bioreactors, except the aerated non-supplemented ones, there was the establishment of a microbial community with the capacity to biodegrade the polyphenolic content of TPOMW; all of the microbial communities, irrespective of the treatment regime, were capable of biodegrading the organic matter content of TPOMW. Although PLFA analysis detected changes in the relative proportions of bacteria and fungi, and of Gram-positive and Gram-negative bacteria, the TTGE profiles showed that the community structure was established rapidly (within 7 d) and that thereafter the predominant microbial species in the bioreactors were stable throughout the incubation period, i.e. not only during the initial phases of maximum polyphenol biodegradation and TOM decrease, but also during the later stages when less biodegradation was occurring. Partial rDNA bacterial and fungal sequence comparisons with sequences in the database gave values that ranged from 84 to 99% and from 92 to 100% respectively, although most values were higher than 97%. These values could change if the complete sequences were compared.

Most of the bacteria, as identified by TTGE, which are most likely to comprise the predominant members of the bacterial community in the TPOMW-straw mixture, are phylogenetically associated with bacteria, such as *Luteibacter* sp., that have previously been isolated from plant material (Johansenn et al. 2005). Moreover, some have been described as plant pathogens or at least associated with plant diseases. For example, *Rhodococcus fascians* and *Clavibacter michiganensis*, which are present in the bioreactors, are known plant pathogens (Young et al. 1996). *Stenotrophomonas maltophila* is another plant pathogen that is able to degrade polyphenolic compounds (Franco et al. 2005). In addition, *Pantoea agglomerans* is associated with the disease known as “olive knots” and was originally isolated from olives trees (Marchi et al. 2006). Actinomycetes belonging to the genus *Curtobacterium* have also been isolated from plant material (Postma et al. 2005). *Clostridium saccharolyticum*, which was isolated from sewage sludge (Murray et al. 1982), as well as *C. xylanolyticum* (Rogers et al. 1991), are both able to biodegrade polymers that could have a role in the bioremediation of TPOMW. The finding of anaerobes such as clostridia in aerated bioreactors could be regarded as an anomaly. However, it should be pointed out that the material in the bioreactors is very heterogeneous and it is quite possible that, despite the aeration, there were anaerobic “pockets” within the organic material that would harbour clostridia that are able to tolerate the intermittent presence of oxygen.

The greatest biodegradation of polyphenols occurred in those bioreactors that were both aerated and received nutrients. These reactors had higher ratios of fungal to bacterial biomass, as demonstrated by the PLFA analyses. Fungi have previously been studied for their capacity to remove polyphenols from olive residues by the release of extracellular enzymes, particularly lignin peroxidases, Mn-dependent peroxidases and laccases, which not only biodegrade polyphenols but also decolourize the olive-waste residues. The rate and extent of the removal of polyphenols depends on the particular fungi present and the nature of the polyphenols. For example, in an investigation of the potential for saprophytic fungi to decolourize TPOMW and biodegrade monomeric polyphenols, fungi such as *Phanerochaete chrysosporium* first removed hydroxytyrosol and tyrosol and later their glucosides: in contrast, *Paecylomyces*

farinosus hydrolyzed the glucosides first and then eliminated the monomeric phenols (Sampedro et al. 2004).

The present paper reports for the first time a molecular biological investigation of fungal communities involved in the process of olive-mill waste bioremediation. In our TTGE analysis of the fungal communities, the intense band in the lower part of the gel in AAN-profiles (band AAN1, Fig. 5) had a similarity of 99% (ITS2 region) with a sequence that belongs to *Penicillium roquefortii* (Table 1). This band also appears in AA-bioreactors, but its presence is not clear in OAN-samples. Given the fact that nutrients and aeration generally stimulated fungal growth and activity, as demonstrated by the PLFA and polyphenol analyses, the PCR-TTGE results can be interpreted as showing that an organism closely related to *P. roquefortii* could be the main candidate for polyphenol biodegradation.

Strains belonging to *Penicillium*, *Geotrichum, Pichia* and *Candida* were the major fungi detected by TTGE. These genera have previously been isolated from different OMWs including TPOMW (Assas et al. 2000, Ettayebi et al. 2003, Millan et al. 2000), demonstrating that they are likely to be part of the natural microbiota of these residues. These fungi show a promising range of biotechnological applications in connection with the treatment of OMWs. For example, olive-mill waste-water has been investigated as a substrate for microbial lipase production (D'Annibale et al. 2005), and the decolourization of black olive mill wastewaters has been studied with the use of lignin peroxidases (Ayed et al. 2005).

The present study describes the first detailed molecular study of the microbiota of TPOMW during the biodegradation of its polyphenol content. It highlights that probably a relatively small number of strains within the indigenous microbiota of the TPOMW is able to biodegrade the polyphenolic content of the waste without the inoculation of specific microorganisms. The results indicate that for improved polyphenol biodegradation, the fungal community should be stimulated by the addition of nutrients and air. However, the contribution of the bacteria able to grow in this waste should be taken into account. We also

demonstrated that the combination of PLFA and PCR-TTGE with specific primers is a powerful tool for evaluating the complex composition of the microbial communities of this waste under different treatment regimes. Further research is necessary to optimize the conditions for improved bioremediation of olive waste, and different biotechnological applications could be coupled to the process.

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Bacterial sequences

OAN15

CCGCNNCATGCTGNATCTGCGNATTACTAGCGATTCCGACTTCATGGAAG
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Fungal sequences.

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Capítulo 3

Production of a metal binding exopolymer by *Paenibacillus jamilae* using two-phase olive-mill waste as fermentation substrate

3.1. ABSTRACT.

The present study investigated the use of the two-phase olive mill waste (TPOMW) as substrate for the production of exopolysaccharide (EPS) by the endospore-forming bacilli *Paenibacillus jamilae*. This microorganism was able to grow and produce EPS in aqueous extracts of TPOMW as unique source of carbon. The effects of substrate concentration and the addition of inorganic nutrients were investigated. Maximal polymer yield in 100 ml batch-culture experiments (2 g l^{-1}) was obtained in cultures prepared with an aqueous extract of 20% TPOMW (w/v). An inhibitory effect was observed on growth and EPS production when TPOMW concentration was increased. Nutrient supplementation (nitrate, phosphate and other inorganic nutrients) did not increase yield. Finally, an adsorption experiment of Pb (II), Cd (II), Cu (II), Zn (II), Co (II) and Ni (II) by the EPS was reported. Lead was preferentially complexed by the polymer with a maximal uptake of 230 mg/g EPS.

3.2. INTRODUCTION.

The olive mill industry generates by-products which are potentially harmful to the environment, and in Mediterranean countries this problem is one of the most serious environmental concerns. At present two processes are used for the extraction of olive oil: the three-phase system and the modern two-phase system. The three-phase system produces three streams: pure olive oil, olive mill wastewater (OMW) and a solid cake-like by-product called *orujo* or olive cake. The two-phase centrifugation system, recently introduced for olive oil extraction, generates olive oil and a semi-solid waste: the two-phase olive mill waste (TPOMW), or *alpeorujo*.

Basically, TPOMW is a thick sludge that contains water and pieces of pit and pulp of the olive fruit. This semi-solid effluent has a moisture content around 65% (Arjona et al. 1996), slightly acidic pH values, and a very high content of organic matter, mainly composed of lignin, hemicellulose and cellulose. It has also a considerable proportion of fats, proteins, water-soluble carbohydrates and a small but active fraction of hydrosoluble (poly) phenolic substances. Amongst plant and microbial nutrients, TPOMW is also rich in potassium, less so in (mainly organic) nitrogen, and poor in phosphorus (Alburquerque e tal. 2004).

The combustion of TPOMW, as a fuel with high calorific value, is the most currently used procedure to eliminate its harmful effects on the environment. However, greater environmental and economic benefits could result from the conversion of this waste to a product of higher added value. In this sense, recent works have been published about direct extraction of high added value products from TPOMW like antioxidants (Fernández-Bolaños et al. 2002) and sugars (Fernández-Bolaños et al. 2004).

On the other hand, valorization of TPOMW by using the residue as a culture medium for microorganisms can provide new resources and will contribute to diminish the environmental impact of this waste (only in Spain, ~3.5-6 million tons/year of TPOMW are generated; Aragón et al. 2000). This biotechnological

approach has led to the production of a wide range of compounds (alcohols, biosurfactants, biogas, vitamins and plant growth hormones) by the use of OMW as fermentation substrate for micoorganisms (Ramos-Cormenzana et al. 1995, Niaounakis et al. 2004). Microbial exopolysaccharides (EPS), a class of high-value polymers with many industrial applications, can be also obtained by fermentation of olive-mill wastes. Xanthan gum, an EPS produced by *Xanthomonas campestris*, and used for food and non-food applications as thickener or viscosifier (Sutherland 1988), was produced using OMW as the culture medium (López et al. 1996).

However, new research must be carried on due to the new properties of the recent introduced TPOMW. As a result of screening EPS-producing bacteria with potential applications in OMW bioremediation, our group described the edospore-forming bacterium *Paenibacillus jamilae*. This strain, isolated from corn-compost treated with OMW, produced an heteropolysaccharide consisting of fucose, xylose, rhamnose, arabinose, mannose, galactose and glucose as sugar components (Aguilera et al. 1991). The aim of the present work was the study of the production of EPS by *P.jamilae* using TPOMW as the carbon source. In this laboratory study, the effect of TPOMW concentration and nutrient addition to the culture media was investigated, as well as the adsorption capacities of the biopolymer obtained on various toxic heavy metals (Pb, Cu, Ni, Co, Zn and Cd).

3.3. MATERIAL AND METHODS.

Microorganism. *Paenibacillus jamilae* CECT 5266 was obtained from the Spanish Type Culture Collection. The strain was maintained on YM agar (Difco, Detroit, USA) slants stored at 4ºC and under cultured at weekly intervals.

TPOMW. TPOMW was obtained from the factory “Aceites Jimena S.A.” (Granada, Spain), collected in plastic containers and stored at -20ºC. The sample was dark brown in colour and had a smooth dough-like consistency, with a high content of water (56%).

Experimental methods. To prepare a liquid medium capable of supporting the microorganism culture, an aqueous extract was obtained from TPOMW. The sludge was suspended in 1000 ml water to achieve the desired concentration level (1-30% w/v). The mixture was then placed in Erlenmeyer flasks and shaken at 100 rev min^{-1} for two hours to dilute the water soluble compounds. The aqueous extracts used as substrate were filtered through no.40 Whatman filter paper to eliminate pieces of pits and other solid material of the olive, and neutralized to pH 7 with 0,1 mol l^{-1} Na OH prior to autoclaving at 121 ºC for 20 min.

Batch culture experiments were carried out in order to study the effect of TPOMW concentration and nutrient addition to the media on growth and EPS production. In these experiments, culture media consisted in 100 ml of the basic medium containing TPOMW aqueous solution supplemented with different combinations of inorganic nutrients. The following salts were used as sources of inorganic nutrients: NH_4Cl (20-190 mM), KH_2PO_4 (5-50 mM), $MgSO_4$ $7H_2O$ (40 mg/l), $CaCl_2$ (10 mg/l), SO_4Fe ($7H_2O$ 20 mg/l), Mn SO4 H_2O (10 mg/l) and KCl (50 mg/l). Media were inoculated with 1 ml of a 24 h *Paenibacillus jamilae* culture in YM broth. Cultures were incubated in 250 ml flasks with rotary shaking at 100 rev. min^{-1} for 5 days at 30 ºC. After culture, biomass and polymer concentration were estimated as described below.

A 2 l jar-fermentor BIOSTAT M (Braun-Biotech) containing 1000 ml of medium was also used for the production of EPS. Media were inoculated with 10 ml of a 24 hours *Paenibacillus jamilae* culture in YM broth. Fermentation conditions (30ºC, pH 7 and 150 rev min^{-1}) were kept constant during 5 days with the addition of 2 ml/min of air by bubbling. For biomass and EPS determinations, 20 ml of culture were sampled at regular intervals.

EPS and biomass extraction. The production of biomass was calculated from dry weight of cells harvested from media by centrifugation at 10,000 rev min^{-1} for 30 min in a Sorvall centrifuge (Delaware, USA). The EPS was recovered from cell-free supernatant by precipitation with two volumes of -20ºC ethanol with a prior addition of 1% (w/v) KCl as the electrolyte. The

solution was kept at -20ºC overnight and centrifuged at 5000 rev min^{-1} for 10 min. The precipitated polymer was dissolved in distilled water and dialyzed exhaustively against distilled water for 48 hours at 4 ºC. Dry weight of polymer was obtained by freeze-drying. The reported biomass and EPS were corrected for the mass of the pellet resulting from aqueous extracts of TPOMW itself. All assays were performed in triplicate.

Metal adsorption experiments. To study the metal uptake capacity of the polymer on different heavy metals, the following procedure was applied. Equilibrium dialysis experiments (Geddie and Sutherland 1993) were carried out in batches as follows: 200 ml solutions (0,1 mM) of the selected cations were prepared in 250 ml flasks using double distilled-deionised water (MilliQ quality). Selected cations were Pb (II), Cd (II), Cu (II), Zn (II), Co (II) and Ni (II) as nitrate salts (Sigma). Multimetallic solutions containing the six selected metals (0,1 mM) were also prepared for studying the competitive effect of various heavy metals on polymer adsorption.

Dialysis tubing (Medicell International MWCO 12-14000 Daltons) was boiled in 2% $NaHCO_3$ and 1 mM EDTA, 1 mM EDTA and finally in deionized water to remove any contaminating metals. It was stored at 4ºC before use. Five millilitres of EPS 0,1% w/v solutions were placed in dialysis tubing containing 200 ml of the appropriate ion solutions. The experiment was performed in triplicate. Flasks were shaken in an orbital shaker (100 rev min^{-1}) at 25ºC, until equilibrium sorption was reached (24h). At the end of the experiment, 10 ml of the solution was sampled and acidified with 1% nitric acid solution for residual metal determination. The final metal concentration was determined by atomic absorption spectrophotometry (Perkin Elmer 5100).

The metal uptake (q) was determined as follows: $q = V\,(Ci\text{-}Cf) / m$, where Ci is the initial metal concentration in solution of volume V , Cf is the equilibrium concentration of metal in solution, and m is the mass of EPS. Appropriate blanks were examined throughout the sorption experiments to correct the glassware and dialysis tubing sorption of metals and other potential side effects.

3.4. RESULTS AND DISSCUSION.

Production of EPS

The effect of TPOMW concentration on growth and EPS production is shown in Fig. 1. A similar trend was obtained for both variables. At low TPOMW concentration, EPS production and growth increased with the concentration of the substrate. The maximal values obtained for both variables, 2 g/l of EPS and 0,25 g/l of cell, were reached with a concentration of 20% of TPOMW. When more than 20%TPOMW was used to prepare the culture media, a decrease of microbial growth and EPS production was obtained. This effect of TPOMW concentration on growth and EPS production could be explained as a consequence of a balance between the nutrients in the aqueous extract used as substrate (like water soluble sugars) and other components with antimicrobial activity typically found in olive-mill wastes (mainly polyphenols). At low TPOMW concentration, there was also a dilution of nutrients, and hence, less carbon was available for EPS production. As TPOMW increased, growth and EPS production rose, but with concentrations higher than 20%, an inhibitory effect was produced. Therefore, a concentration of 20% TPOMW was used to prepare the culture media to carry out the following experiments of this work. Previous works with olive-mill wastewater as substrate for xanthan production by *Xanthomonas campestris* found a similar effect, with optima of 40% of olive mill wastewater (three phases system) for polymer production (López and Ramos-Cormenzana 1996). Xanthan was also obtained from fermentation of aqueous extracts of TPOMW with a production of 3g l^{-1} using 10% TPOMW supplemented with nitrogen (López et al. 2001).

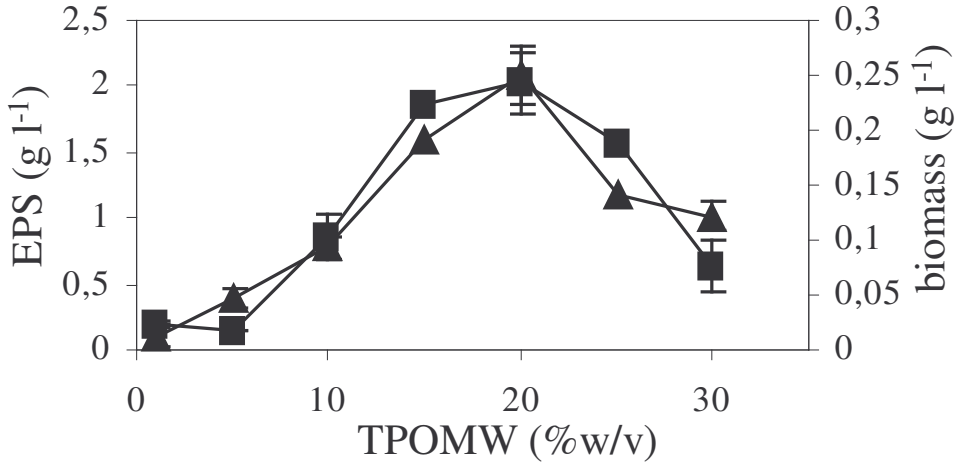

Fig.1 Effect of varying TPOMW concentration on biomass (▲) and EPS (■) production.

Table 1 Effect of the addition of NH_4Cl (N in the table), yeast extract (YE) and KH_2PO_4 (P) on growth and EPS production. Concentrations of nutrients are expressed in mM (brackets) and in g l^{-1} in the case of yeast extract. Data are the mean of 3 replicates ± standard deviation.

Experiment	EPS (g l^{-1})	Biomass (g l^{-1})
control	1,73 ± 0,07	0,23 ± 0,03
N (20)	1,69 ± 0,09	0,21 ± 0,05
N (40)	1,41 ± 0,33	0,27 ± 0,07
N (60)	1,56 ± 0,42	0,30 ± 0,04
N (90)	1,57 ± 0,25	0,26 ± 0,02
N (190)	1,45 ± 0,19	0,43 ± 0,03
YE (1)	1,58 ± 0,22	0,28 ± 0,02
N (20)+YE(1)	1,62 ± 0,05	0,31 ± 0,05
P (5)	1,66 ± 0,27	0,50 ± 0,07
P (10)	1,71 ± 0,17	0,37 ± 0,05
P (20)	1,9 ± 0,28	0,19 ± 0,10
P (50)	1,82 ± 0,30	0,19 ± 0,05

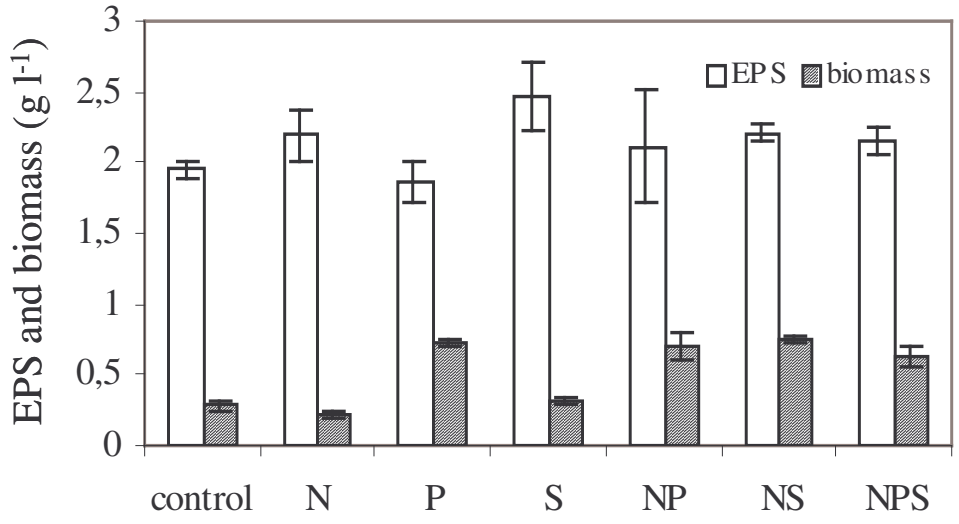

Fig.2 Effect of the combined addition of a source of nitrogen, phosphorus and other inorganic salts on growth and EPS production. The medium culture, prepared with an aqueous extract of 20% TPOMW (w/v), was supplemented with solutions N, P and S with the following combinations: N, P, N + P, N + S, N + P + S. These solutions consisted in: solution "N": NH_4Cl 90 mM; solution "P": KH_2PO_4 20 mM; solution "S": $MgSO_4$ $7H_2O$ 40 mg/l, $CaCl_2$ 10 mg/l, $FeSO_4$ 7 H_2O 20 mg/l, Mn SO_4 H_2O 10 mg/l and KCl 50 mg/l. A control without extra-addition of nutrients was included.

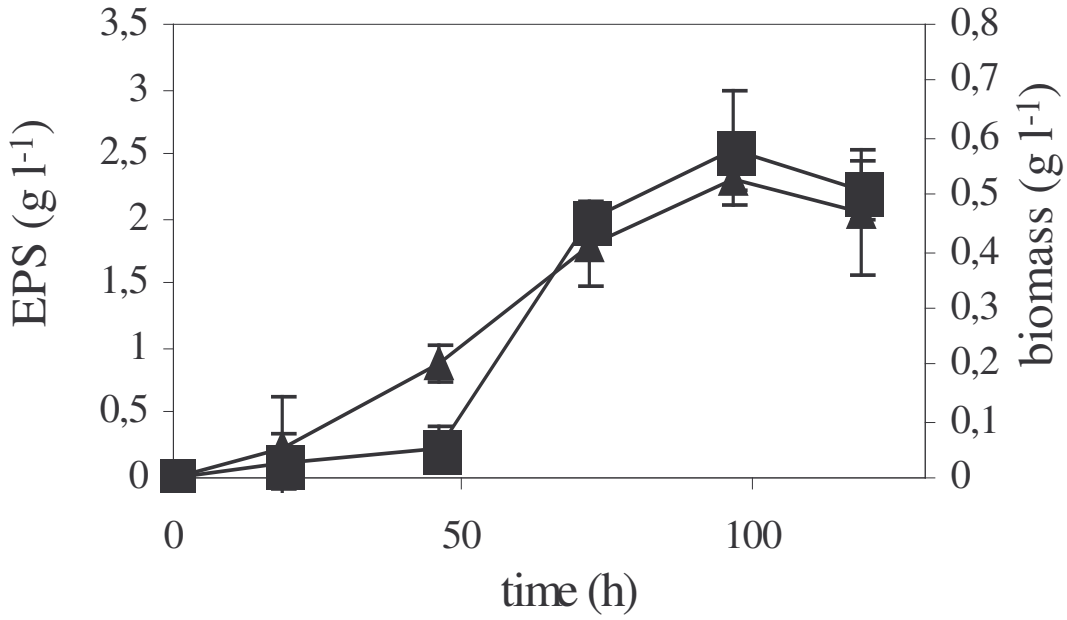

Fig.3 Kinetic of biomass (▲) and EPS (■) production in 1-l fermentation experiment. The medium culture, prepared with an aqueous extract of 20% TPOMW (w/v), was supplemented with NH_4Cl (90 mM), KH_2PO_4 (20 mM), $MgSO_4$ $7H_2O$ (40 mg/l), $CaCl_2$ (10 mg/l), $FeSO_4$ 7 H_2O (20 mg/l), Mn SO_4 H_2O (10 mg/l) and KCl (50 mg/l).

The addition of a source of nitrogen and phosphorous at different concentrations produced no important effects on growth and EPS production (Table 1). However, with 200 mM of NH_4Cl, a slight increase in biomass (0,43

g/l compared with 0,23 g/l in the control cultures) was obtained, although this effect was not followed by an increase in EPS production.

In order to check the possibility of increasing yield, the basic medium prepared with 20%TPOMW was amended with the combined addition of three solutions of inorganic salts (Figure 2) that contribute with a source of N, P, S, Mg, Ca, Fe, Mn and K, that have been reported as substantially important nutrients for the production of EPS by microorganisms (Sutherland 1990). The results obtained in this experiment showed that an slight increase in the yield of cells was obtained with the combined addition of inorganic nutrients. The production of EPS however was approximately the same in all treatments (between 2-2,5 g/l).

Figure 3 shows typical time course of cell growth and EPS production from *P. jamilae* in a jar fermentation reactor. The EPS production was mainly associated to the stationary phase of growth. The production of EPS in this fermentation system of 1 litre was slightly higher than in the 100 ml cultures of the batch experiments. This effect was most likely due to the general optimization of the fermentation process achieved in the 1-litre-system.

Metal uptake by the EPS

EPS are recommended as surface-active agents for the removal of heavy metals because of their extensive capacity (Veglio et al. 1997, Kaplan et al. 1987, Norberger et al. 1982, Pagnanelli et al. 2000). The EPS used for this assay was obtained using 20% TPOMW supplemented with nutrients in the 1-l fermentation process described previously. Selected metals tested in this study chosen for their important industrial uses and potential toxicity, were Pb, Cu, Ni, Co, Zn and Cd. Two kinds of sorption systems were tested. The one-metal sorption system consisted in sorption experiments with only one of the metals in solution, in order to study the ability of the EPS to uptake each metal individually. To obtain a first evaluation about the competitive effect of various heavy metals on EPS adsorption, a six-metal sorption system was carried out. Although metal solutions were prepared with the same concentration in a molar basis (0,1 mM), the results are presented on the basis of mass (mg) metal

uptake per gram EPS, as this unit is more frequently used in quantifying respective metal capacities in real terms.

Lead uptake, 228 mg/g EPS, was greater than that of the other metals tested (Fig.4). The EPS studied showed the following order of metal uptake: Pb >> Cd > Cu > Zn >> Ni > Co. The different affinity of metals for the EPS can be attributed to charge density, attractive interaction and types of conformation of polymer with adsorbed ions (Salehizadeh and Shojaosadati 2003). Other researchers reported high affinity of lead to bacterial exopolymers. The exopolymer produced by an hydrothermal bacteria (*Alteromonas macleodii* subsp *fijiensis*) adsorbed 316 mg/g of lead in optimal experimental conditions (Loaëc et al. 1997). However, a comparison of the reported data on EPS-heavy metal uptake is still difficult because of the different methodologies used, experimental conditions, range of concentrations of the metals, etc. In the multimetal sorption system, the quantity of lead removed was also high (164 mg/g) but to a lesser extent than in the one-metal system (228 mg/g). In the case of the other metals, the uptake by the polymer was substantially inhibited in the mixture.

The actual mechanisms of heavy metal uptake, especially multicomponents, are not well understood thus further detailed studies are necessary (Salehizadeh and Shojaosadati 2003, de Carvalho et al. 1995, Jang et al. 1995).

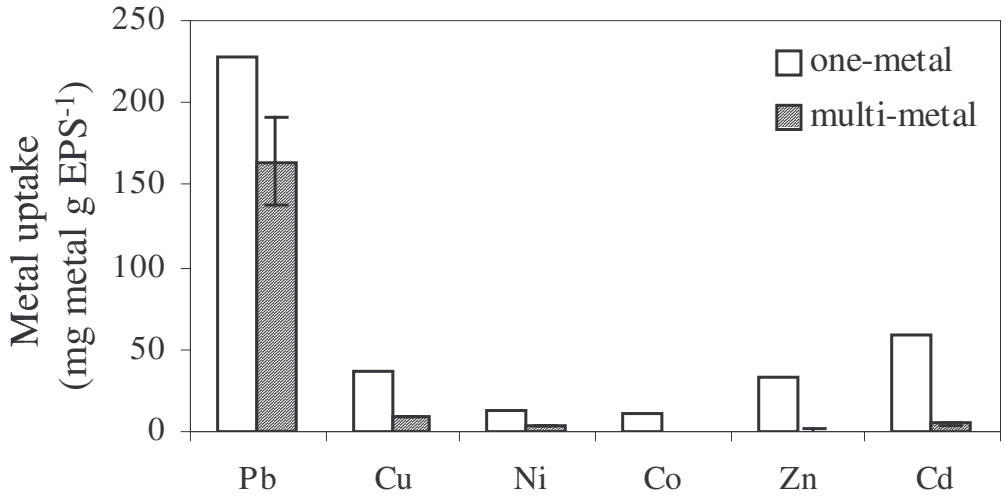

Fig.4 Metal uptake by the EPS produced by *P. jamilae* using TPOMW aqueous extract as substrate for growth and EPS production. Metal uptake is shown in one-metal and multi-metal sorption systems.

3.5. CONCLUSIONS

The performed study made evident the possibility of using aqueous extracts of TPOMW as substrate for the production of EPS by *Paenibacillus jamilae*. No effects of substantial significance were found of inorganic nutrient supplements on EPS yields. It can be concluded from these results that under the experimental conditions tested, the EPS produced in this study complexed specifically lead. The results obtained in the assays of metal uptake by this "ecologically attractive" biosorbent could base further investigation on possible applications in the environmental industry.

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Capítulo 4

Biosorption of heavy metals by the exopolysaccharide produced by *Paenibacillus jamilae*

4.1. ABSTRACT.

This paper deals with the interaction of several heavy metals (Pb, Cd, Co, Ni, Zn and Cu) with the exopolysaccharide (EPS) produced by *Paenibacillus jamilae*. The biochemical composition of the EPS was determined. Glucose was the most abundant neutral sugar, followed by galactose, rhamnose, fucose and mannose. The polymer presented a high content in uronic acids (28.29%). The FTIR spectrum of the polymer evidenced the presence of carboxyl groups, which may serve as binding sites for divalent cations. Lead biosorption was tenfold higher (in terms of mg of metal adsorbed per g of EPS) than the adsoprtion of the rest of metals. The EPS precipitated Fe (III), but the EPS-metal precipitate did not form with Fe (II), Pb (II), Cd (II), Co (II), Ni (II), Cu (II) and Zn (II).

4.2. INTRODUCTION

One of the major challenges in environmental biotechnology is the bioremediation of heavy metal pollution. Health and environmental hazards presented by heavy metals have stimulated the imposition of stricter regulations and the demand of new mechanisms to remove these pollutants. The search for novel technologies has been directed to the application of biosorption, which constitutes an attractive and economical alternative to common by-applied physicochemical remediation methods. Biosorption consists in the removing of heavy metals from dilute aqueous solutions based on the property of certain kinds of inactive and dead biomasses to bind and accumulate these pollutants by different mechanism such as physical adsorption, complexation, ion exchange and surface-micro-precipitation (Volesky, 2001).

The potential use of biomass of microorganisms in the treatment of heavy metal-contaminated wastewaters and in the recovery of metals in mining wastes or in metallurgical effluent is of special importance (Loren 1979, Gadd 1996). In this regard, extracellular polysaccharides (EPSs) secreted by bacteria, fungi and algae are recommended as surface active agents for heavy metal removal because of their extensive capacity (Veglio, 1997; Kaplan, 1987; Pagnanelli, 2000). The adsorption of heavy metal by EPS is a metabolism-independent process, and it is attributed to interaction between metal cations and negative charges of acidic functional groups of EPS (Kim et al. 1996 en I4). The understanding of the mechanisms by which microorganisms accumulate metal is crucial to the development of microbial processes for concentration, removal and recovery of metals from aqueous solutions (Ahluwalia et al. 2006).

The interaction between bacterial EPSs and metal ions have stimulated specific research interest due to its important ecological and practical implications. Different combinations of metal ions, microorganisms and experimental conditions have been investigated. For example, investigations focused on adsorption of Cu, Mn, Pb and Hg by exopolysaccharide-producing cyanobacteria (Paperi et al. 2006; Freire-Nordi et al. 2005), Pb, Cu and Zn removal by EPS produced by *Bacillus firmus* (Salehizadeh et al. 2003), adsorption of Cu, Cd, Zn and Ni by the exopolysaccharide producing

Pseudomonas sp. CU-1 (Lau et al. 2005) and La fixation by the exopolymer of *Myxococcus xanthus* (Merroun et al. 2003).

Bioremediation technologies in general should be relatively inexpensive and simple because of the low value associated with their commercial applications (Nies 1999 en gutnick et al 2000). In this sense, we recently described the production of a heavy metal-binding EPS by *Paenibacillus jamilae*, a bacterium that is able to use olive-mill wastes (agricultural wastes with antimicrobial and fitotoxic activity), as the fermentation substrate for the production of the polymer (Morillo et al. 2006). However, a better knowledge of the chemistry and biosorption capabilities of this new bacterial polymer is necessary to perform possible applications in the environmental industry. On the base of the previous promising results, the aim of this study was therefore to investigate the specific interactions of the EPS produced by *P. jamilae* with a number of various heavy metals (Pb, Cd, Co, Ni, Zn, Cu and Fe), and also to provide information about the chemical composition of the EPS.

4.3. MATERIALS AND METHODS

Microorganism. *Paenibacillus jamilae* CECT 5266 was obtained from the Spanish Type Culture Collection. The strain was maintained on YM agar (Difco, Detroit, USA) slants stored at 4ºC and under cultured at weekly intervals.

Exopolysaccharide production. A 2 l jar-fermentor BIOSTAT M (Braun-Biotech) containing 1000 ml of culture medium was used to carry out the production of EPS by *P. jamilae*. The composition of the medium was: sucrose (80 g), KNO_3 (5.1 g), K_2HPO_4 (1 g), KH_2PO_4 (0.8 g), NaCl (1 g), $MgSO_47H_2O$ (40 mg), $CaCl_2$ (10 mg), $FeSO_47H_2O$ (20 mg), $MnSO_41H_2O$ (10 mg), yeast extract (Difco) (0.1 g), proteose peptone (Difco) (0.1 g) and distilled water (1000 ml). The medium was inoculated with 10 ml of a culture of *P. jamilae* in exponential phase (cultured in the same medium). Fermentation conditions (30ºC, pH 7 and 150 rev min^{-1}) were kept constant for 4 days with the addition of 2 ml min^{-1} of sterile air by bubbling.

Isolation of the exopolysaccharide. The culture medium was diluted with 1000 ml of distilled water and bacterial cells were harvested by centrifugation at

10000 rev min^{-1} for 30 min in a Sorvall centrifuge (Delaware, USA). The EPS was recovered from cell-free supernatant by precipitation with two volumes of -20 ºC ethanol with a prior addition of 1% (w/v) KCl as the electrolyte. The solution was kept at -20 ºC overnight and centrifuged at 5000 rev min^{-1} for 10 min. The precipitated polymer was dissolved in distilled water and dialyzed extensively (Medicell International MWCO, 12000 to 14000 Da) against distilled water for 48 h at 4 ºC. Dry weight of polymer was obtained by freeze-drying.

Compositional analysis of exopolysaccharide. The biochemical composition of EPS was determined by the following spectrophotometric methods. Total carbohydrates were analyzed by the phenol-sulphuric method (Dubois *et al.*, 1956) with D-glucose as the standard. Uronic acids were measured by the carbazole-sulfuric acid method (Dische, 1962) with D-glucuronic acid as standard. Total protein was analyzed by the method of Bradford (1976) with bovine serum albumin as the standard. Hexosamine was determined by the method of Johnson (1971). Pyruvic acid was quantified by the enzymatic assay described by Duckworth and Yaphe (1970). Acetyl groups were assayed using a modified colorimetric procedure of Hestrin (1949). All determinations were performed in triplicates.

Elemental composition of the EPS (content of total carbon, total nitrogen and total hydrogen) was determined using an elemental analyzer Fisons Carlo Ebra E 1108. For the analyses of sugars, the polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 ºC for 120 minutes and evaporated to dryness under nitrogen stream. Carbohydrate composition was analysed by gas chromatography (Carlo Ebra serie 8000 model 8060) equipped with a spectrometer Platform II (Micromass Instruments). A stainless steel SPB 2380 column was used and the temperature program was 125 ºC for 2 min and 125 to 280 ºC at 5 ºC min^{-1}.

Metal adsorption experiments. To study the metal uptake capacity of the polymer on different heavy metals, the following procedure was applied. Equilibrium dialysis experiments (Geddie *et al.* 1993) were carried out in batches as follows: 200 ml solutions of the selected metal ions were prepared in 250 ml flasks using double distilled-deionised water (MilliQ quality). Selected cations were Pb (II), Cd (II), Cu (II), Zn (II), Co (II) and Ni (II) as nitrate salts

(Sigma). The concentration of the metal ions varied in the different experiments of this study, within the range 0.01-1 mM. Multimetallic solutions containing the six selected metals (0.1 mM) were also prepared for the study of the competitive effect of various heavy metals on polymer adsorption. The pH of the EPS and of the metal solutions was maintained at 5.5, (which is close to the pH of real waste waters containing heavy metals) by adding HCl 1 mol l^{-1} when necessary.

Before performing the sorption experiments, dialysis tubing (Medicell International MWCO 12000-14000 daltons, 100mm length and 33mm diameter) was boiled in 2% $NaHCO_3$ and 1 mM EDTA, 1 mM EDTA and finally in deionized water to remove any contaminating metal. It was stored at 4ºC before use.

Aliquots of EPS solution (5 ml of EPS 0.1% w/v) were confined in the dialysis tubing, and then the tubes were introduced in the 250 ml flasks containing 200ml of the metal solutions. Appropriate blanks, in which the dialysis tubing contents were aliquots of 5 ml of double distilled-deionised water, were examined throughout all the sorption experiments to correct the glassware and dialysis tubing sorption of metals and other potential side effects. Flasks were shaken in an orbital shaker (100 rev min^{-1}) at 25ºC. The contact time between metal and EPS solutions was 24 h in all experiments except for the study of the kinetics of adsorption, where it was variable changing from 5 min to 24 h. At the end of the experiment, 10 ml of the metal solution was sampled and acidified with 1% nitric acid solution for residual metal determination. The experiments were performed in triplicate.

The final metal concentration was determined by atomic absorption spectrophotometry (Perkin Elmer 5100). The metal uptake (q) was determined as follows: $q = V\,(Ci\text{-}Cf) / m$, where Ci is the initial metal concentration in solution of volume V , Cf is the equilibrium concentration of metal in solution, and m is the mass of EPS.

Precipitation studies. The precipitation experiments were carried out following the methodology of Corzo *et al.* (1994) with minor modifications. Appropriate volumes of metal ions (0-50 µl) were mixed in Eppendorf tubes with 1ml of EPS solution (0.1% w/v). EPS solutions were made in acetic acid buffer (10 mM) at pH 3 and pH 6. After the incubation time (4 h), the tubes were centrifuged at

13000 g for 5 min. Supernatant samples (0.2 ml) were picked up in duplicate to new Eppendorf tubes to measure EPS remaining in solution. EPS was measured as total carbohydrates, analyzed by a modification of the phenol-sulphuric method (Dubois et al., 1956): 0.2 ml of sample solution was diluted to 1ml with water, and 50µl of phenol (80% in water, w:v) were added. After stirring, samples were incubated for 1 h in a water bath at 60ºC. H_2SO_4 (1 ml) was added and samples were left at room temperature for 30 min. Finally, the absorbance at 480 nm was read. All experiments were performed in triplicate.

4.4. RESULTS

Chemical characterisation of the EPS.

A yield of 10 g l^{-1} of EPS was obtained by cultivating cells of *P. jamilae* in the fermentation system during 4 days. The culture medium used to obtain the EPS was rich in carbohydrates (sucrose) to facilitate the production of the EPS by the bacteria. The results of chemical analysis of the EPS produced by *P. jamilae* are shown in Table 4.1. The neutral sugar composition of the EPS, analysed using gas chromatography, shows the percentage relative contribution of hexoses (glucose, galactose), deoxyhexoses (rhamnose, fucose) and pentoses (mannose). Glucose and mannose were the most abundant neutral sugars in differential amounts.

Table 4.1. Biochemical composition (A), elemental composition (B) and neutral monosaccharides composition (C) of the EPS produced by *P. jamilae*.

(A) Biochemical composition[a]		(B) C:N:H[a]		(C) Neutral carbohydrates[b]	
CH	62,86	C (%)	57,89	Glucose	54,6
Pro	1,05	N (%)	1,42	Mannose	25,6
Ace	4,13	H (%)	6,46	Galactose	12,9
Uro	28,29	C/N mole ratio	50,95	Rhamnose	3,1
Pyr	8,7			Fucose	3,8
Hex	2,81				

CH: total carbohydrates; Pro: proteins; Ace: acetyls; Uro: uronic acid; Pir: pyruvic acid; Hex: hexosamines.
[a] Results are expressed as percentages of total dry weight of the polymer.
[b] Data are presented as percentage of total uncharged monosaccharides detected by GC-MS.

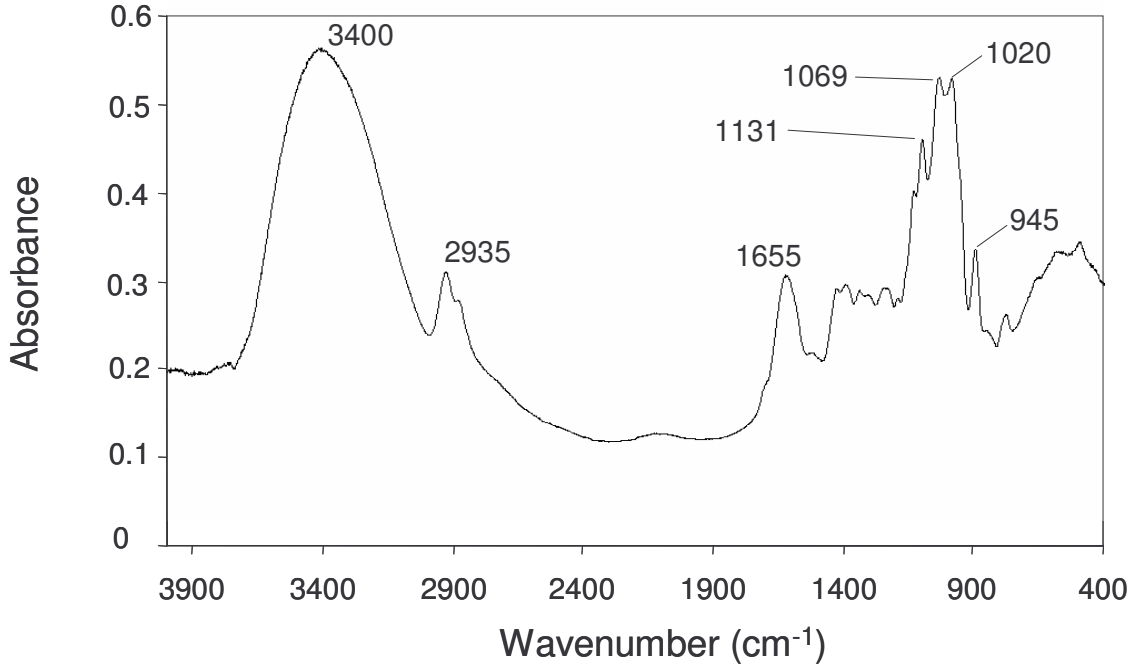

Fig 4.1. Fourier-transformed infrared (FTIR) spectrum of the EPS produced by *P. jamilae.*

Table 4.2. Langmuir isotherm model constants for adsorption of Pb, Cd, Co, Ni, Cu and Zn on the EPS. unidades

Metal ion	Langmuir adsorption isotherm		
	Q	b	r2
Pb	303.03	5.45	0.969
Cd	20.49	2.41	0.962
Co	30.12	7.87	0.726
Ni	17.66	10.24	0.565
Cu	7.81	*0.591*	0.955
Zn	12.31	*0.473*	0.952

The FTIR spectrum (400-4000 cm^{-1}) of the polymer is presented in Fig. 4.1. There were several distinct, characteristic absorption peaks of chemical groups contained in the EPS, the most intensive of them in the range 3400, 2935, 1655, 1131, 1069, 1020 and 945 cm^{-1}.

Interactions between EPS and heavy metal ions.

Adsorption isotherms.

Fig. 4.2 indicates the metal uptake isotherms for Pb, Cd, Co, Ni, Cu and Zn ions plotted against final metal concentration in aqueous solutions. Lead adsorption by the EPS produced by *P. jamilae* was tenfold higher (in terms of mg of metal adsorbed per g of EPS) than the adsorption of the rest of metals. The maximum binding ability of the EPS for the six metals calculated from the Langmuir´s linear plot is shown in Table 4.4. The EPS showed the following order of metal uptake: Pb >> Co > Cd > Ni > Zn > Cu.

Competitive biosorption.

We evaluated the competitive effect of various heavy metals on EPS adsorption. The specific adsorption of each metal was compared in one-metal and multi-metal sorption systems. One-metal systems consisted in sorption experiments with only one of the metals in solution with initial concentrations of 0.1 mM. The multi-metal sorption system was performed in the exactly same manner but with a mixture of the six metal ions at 0.1 mM. Results of the competitive biosorption experiments are summarized in Table 4.5. In both systems, lead was by far the metal that presented higher adsorption to the EPS in both one-metal and multi-metal systems. Besides, lead adsorption was not influenced by the presence of the other ions in solution ($P<0.005$). For the rest of the ions, the specific biosorption to the EPS was statistically reduced in the multi-metal system in comparison with the results obtained from the one-metal system ($P<0.05$), with the exception of Ni ($P=0.08543$). Adsorption of Cu and Co was not detected in the multi-metal system.

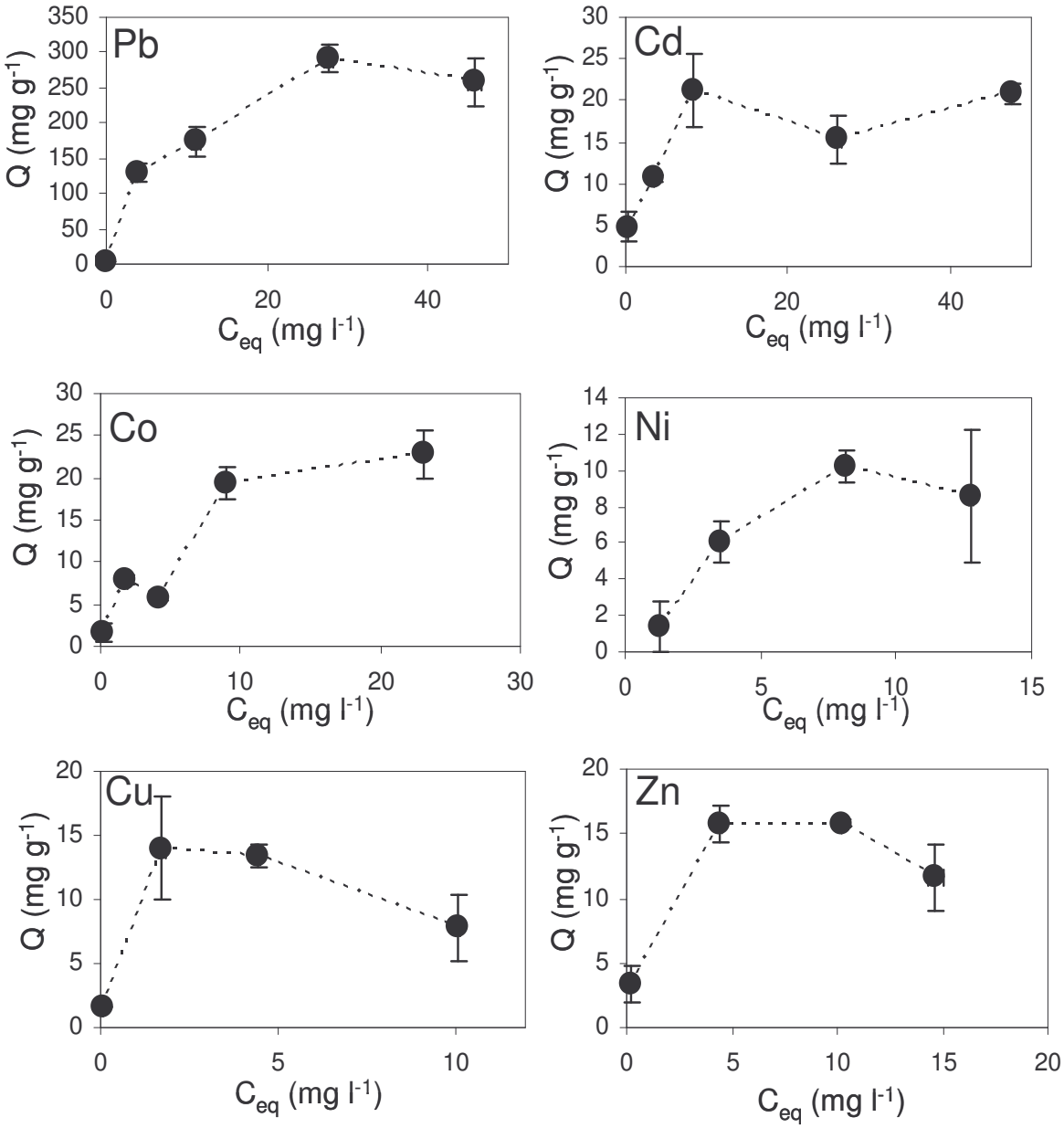

Fig. 4.2. Metal biosorption isotherms for EPS produced by *P. jamilae.*

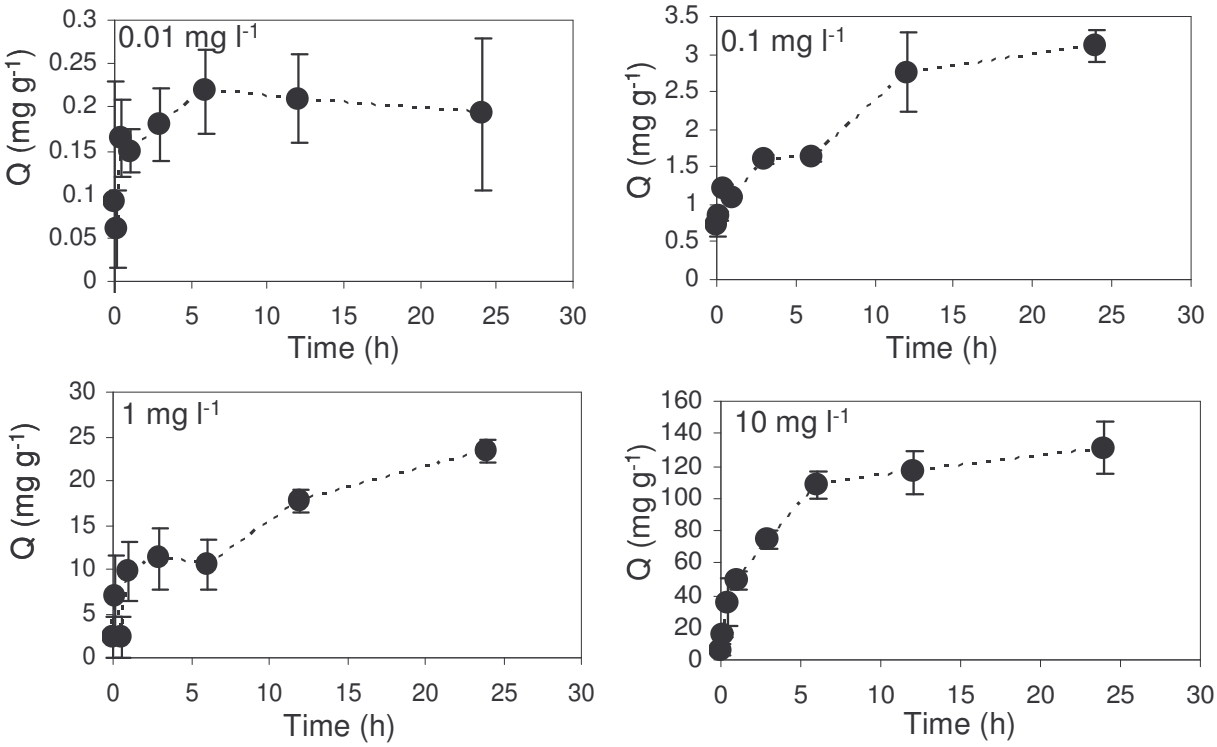

Fig 4.3. Time course of lead removal (q) by EPS produced by *P. jamilae* expressed as mg of Pb removed per g of polymer.

Table 4.3. Specific biosorption of heavy metals by the EPS in one-metal and multi-metal sorption systems.

	One-metal system	Multi-metal system
Metal	Adsorption	Adsorption
Pb	189.53 ± 30.92	200.33 ± 40.00
Cd	21.93 ± 3.44	5.33 ± 2.57
Co	6.83 ± 1.80	nd[1]
Ni	5.40 ± 1.31	3.03 ± 0.21
Cu	13.40 ± 0.60	nd[1]
Zn	15.73 ± 1.01	1.43 ± 1.27

Biosorption is expressed as mg of metal per g of EPS (average ± standard deviation).
[1] Not detected.

Lead biosorption kinetics and effect of pH on lead biosorption.

Of the metals used to study the capability of the EPS to remove metals from aqueous solution, lead was by far the cation that presented the greatest biosorption to the EPS. In view of these results, we studied the kinetics of lead removal by the EPS and the effect of the pH on this process.

The kinetics of lead removal by the EPS was studied at the following initial metal concentrations: 0.01, 0.1, 1 and 10 mg l^{-1} (Fig. 3). The amount of lead removed increased with time until the sorption capacity of the EPS reached its saturation point. Saturation time was dependent on the initial lead concentration. At the lower lead concentration (0.01 mg l^{-1}), saturation of the EPS occurred after 5 hours. However, more than 12 hours were needed to reach the saturation point in the other three assays with higher concentrations on the metal.

The effect of pH on lead adsorption to the EPS was tested at the initial pH values 3, 4.5 and 6. At higher pH values, a white precipitate was observed in

the lead aqueous solutions due to the formation of insoluble hydroxides, making metal adsorption experiments very difficult. The pH values at the end of the adsorption did no change significantly from the initial values. A higher amount of lead was adsorbed at pH 6 (213.66 mg Pb g EPS^{-1}) than at pH 4.5 (133.0 mg g^{-1}) and pH 3 (89.66 mg g^{-1}). These differences were statistically significant between the values obtained for pH 6 and the data of the two lower pHs ($p<0.05$). However, the lead adsorptions obtained at pH 3 and pH 4.5 were not statistically different ($p>0.1$).

Precipitation experiments.

We studied the precipitation of the EPS by the six heavy metals previously used to characterize the adsorption capabilities of the polymer. Additionally, we included Fe (II) and Fe (III) in the precipitation assays, as iron have been shown to precipitate others EPS of bacterial origin (Corzo et al. 1994). Therefore, the following ions were tested in the precipitation experiments: Pb (II), Cd (II), Co (II), Ni (II), Cu (II), Zn (II), Fe (II) and Fe (III). We studied the precipitation of the EPS by the metals at different ionic concentrations in the milimolar range (0-5 mM). Also the effect of the pH was evaluated (pH 3 and pH 6).

Within all these cations, only Fe (III) produced a significant precipitation of the polymer (more than 50% at pH 6). Fe (III) was also the unique cation that produced the precipitation of the EPS at the two pHs assayed. When the solutions of Fe (III) and EPS were combined, the solution became cloudy, and the precipitate could be easily removed by centrifugation, leaving a clear supernatant. The reactions were fast, and most of the precipitate was produced in less than 5 min. Fig. 4.4. shows the results of the precipitation of the polymer by Fe (III). In general, the precipitation of the EPS by Fe (III) increased with the concentration of the cation until the process reached a saturation point at 1-1.5 mM. The pH affected also the precipitation of the polymer by Fe (III). Ni (II) and Zn (II) also produced a slight precipitation of the EPS at pH 3, but the amount of the polymer precipitated by this two metals at 5 mM did not exceed in any case 10% of total EPS (9 ± 3.9% and 6.7 ± 0.52% for Zn and Ni respectively).

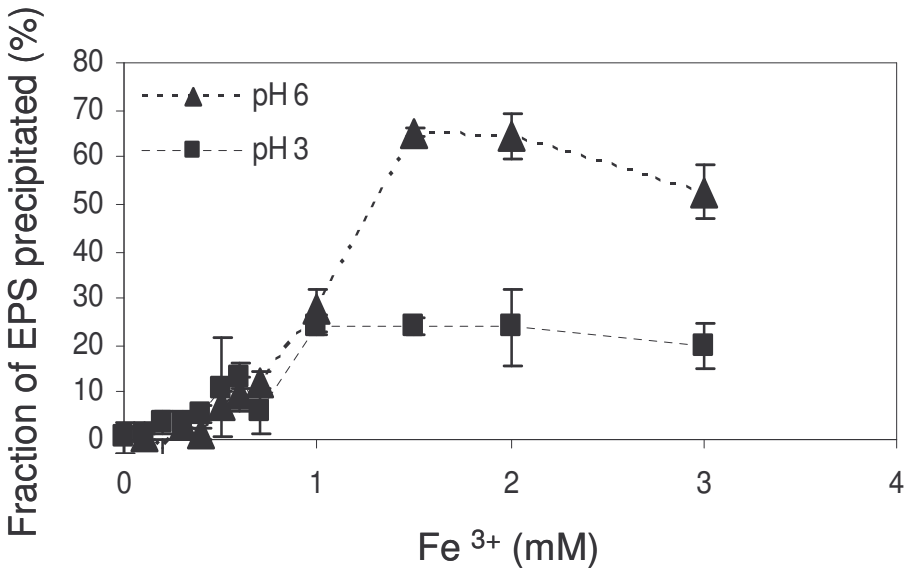

Fig. 4.4. Precipitation of the EPS by Fe (III). Experiments were performed at pH 3 and pH 6.

4.5. DISCUSSION

It is accepted that bacterial EPSs are able to interact with toxic metals by various mechanisms including van der Waals´ forces, electrostatic attraction and surface complexation (Gutnick and Bach 2000, Guibaud 2006). The results presented in this study show that the exopolysaccharide produced from *P. jamilae* presented binding-metal characteristics. Dialysis adsorption experiments evidenced that the polymer was able to remove different amounts of all the tested metallic ions [Pb (II), Cd (II), Co (II), Ni (II), Cu (II) and Zn (II)] from the aqueous solutions. The adsorption isotherms showed that Pb (II) ions presented a stronger interaction with the EPS than the other tested metals, with a maximum binding capacity (Q) of 303.03 mg of metal g^{-1} EPS. In the competitive biosorption experiments, the amount of lead adsorbed to the polymer appeared to be not influenced by the presence of the other five metals in solution at the same concentration (in a molar basis). In the case of the other metals, the uptake by the polymer was substantially inhibited in the mixture. Therefore, it can be concluded from these results that under the experimental conditions tested, the EPS produced in this study adsorbed specifically lead. Other researchers reported high affinity of lead to microbial exopolymers. For

example, the bacterial exopolymer produced by an hydrothermal bacteria (*Alteromonas macleodii* subsp *fijiensis*) adsorbed 316 mg/g of lead in optimal experimental conditions (Loaëc *et al*. 1997).

The comparison of the reported data on EPS-heavy metal uptake remains a difficult task, because of the wide taxonomic range of organisms tested (bacteria, fungi and algae), and the different methodologies, experimental conditions, and concentrations of the metals used. Different experimental factors that could alter the molecular environment of the binding sites, like the anion of the salt that it is used to prepare the metallic solutions (Pulsawat et al. 2003) or the pH of the solutions (Guibaud et al. 2006) can significantly affect the metal adsorption capacity. Also, the kind of the confining systems that are used to prepare the EPS-based filters is an important factor to take into account (Paperi et al. 2006).

Despite the relatively few functional groups potentially involved in cation binding, microbial polymers differ widely both in specificity and in their metal-binding capacity (Gutnick and Bach 2000). This different affinity of metals for the EPS can be attributed to charge density, attractive interaction and types of conformation of polymer with adsorbed ions (Salehizadeh *et al.* 2003). Therefore, information about the chemical composition of the EPS is helpful in order to understand the nature of the metal ion-EPS interactions. The elemental composition of the EPS (C/N=50.95 mol:mol) was compatible with the data obtained from spectrophotometric analysis. The polymer was basically a heteropolysaccharide rich in acidic sugars with a small (1.05% w/w) fraction of proteins. The high content (28.29%) in uronic acid (acidic polysaccharides) detected was in the range of other bacterial EPS with metal binding capacities (Salehizadeh *et al.* 2003).

The confirmation of the presence of functional groups involved in the binding of cations by the EPS is important. FTIR is able to simultaneously measure the vibrations of different functional groups in biological molecules. A number of studies have shown the usefulness of FTIR spectroscopy technique in the field of microbiology (Pradhan et al. 2007, Tindall et al. 2002, Galichet et al. 2001),

and more specifically in the study of bacterial EPSs (Guibaud et al. 2003, Bramhachari et al. 2006, Sheng et al. 2006). In this study, the analysis of the FTIR-spectrum confirmed the biochemical composition of the EPS, and more specifically the presence of ionisable functional groups able to react with the metal ions. The FTIR spectrum of the EPS produced by *P.jamilae* presented characteristic bands that could be attributed to functional groups existing in biological molecules, such as wide stretching hydroxyl group at 3400 cm^{-1} and a weak C-H stretching peak of methyl group at 2935 cm^{-1} (Bramhachari et al. 2006, Guibaud et al. 2003). Further, a band at 1655 cm^{-1} could be identified, corresponding to the vibration of the -CONH- group of Amide I in proteins (Schmitt and Fleming 1998, Pradhan et al. 2007, Sheng et al. 2006).[confirmar pico de (Amida II)] At wave numbers 950-1200 cm^{-1}, a broad stretching of C-O-C, C-O corresponded to carbohydrates (Lin and Rayson 1998 (en pradh), Pradhan et al. 2007). The peaks at 1000-1125 range are characteristic of uronic acid, *o*-acetyl ester linkage bond (Bramhachari et al. 2006).

We demonstrated that the EPS produced by *P.jamilae* precipitates in the presence of Fe (III), and that the complex can be easily separated from the aqueous solution by centrifugation. The EPS-metal precipitate did not form with Fe (II), Pb (II), Cd (II), Co (II), Ni (II), Cu (II) and Zn (II). Hence it can be conclude that despite the high binding of the EPS to Pb (II), the complex between this metal and the polymer remains “soluble”. The precipitation of EPS in the presence of Fe (III) showed a non-linear dependence on the concentration of the metal ion. This behaviour of the EPS-metal interaction was similar to the previously obtained in other metal-EPS interactions, like the coprecipitation of the EPS produced by *Bradyrhizobium* (*Chamaecytisus*) strain BGA-1 with Fe(III) (Corzo et al. 1994) and the interaction of the same polymer with Th (IV) (Díaz-Marrero et al. 2004). These authors suggested that the metal cations could interact with two carboxylate groups from different chains of the polysaccharide, acting as an electrostatic bridge between them, producing finally a mesh of polymer large enough to precipitate (Díaz-Marrero et al. 2004). Apparently, the formation of the complex between Fe (III) and the EPS produced by *P. jamilae* could be explained by a similar mechanism.

Due to the important ecological and practical implications of the interaction between EPSs and heavy metals, further detailed studies should be necessary to better understand these interactions and develop technologies directed to the bioremediation of heavy metal pollution.

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Conclusiones

The main general conclusions of this Thesis are:

1. The indigenous microbiota of alpeorujo, through stimulation of the fungal fraction, is able to degrade the polyphenolic content without the inoculation os specific microorganisms.

2. Predominant fungi identified included members of the genera *Penicillium*, *Candida*, *Geotrichum*, *Pichia*, *Cladosporium* and *Aschochyta.*

3. The combination of PLFA and PCR-TTGE with specific primers is a powerful tool for evaluating the complex composition of the microbial communities of alpeorujo waste under different treatment regimes.

4. *Paenibacillus jamilae* is able to grow and produce EPS in aqueous extracts of alpeorujo as unique source of carbon.

5. Maximal polymer yield in 100 ml batch-culture experiments (2 g l^{-1}) was obtained in cultures prepared with an aqueous extract of 20% TPOMW (w/v).

6. Lead biosorption was tenfold higher (in terms of mg of metal adsorbed per g of EPS) than the adsoprtion of the rest of metals.

Las principales conclusiones generales de la presente tesis doctoral son:

1. La microbiota indígena del alpeorujo, a través de la estmulación de la fracción fúngica mediante la adición de nutrientes (N y P), es capaz de degradar el contenido fenólico sin la inoculación de microorganismos específicos.

2. Los hongos predominantes identificados en el proceso de bioremediación pertenecieron a los géneros *Penicillium*, *Candida*, *Geotrichum*, *Pichia*, *Cladosporium* and *Aschochyta.*

3. La combinación de las técnicas PLFA y PCR-TTGE con primers específicos es una herramienta útil para evaluar la composición compleja de las comunidades microbianas del alpeorujo en diferentes condiciones experimentales.

4. *Paenibacillus jamilae* puede crecer y producir EPS a partir de extractos acuosos de alpeorujo.

5. La máxima producción de polímero (2 g l^{-1}) se obtuvo en cultivos preparados con un extracto acusos de alpeorujo al 20%.

6. La biosorción de plomo fue diez veces mayor (en términos de mg de metal absorbido por g de EPS) que la adsorción del resto de los cationes.