UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Enxeñaría Química

Laccase-based technologies to remove organic pollutants from soils and wastewaters

Memoria presentada por:

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Para optar al grado de Doctor por la
Universidad de Santiago de Compostela

Santiago de Compostela, Septiembre de 2015

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Informan:

Que la memoria titulada "Laccase-based technologies to remove organic pollutants from soils and wastewaters" para optar al grado de Doctor de Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, que presenta Doña Adriana Arca Ramos, ha sido realizada bajo nuestra inmediata dirección en el Departamento de Ingeniería Química de la Universidad de Santiago de Compostela.

Y para que así conste, firman el presente informe en Santiago de Compostela, el 14 de Septiembre de 2015.

Mª Teresa Moreira

Gemma Eibes

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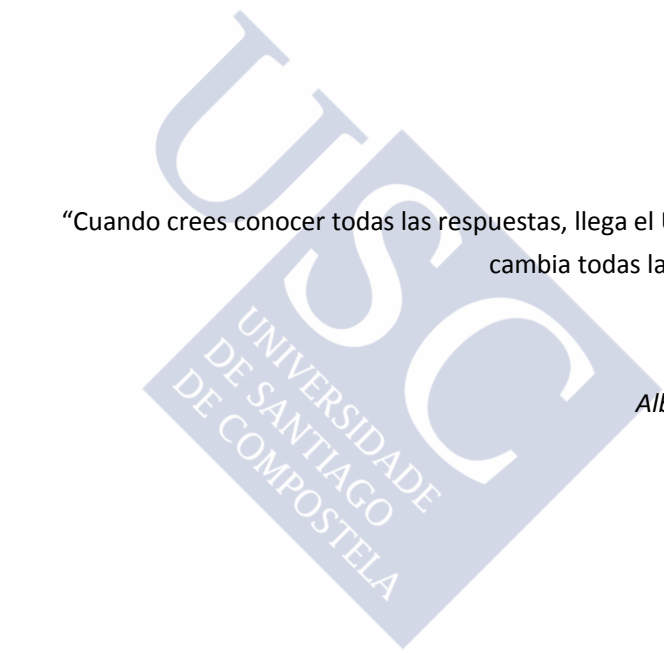

"Cuando crees conocer todas las respuestas, llega el Universo y te cambia todas las preguntas"

Albert Espinosa

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RESUMEN

La contaminación del suelo y del agua es un problema ambiental a nivel mundial. Por ello, el desarrollo e implementación de tratamientos ecológicos y de bajo coste para la descontaminación de suelos y aguas residuales constituye una prioridad. En este sentido, el empleo de agentes biológicos, tales como los hongos de podredumbre blanca, para la degradación de contaminantes ambientales es una alternativa interesante. Estos microorganismos han demostrado la capacidad de eliminar un amplio rango de compuestos xenobióticos mediante la acción de las enzimas extracelulares modificadoras de la lignina, tales como peroxidasas y lacasas. Las lacasas (oxidasas con contenido en cobre, EC 1.10.3.2) son biocatalizadores prometedores debido a su amplia especificidad de sustrato y bajos requerimientos catalíticos: usan oxígeno del aire, en lugar de peróxido de hidrógeno requerido por las peroxidasas, y liberan agua como único subproducto. Tanto las lacasas como el sistema lacasa-mediador se han usado con éxito para eliminar un amplio rango de contaminantes y la toxicidad asociada. En la presente investigación se exploró el potencial oxidativo de las lacasas para desarrollar tecnologías en continuo o semicontinuo dirigidas a la eliminación de contaminantes orgánicos, tales como Hidrocarburos Aromáticos Policíclicos (HAP) y contaminantes orgánicos emergentes en matrices ambientales reales.

En la primera sección de la Tesis, se investigó el uso de lacasas para degradar HAPs. Estos contaminantes generan gran preocupación debido a su potencial mutagénico y cancerígeno, y alta persistencia en el medio ambiente. Su biodegradación natural está restringida por dos factores: la baja solubilidad en agua y una alta hidrofobicidad, que hacen de los HAPs contaminantes persistentes en el suelo. La extracción con disolventes o surfactantes es una alternativa para eliminar HAPs de suelos. Sin embargo, esta técnica por sí sola no

destruye los contaminantes, sino que solo consigue su transferencia a otra fase. La eliminación enzimática con lacasa podría ser una opción factible, pero requiere la adición de surfactantes o disolventes para incrementar la biodisponibilidad del contaminante a la acción enzimática. La combinación de la extracción con disolventes y la tecnología basada en reactores bifásicos puede constituir un proceso prometedor de remediación dirigido a eliminar y degradar compuestos hidrofóbicos de suelos. Los reactores bifásicos se basan en el uso de un disolvente inmiscible en agua y biocompatible, en contacto con una fase acuosa que contiene el biocatalizador. El disolvente se usa para disolver altas concentraciones del compuesto objetivo, que luego se transfiere a la fase acuosa en concentraciones establecidas por el coeficiente de reparto. Dado que el biocatalizador, microorganismo o enzima, sólo es capaz de eliminar el compuesto en la fase acuosa, este se va transfiriendo desde la fase orgánica para re-establecer el equilibrio, hasta que la fase orgánica queda libre del contaminante.

En el **Capítulo 2** se evaluó la degradación de antraceno, el compuesto modelo de baja solubilidad, en reactores con lacasa. Se consideraron tres configuraciones diferentes: micelar, bifásica y la combinación de ambas, con el objetivo de operar con concentraciones de antraceno considerablemente superiores a la restringida por la solubilidad en la fase acuosa. En el sistema micelar, el empleo de un surfactante (Triton X-100) en una concentración por encima de la concentración micelar crítica (CMC) incrementó la solubilidad del antraceno y facilitó su degradación. Además, este surfactante ejerció un efecto beneficioso sobre la estabilidad de la lacasa y la protegió frente a la acción oxidativa del mediador 1-hidroxibenzotriazol (HBT). Como segunda opción, se consideró el uso de un reactor bifásico para permitir el tratamiento de altas cargas de antraceno. La fase orgánica consistió en aceite de silicona (10% v:v) saturado con antraceno, mientras que la fase acuosa comprendió el sistema lacasa-mediador. Sin embargo, la velocidad de degradación de antraceno en este reactor bifásico fue insatisfactoriamente lenta, debido a la baja solubilidad de antraceno en la fase acuosa y a la limitada transferencia de masa de antraceno desde la fase orgánica a la acuosa. Con el objetivo de superar estos inconvenientes, se propuso la combinación de un reactor bifásico con la adición del surfactante Triton X-100 (1% v:v) en la fase acuosa. Este sistema logró la

degradación de antraceno a una velocidad de conversión elevada: 16 µmol/L_R·h, demostrando que el empleo de un reactor bifásico con surfactante puede ser una solución para superar las limitaciones relacionadas con la transferencia del HAP desde la fase orgánica a la acuosa. La antraquinona se identificó como el principal producto de degradación del antraceno, aunque en concentración inferior a las estequiométrica. Se desarrolló un modelo para la degradación de antraceno por el sistema lacasa-mediador. Este se basó en la premisa de que el HAP debe primero disolverse en la fase acuosa para estar disponible al ataque de la lacasa. El modelo consistió en dos ecuaciones diferenciales para considerar los balances de masa en ambas fases, orgánica y acuosa. La constante cinética de pseudo-primer orden (k) y el coeficiente global de transferencia de masa (K_La) fueron estimados mediante un análisis de regresión por mínimos cuadrados. El elevado valor obtenido para el K_La: 788,1 h^{-1}, probó que el Triton X-100 mejoraba la transferencia de masa de antraceno desde el aceite de silicona a la fase acuosa. La desactivación enzimática ocurrió en dos etapas y pudo modelarse mediante un modelo biexponencial de tres parámetros. Finalmente, se demostró la posibilidad de reutilizar la fase acuosa en varios ciclos hasta agotar su potencial oxidativo. Además, se demostró la factibilidad de reutilizar el aceite de silicona para disolver más antraceno en tres ciclos consecutivos de operación en el reactor bifásico, con porcentajes de eliminación de antraceno elevados.

A pesar de los prometedores resultados obtenidos en el Capítulo 2, el empleo del aceite de silicona como fase orgánica en el reactor bifásico implica una serie de inconvenientes, como alto coste y disponibilidad limitada. Además, el hecho de no ser biodegradable puede constituir una limitación para su aplicación como agente de extracción en suelos. El uso de aceites vegetales biodegradables como fase orgánica en reactores bifásicos, en lugar de aceites minerales, conlleva un alto número de ventajas, tales como menor coste, menor toxicidad y una alta eficacia en la extracción de contaminantes hidrofóbicos de suelos. De hecho, en investigaciones anteriores se demostró que los aceites vegetales se pueden aplicar en procesos de remediación dirigidos a la extracción de contaminantes orgánicos. Por tanto, con el objetivo de hacer del reactor bifásico una opción viable, más próxima a una aplicación real, en el **Capítulo 3** se investigó la factibilidad de oxidar antraceno por lacasa empleando aceite de

orujo de oliva o de girasol como fase orgánica en el reactor bifásico con surfactante. La mayor velocidad de oxidación de antraceno se obtuvo con aceite de orujo de oliva. Además, se investigó la influencia de varios factores, tales como el nivel de oxígeno, concentración de mediador y adición de lacasa para maximizar la eliminación de antraceno. Se observó que la concentración de oxígeno disuelto y la de HBT limitaban la velocidad y avance del proceso de oxidación. La estrategia consistente en su adición en pulsos condujo a la eliminación total de antraceno tras 48 h, con una velocidad de conversión de 30,3 $\mu mol/L_R \cdot h$. Los resultados de degradación de antraceno en el reactor bifásico bajo la estrategia más favorable fueron modelados mediante la aplicación de balances de masas en la fase orgánica y acuosa. Para este sistema, el coeficiente global de transferencia de masa K_La resultó dos veces superior al obtenido para el mismo reactor bifásico operado con aceite de silicona. Este hecho fue consecuencia de la menor tensión interfacial del sistema aceite de orujo-1% Triton X-100 en comparación con el sistema aceite de silicona-1% Triton X-100. La constante cinética de pseudo-primer orden también se vio incrementada respecto al trabajo previo con aceite de silicona debido, probablemente, a la mayor concentración de mediador en el medio de reacción, así como a la presencia de oxi-radicales resultantes de la oxidación lipídica del aceite de orujo.

Hasta aquí los resultados sugerían la potencial aplicabilidad de los reactores bifásicos con enzimas oxidativas como un tratamiento adecuado para la regeneración de aceites vegetales contaminados con compuestos hidrofóbicos. Sin embargo, todavía existía la necesidad de combinar el proceso de extracción del suelo contaminado y la regeneración del disolvente en el reactor bifásico. Por este motivo, en el **Capítulo 4** se investigó un proceso novedoso para la eliminación de antraceno de suelo. El proceso se inició con la extracción de antraceno, presente en una alta concentración (1004 mg/kg), del suelo, mediante el uso de aceite de orujo de oliva como agente de extracción. A continuación se trató la fracción orgánica en un reactor bifásico con surfactante operado con el sistema lacasa-mediador. Los principales resultados de este estudio mostraron una alta eficacia de extracción de antraceno tanto por aceite de orujo fresco, como regenerado (superior al 84%) y la eliminación casi completa del contaminante tras 48 h de operación en el reactor bifásico.

Además, la posibilidad de reutilizar tanto la fase orgánica como la acuosa en sucesivos ciclos de degradación de antraceno en el reactor bifásico evidenció los bajos requerimientos del sistema de degradación.

La segunda sección de la Tesis se centró en la aplicación del potencial catalítico de la lacasa para eliminar contaminantes orgánicos emergentes (EOCs, por sus siglas en inglés) de sistemas acuosos. El término EOC abarca una amplia gama de productos químicos utilizados en todo el mundo y sus metabolitos, presentes en las aguas en concentraciones traza, y no sujetos todavía a las normativas existentes de calidad del agua, o en proceso de regulación. Su presencia en las masas de agua genera preocupación ya que constituyen una posible amenaza para los ecosistemas ambientales y la salud humana. El compuesto químico industrial bisfenol A (BPA), que se sabe que es un disruptor endocrino, y los compuestos con actividad farmacéutica (PhACs, por sus siglas en inglés) son ejemplos de EOCs detectados con frecuencia en aguas residuales, consideradas como la principal fuente de entrada de los EOCs al medio ambiente. La aplicación de las lacasas en tratamientos terciarios de agua residual requiere la retención del biocatalizador en el reactor y para cumplir dicho requisito se pueden considerar dos opciones.

La primera opción se basa en el uso de la enzima libre en un Reactor Enzimático de Membrana (REM). Esta opción se investigó en el **Capítulo 5** para llevar a cabo la transformación de BPA catalizada por lacasa. En primer lugar se evaluó el efecto de parámetros clave, como tipo de lacasa, pH o actividad enzimática en experimentos en discontinuo. La lacasa de *Trametes versicolor*, de alto potencial redox, fue más eficiente en la oxidación del BPA que la lacasa de *Myceliophthora thermophila*, una lacasa de bajo potencial redox, pero de bajo coste. El pH afectó de manera significativa a la actividad enzimática: a pH neutro la lacasa fue más estable que a pH ácido, pero la velocidad de eliminación del BPA fue inferior. La transformación del BPA por lacasa también dependió de la actividad enzimática inicial, de modo que las velocidades de transformación más elevadas se obtuvieron para la mayor actividad enzimática evaluada, que fue de 1000 U/L. A continuación, las mejores condiciones establecidas a partir de los experimentos en discontinuo (pH 6 y 1000 U/L de lacasa de *T. versicolor*) fueron aplicadas en el REM en continuo. La configuración del reactor consistió en un reactor de tanque agitado acoplado a

una membrana cerámica, lo que impidió la adsorción del contaminante y permitió la recuperación y recirculación de la lacasa. Se llevaron a cabo experimentos con una concentración de BPA de 75 µg/L y diferentes tiempos de residencia hidráulico (TRH): 2,5, 5, 7,5 y 10 h. Los porcentajes de eliminación del BPA estuvieron por encima del 93% para todos los TRH evaluados, y para el TRH de 10 h, se logró la conversión completa del BPA, manteniéndose a la vez una actividad enzimática bastante constante durante el período de operación de 50 h. Finalmente, se operó el REM con efluente secundario de una estación depuradora de agua residual municipal (EDAR) y la misma concentración de BPA para evaluar el efecto de la matriz real sobre la acción catalítica de la lacasa. En este caso, la eliminación del BPA se mantuvo alta (por encima del 94.5%), mientras que la presencia de coloides, ciertos iones y la formación de precipitados sobre la membrana posiblemente afectaron a la estabilidad enzimática, haciendo necesaria la adición periódica de enzima. Se vio que la polimerización y la degradación eran los mecanismos probables de transformación de BPA por la lacasa.

Una segunda opción para permitir la reutilización de la enzima reside en el uso de lacasas insolubilizadas o inmovilizadas para facilitar su retención en sistemas en continuo. Además, la inmovilización de enzimas demostró ser una herramienta útil para incrementar su estabilidad. En el **Capítulo 6**, lacasas de *Trametes versicolor* (*TvL*) y *Myceliophthora thermophila* (*MtL*), fueron inmovilizadas y coinmovilizadas en nanopartículas de sílice ahumada (fsNP) utilizando glutaraldehido como agente entrecruzante. Para las dosis óptimas de *TvL*, *MtL y* lacasas coinmovilizadas, se obtuvieron cargas enzimáticas de 1,78±0,07, 0,69±0,03 y 1,10±0,01 U/mg fsNP, respectivamente. En general, los conjugados de lacasa-fsNP mostraron una mayor resistencia que las lacasas libres frente a pH ácido, y también mayor estabilidad de almacenamiento, especialmente cuando se incubaron en efluente secundario de una EDAR. Se evaluó la capacidad de los conjugados lacasa-fsNP para eliminar una mezcla de concentraciones conocidas de 14C-BPA y del compuesto farmacéutico 14C-diclofenaco sódico (DCF) de un efluente secundario en experimentos en discontinuo. La eficiencia catalítica fue altamente dependiente del origen del biocatalizador y del estado. La lacasa de *T. versicolor*, en forma libre alcanzó un porcentaje de transformación de BPA 4-veces superior que el de la lacasa libre

de *M. thermophila.* En comparación con las lacasas libres, las enzimas inmovilizadas condujeron a velocidades de transformación de BPA mucho menores. Por ejemplo, tras 24 h, los porcentajes de transformación de BPA por 1000 U/L por una mezcla de enzimas libres y co-inmovilizadas fueron 67,8 ± 5,2 y 27,0 ± 3,9%, respectivamente. No obstante, el uso de 8000 U/L de lacasa coinmovilizada condujo a eliminación casi completa del BPA, a pesar de las desfavorables condiciones para la acción catalítica de lacasa (pH~ 8,4). No se observó transformación de DCF por ninguno de los sistemas enzimáticos, lo que evidenció su carácter recalcitrante frente a la oxidación por lacasa en condiciones reales.

En el **Capítulo 7** se investigó la insolubilización de lacasa como CLEAs para incrementar la estabilidad y reuso del biocatalizador. Dado que los CLEAs convencionales presentan generalmente malas propiedades mecánicas, se propuso el uso de microesferas magnéticas mesoporosas de sílice (MMSMB) como soporte para producir CLEAs mecánicamente resistentes y magnéticamente separables (MCLEAs). Se investigó el efecto de varios parámetros como el tiempo de entrecruzamiento, adición de albúmina de suero bovino (ASB), pH, concentración de glutaraldehido y el ratio de lacasa: MMSMB. Las mejores condiciones evaluadas: 2 h de tiempo de entrecruzamiento, ausencia de ASB, pH 7, glutaraldehido en concentración 5 mM y una relación de lacasa: MMSMB de 1:2 (w:w), permitieron la rápida preparación de MCLEAs con alta carga de enzima: 1,53 U de lacasa/mg MCLEAs. Los MCLEAs presentaron mayor estabilidad frente a pH ácido, presencia de químicos desnaturalizantes y matriz real de agua residual, en comparación con la enzima libre. Además, el novedoso biocatalizador exhibió una buena estabilidad operacional, manteniendo hasta un 70% de su actividad inicial tras 10 ciclos consecutivos de reacción, operados con separación magnética. Con el objetivo de evaluar la capacidad catalítica de los MCLEAs, estos se aplicaron para la eliminación de una mezcla de 10 compuestos farmacéuticos: acetaminofeno, ketoprofeno, ciclofosfamida, ácido mefenámico, cafeína, indometacina, naproxeno, fenofibrato, trimetoprim e ibuprofeno en concentraciones en el rango de 10-50 µg/L de un efluente secundario real. Los MCLEAs mostraron la capacidad de transformar el compuesto fenólico acetaminofeno y ciertos PhACs no fenólicos como el ácido mefenámico, indometacina y fenofibrato, con una eficiencia

similar o incluso superior a la de la lacasa libre. Los otros PhACS no sufrieron transformación apreciable ni por la lacasa libre ni por los MCLEAs.

RESUMO

A contaminación do solo e da auga é un problema ambiental a nivel mundial. Polo tanto, o desenvolvemento e posta en marcha de tratamentos ecolóxicos e de baixo custo dirixidos á descontaminación de solos e de augas residuais é unha prioridade. Neste sentido, o emprego de axentes biolóxicos como fungos de podremia branca para a degradación de contaminantes ambientais é unha alternativa interesante. Estes microorganismos demostraron a súa capacidade para eliminar un amplo rango de compostos xenobióticos mediante a acción das enzimas extracelulares modificadoras da lignina, tales como peroxidasas e lacasas. As lacasas (oxidasas con contido en cobre, EC 1.10.3.2) son biocatalizadores prometedores debido á súa ampla especificidade de substrato e baixos requirimentos catalíticos: usan osíxeno do aire, en lugar do peróxido de hidróxeno requirido polas peroxidasas, e liberan auga como único subproduto. Tanto as lacasas como o sistema lacasa-mediador empregáronse con éxito para eliminar un amplo rango de contaminantes e a toxicidade asociada.

Na presente investigación explorouse o potencial oxidativo das lacasas para desenvolver tecnoloxías dirixidas á eliminación en continuo ou semi-continuo de contaminantes orgánicos, tales como hidrocarburos aromáticos policíclicos (HAPs) e contaminantes orgánicos emerxentes en matrices ambientais reais.

Na primeira sección da tese, investigouse o uso da lacasa para degradar HAPs. Estes contaminantes xeran preocupación debido ó seu potencial mutaxénico e carcinoxénico, e á alta persistencia no medio ambiente. A súa degradación natural está limitada por dous factores: baixa solubilidade e unha alta hidrofobicidade que fai dos HAPs contaminantes persistentes en solos. A

extracción con disolventes ou tensioactivos é unha alternativa para a eliminación de HAPs do solo. Con todo, esta técnica por si soa non logra destruír os contaminantes, senón que soamente consigue a súa transferencia a outra fase. A eliminación enzimática coa lacasa podería ser unha opción factible, pero require a adición de tensioactivos ou disolventes para aumentar a biodispoñibilidade dos contaminantes ó ataque enzimático. A combinación da extracción con disolventes e a tecnoloxía baseada en reactores bifásicos podería constituír un proceso prometedor para eliminar e degradar compostos hidrófobos presentes en solos. Os reactores bifásicos baséanse no uso dun disolvente inmiscible en auga e biocompatible, en contacto cunha fase acuosa que contén o biocatalizador. O disolvente úsase para disolver altas concentracións do composto obxectivo, que logo se transfire á fase acuosa en concentracións establecidas polo coeficiente de reparto. Posto que o catalizador, microorganismo ou enzima, só é capaz de eliminar o composto na fase acuosa, este vaise transferindo desde a fase orgánica para restablecer o equilibrio, ata que a fase orgánica queda libre do contaminante.

No **Capítulo 2** avaliouse a degradación do antraceno, o composto modelo de baixa solubilidade, en reactores con lacasa. Consideráronse tres configuracións diferentes: micelar, bifásica e a combinación de ambas, coa finalidade de operar con concentracións de antraceno considerablemente superiores á restrinxida pola solubilidade na fase acuosa. No sistema micelar, o emprego dun tensioactivo (Triton X-100) nunha concentración superior á concentración micelar crítica (CMC) incrementou a solubilidade do antraceno e facilitou a súa degradación. Ademais, este tensioactivo exerceu un efecto beneficioso para a estabilidade da lacasa e protexeuna contra a acción oxidativa do mediador 1-hidroxibenzotriazol (HBT). Como segunda opción, considerouse o emprego dun reactor bifásico para permitir o tratamento de altas cargas de antraceno. A fase orgánica consistiu en aceite de silicona (10% v:v) saturado con antraceno, mentres que a fase acuosa comprendeu o sistema lacasa-mediador. Con todo, a velocidade de degradación do HAP no reactor bifásico resultou insatisfactoriamente lenta, debido á baixa solubilidade do antraceno na fase acuosa e á limitada transferencia de masa de antraceno desde a fase orgánica á acuosa. Co obxectivo de superar estas limitacións, propúxose a combinación dun reactor bifásico coa adición do tensioactivo Triton X-100 (1% v:v) na fase acuosa.

Este sistema logrou a degradación de antraceno a unha velocidade de conversión elevada: 16 µmol/L_R·h, demostrando que o emprego dun reactor bifásico con tensioactivo pode ser unha solución para superar as limitacións vinculadas coa transferencia de masa do HAP desde a fase orgánica á fase acuosa. A antraquinona identificouse como o principal produto de degradación do antraceno, aínda que en concentración inferior á estequiométrica. Desenvolveuse un modelo para a degradación do antraceno polo sistema lacasa-mediador. Este baseouse na premisa de que o HAP debe de disolverse na fase acuosa para estar dispoñible ó ataque catalítico da lacasa. O modelo compúxose de dúas ecuacións diferenciais para considerar os balances de masa en ambas fases, orgánica e acuosa. A constante cinética de pseudo-primeira orde (*k*) e o coeficiente global de transferencia de masa (K_La) estimáronse mediante unha análise de regresión por mínimos cadrados. O elevado valor obtido para o K_La: 788,1 h^{-1}, probou que o Triton X-100 melloraba a transferencia de masa do antraceno desde o aceite de silicona á fase acuosa. A desactivación enzimática ocorreu en dúas etapas e puido modelarse mediante un modelo biexponencial de tres parámetros. Finalmente, demostrouse a posibilidade de reutilizar a fase acuosa en varios ciclos ata extinguir o seu potencial oxidativo. Ademais, demostrouse a viabilidade de reutilizar o aceite de silicona para disolver máis antraceno en tres ciclos consecutivos no reactor bifásico con porcentaxes de eliminación de antraceno elevados.

A pesar dos prometedores resultados obtidos no Capítulo 2, o emprego do aceite de silicona como fase orgánica no reactor bifásico implicou unha serie de inconvenientes, como alto custo ou dispoñibilidade limitada. Ademais, o feito de non ser biodegradable pode constituír unha limitación para a súa aplicación como axente de extracción en solos. O uso de aceites vexetais biodegradables como fase orgánica en reactores bifásicos, en lugar de aceites minerais, implica un gran número de vantaxes, tales como menor custo, menor toxicidade e alta eficacia na extracción de contaminantes hidrofóbicos de solos. De feito, en investigacións anteriores demostrouse que os aceites vexetais poden ser aplicados en procesos de remediación dirixidos á extracción de contaminantes orgánicos. Así pois, a fin de facer do reactor bifásico unha opción viable, máis próxima a unha aplicación real, no **Capítulo 3** investigouse a viabilidade de oxidar antraceno por lacasa usando aceite de bagazo de oliva ou de xirasol como

fase orgánica no reactor bifásico con tensioactivo. A maior velocidade de oxidación de antraceno obtívose co aceite de bagazo de oliva. Ademais, investigouse a influencia de varios factores, tales como nivel de osíxeno, concentración de mediador e adición de lacasa para maximizar a eliminación do antraceno. Observouse que a concentración de osíxeno disolto e a de HBT limitaban a velocidade e o avance do proceso de oxidación. A estratexia consistente na súa adición en pulsos conduciu á eliminación total do antraceno tras 48 h, cunha velocidade de conversión de 30,3 µmol/L_R.h. Os resultados de degradación de antraceno no reactor bifásico con tensioactivo baixo a estratexia máis favorable modeláronse mediante a aplicación de balances de masa na fase orgánica e acuosa. Para este sistema, o coeficiente global de transferencia de masa, K_La resultou dúas veces superior ó obtido para o mesmo reactor bifásico operado con aceite de silicona. Este feito foi consecuencia da menor tensión interfacial do sistema aceite de bagazo-Triton X-100 en comparación co sistema aceite de silicona-1% Triton X-100. A constante cinética de pseudo-primeira orde tamén se viu incrementada respecto ó traballo previo con aceite de silicona, probablemente debido á maior concentración de mediador no medio de reacción así como á presenza de oxi-radicais resultantes da oxidación lipídica do aceite de bagazo.

Ata o de agora, os resultados suxerían a potencial aplicabilidade dos reactores bifásicos con enzimas oxidativas como un tratamento axeitado para a rexeneración de aceites vexetais contaminados con compostos hidrofóbicos. Con todo, aínda existía a necesidade de combinar o proceso de extracción do solo contaminado e a rexeneración do disolvente no reactor bifásico. Por este motivo, no **Capítulo 4** investigouse un proceso novidoso para a eliminación de antraceno do solo. O proceso iniciouse coa extracción de antraceno, presente nunha alta concentración (1004 mg/kg), do solo, mediante o uso do aceite de bagazo como axente de extracción. A continuación, a fracción orgánica tratouse nun reactor bifásico con tensioactivo operado co sistema lacasa-mediador. Os principais resultados deste estudio mostraron unha alta eficacia de extracción de antraceno, tanto polo aceite de bagazo fresco, como rexenerado (superior ó 84%) e case a eliminación completa do contaminante no reactor bifásico tras 48 h. Ademais, a posibilidade de reutilizar tanto a fase orgánica como a acuosa en

ciclos sucesivos de degradación do antraceno no reactor bifásico evidenciou os baixos requirimentos do sistema de degradación.

A segunda parte da Tese centrouse na aplicación do potencial catalítico da lacasa para eliminar contaminantes orgánicos emerxentes (EOC, polas siglas en inglés) de sistemas acuosos. O termo EOC comprende unha ampla gama de produtos químicos utilizados en todo o mundo e os seus metabolitos, comunmente presentes en auga a concentracións traza e non suxeitos aínda a normativas existentes de calidade da auga, ou en proceso de regulación. A súa presenza en masas de auga xera preocupación, xa que supoñen unha posible ameaza para os ecosistemas ambientais e a saúde humana. O composto químico industrial bisfenol A (BPA), que se sabe que é un disruptor endocrino, e os compostos con actividade farmacéutica (PhACs, polas siglas en inglés) son exemplos de EOCs detectados con frecuencia en augas residuais, consideradas como a principal fonte de entrada dos EOCs ó medio ambiente. A aplicación das lacasas en tratamentos terciarios de auga residual require a retención do biocatalizador no reactor e para responder a esta esixencia pódense considerar dúas opcións.

A primeira opción baséase no uso da enzima libre nun reactor enzimático de membrana (EMR). Esta opción foi investigada no **Capítulo 5** para levar a cabo a transformación do BPA catalizada pola lacasa. En primeiro lugar, avaliouse o efecto de parámetros chave, tales como o tipo de lacasa, pH ou actividade enzimática en experimentos en discontinuo. A lacasa de *Trametes versicolor*, de alto potencial redox, foi máis eficiente na oxidación do BPA que a lacasa de *Myceliophthora thermophila*, unha lacasa de baixo potencial redox, pero de baixo custo. O pH afectou de xeito significativo á actividade enzimática: a pH neutro a lacasa foi máis estable que a pH ácido, pero a velocidade de eliminación do BPA foi inferior. A transformación do BPA catalizada pola lacasa tamén dependeu da actividade enzimática inicial, e as velocidades de transformación máis altas obtivéronse para a maior actividade enzimática avaliada, que foi de 1000 U/L. As mellores condicións establecidas a partir dos experimentos en discontinuo (pH 6 e 1000 U/L de lacasa de *T. versicolor*) foron logo aplicadas no REM en continuo. A configuración do reactor consistiu nun reactor de tanque axitado acoplado a unha membrana cerámica, o que impediu a adsorción do contaminante e permitiu a recuperación e

recirculación da lacasa. Leváronse a cabo experimentos cunha concentración de BPA de 75 µg/L e diferentes tempos de residencia hidráulico (TRH): 2,5, 5, 7,5 e 10 h. As porcentaxes de eliminación do BPA estiveron por encima do 93% para todos os TRH avaliados, e para o TRH de 10 h lograse a conversión completa do BPA, manténdose ó mesmo tempo unha actividade enzimática bastante constante durante o período de operación de 50 h. Finalmente, operouse o REM con efluente secundario real dunha estación depuradora de auga residual municipal (EDAR) e a mesma concentración de BPA para avaliar o efecto da matriz real sobre a acción catalítica da lacasa. Neste caso, a eliminación do BPA mantívose alta (por encima do 94,5%), mentres que a presenza de coloides, certos ions e a formación de precipitados sobre a membrana posiblemente afectaron á estabilidade enzimática, facendo necesaria a adición periódica da lacasa. Viuse que a polimerización e a degradación eran os mecanismo probables de transformación de BPA pola lacasa.

Unha segunda opción para permitir a reutilización da enzima reside no uso de lacasas insolubilizadas ou inmobilizadas para facilitar a súa retención en sistemas en continuo. Ademáis, a inmovilización de enzimas demostrou ser unha ferramenta útil para incrementar a súa estabilidade. No **Capítulo 6**, lacasas de *Trametes versicolor* (*TvL*) e *Myceliophthora thermophila* (*MtL*), foron inmobilizadas e coinmobilizadas en nanopartículas de sílice afumada (fsNP) utilizando glutaraldehido como axente entrecruzante. Para as doses óptimas de *TvL*, *MtL* e lacasas coinmobilizadas, obtivéronse cargas enzimáticas de 1,78±0,07, 0,69±0,03 e 1,10±0,01 U/mg fsNP, respectivamente. En xeral, os conxugados de lacasa-fsNP mostraron unha maior resistencia que as lacasas libres fronte a un pH ácido, e tamén maior estabilidade de almacenamento, especialmente cando se incubaron en efluente secundario dunha EDAR. Avaliouse a capacidade dos conxugados de lacasa-fsNP para eliminar unha mestura de concentracións coñecidas de 14C-BPA e do composto farmacéutico 14C-diclofenaco sódico (DCF) dun efluente secundario en experimentos en discontinuo. A eficiencia catalítica foi altamente dependente da orixe do biocatalizador e do estado. A lacasa de *T. versicolor* en forma libre acadou unha porcentaxe de transformación de BPA 4-veces superior que a da lacasa libre de *M. thermophila.* En comparación coas lacasas libres, as enzimas inmobilizadas deron lugar a velocidades de transformación de BPA moito máis baixas. Por exemplo, tras 24 h, a porcentaxe

de transformación BPA por 1000 U/L dunha mestura de enzimas libres e coinmobilizadas foron 67,8 ± 5,2 e 27,0 ± 3,9%, respectivamente. Con todo, o uso de 8000 U / L de lacasa coinmobilizada levou á eliminación case total do BPA, a pesar das condicións desfavorables para a acción catalítica da lacasa (pH ~ 8,4). Non se observou transformación do DCF por ningún dos sistemas enzimáticos, mostrando o seu carácter recalcitrante fronte á oxidación pola lacasa en condicións reais.

No **Capítulo 7**, investigouse a insolubilización da lacasa como CLEAs para incrementar a estabilidade e reuso do biocatalizador. Posto que os CLEAs convencionais presentan xeralmente malas propiedades mecánicas, propúxose o uso de microesferas magnéticas de sílice mesoporosa (MMSMB) como soporte para producir CLEAs mecanicamente resistentes e magneticamente separables (MCLEAs). Investigouse o efecto de varios parámetros como o tempo de entrecruzamento, adición de albumina de soro bovino (ASB), pH, concentración de glutaraldehido e a razón de lacasa:MMSMB. As mellores condicións avaliadas, 2 h de tempo de entrecruzamento, ausencia de ASB, pH 7, glutaraldehido en concentración 5 mM e unha razón de lacasa: MMSMB de 1:2 (w: w), permitiron a rápida preparación de MCLEAs con alta carga de enzima: 1,53 U de lacasa/mg MCLEAs. Os MCLEAs presentaron maior estabilidade fronte a pH ácido, presenza de químicos desnaturalizantes e matriz real de auga residual, en comparación coa enzima libre. Ademais, o novidoso biocatalizador exhibiu unha boa estabilidade operacional, mantendo ata o 70% da súa actividade inicial tras 10 ciclos consecutivos de reacción operados con separación magnética. Co fin de avaliar a capacidade catalítica dos MCLEAs, estes aplicáronse para a eliminación dunha mestura de 10 compostos farmacéuticos: acetaminofeno, ketoprofeno, ciclofosfamida, ácido mefenámico, cafeína, indometacina, naproxeno, fenofibrato, trimetoprim e ibuprofeno en concentracións no rango de 10-50 µg/L dun efluente secundario real. Os MCLEAs mostraron a capacidade de transformar o composto fenólico acetaminofeno e certos PhACs non fenólicos, como o ácido mefenámico, indometacina e fenofibrato, cunha eficiencia similar ou incluso superior á da lacasa libre. Os restantes PhACs non sufriron transformación apreciable nin pola lacasa libre, nin polos MCLEAs.

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SUMMARY

Pollution of soil and water is an environmental issue worldwide. Thereby, the development and implementation of low-cost and eco-friendly treatments for the decontamination of polluted sites and wastewater is a priority. In this regard, the use of biological agents as white rot fungi to degrade and detoxify environmental contaminants is a potential alternative. These microorganisms have been reported to remove a wide range of xenobiotics by the action of the extracellular lignin-modifying enzymes, such as peroxidases and laccases. Laccases (copper-containing oxidases, EC 1.10.3.2) are promising biocatalysts due to their oxidative versatility and low catalytic requirements: they use oxygen from air, instead of hydrogen peroxide required by peroxidases, and release water as the sole by-product. Laccases as well as laccase-mediator systems have been successfully used to oxidatively detoxify and remove a great number of contaminants. The potential of laccase was explored in the current research aiming to develop continuous or semicontinuous technologies to remove pollutants of concern, such as Polycyclic Aromatic Hydrocarbons (PAHs) and Emerging Organic Contaminants (EOCs), from real environmental matrices.

In the first section of the Thesis, the use of laccase was investigated to degrade PAHs. These chemicals are of environmental concern because of their genotoxic and carcinogenic potential and high persistence in the environment. Their natural biodegradation is restricted by two major factors: low water solubility and high hydrophobicity, which make PAHs persistent pollutants in soil. Extraction with solvents or surfactants is an alternative to remove PAHs from soils. However, this technique by itself does not degrade the pollutants, but only attains their transfer to another phase. Enzymatic remediation with laccase may be a feasible alternative, but requires the addition of surfactants or solvents to increase the pollutant bioavailability for the enzymatic action. Coupling

solvent extraction and the technology based on Two Phase Partitioning Bioreactors (TPPB) may constitute a promising remediation process to remove and degrade hydrophobic pollutants present in soils. The TPPB concept is based on the use of a water immiscible and biocompatible organic solvent in contact with an aqueous phase that contains the biocatalyst. The solvent is used to dissolve high concentrations of the target compound, which then partitions into the aqueous phase at levels determined by its partition coefficient. As the biocatalyst, microorganisms or enzymes, are only able to degrade the pollutant in the aqueous phase, this one diffuses from the organic phase to restore the equilibrium. Subsequently, substrate delivery is maintained until the organic phase becomes completely depleted.

In **Chapter 2**, the degradation of anthracene, a poorly soluble model compound, in laccase-based reactors was evaluated. Three different configurations were considered: micellar, biphasic and the combination of both, in order to allow the operation with concentrations of anthracene considerably higher than that restricted by the solubility in aqueous phase. In micellar system, the use of a surfactant (Triton X-100) at concentration above critical micelle concentration (CMC) enhanced anthracene solubility and facilitated its degradation. Moreover, this surfactant exerted a beneficial effect on the laccase stability and protected it from the oxidative action of the mediator 1-hydroxybenzotriazole (HBT). In the second approach, a TPPB was considered to allow the enzymatic treatment of high loads of anthracene. The organic phase consisted of silicone oil (10% v:v) saturated with anthracene, whereas the aqueous phase comprised the laccase mediator system. Nevertheless, the degradation rate of anthracene in this TPPB was unsatisfactorily slow, due to the low solubility of anthracene in the aqueous phase and the limited mass transfer of anthracene from the organic to the aqueous phase. In order to overcome these limitations, the combination of a TPPB with the addition of the surfactant Triton X-100 (1% v:v) in the aqueous phase was proposed. This system achieved the degradation of anthracene at a high conversion rate: 16 $\mu mol/L_R \cdot h$, demonstrating that the use of a surfactant-assisted TPPB may be a solution to overcome problems related to the transfer of the PAH from the organic phase to the aqueous phase. Anthraquinone was obtained as the main oxidation product of anthracene, but in a lower concentration than that predicted by the

stoichiometry. A model for anthracene degradation by laccase-mediator system was developed. This was based on the assumption that PAH must be first dissolved in the aqueous phase to be available for the catalytic oxidation of laccase. The model coupled two differential equations to consider the mass balances in both organic and aqueous phases. The first order kinetic constant (*k*) and the overall mass transfer coefficient (K_La) were estimated by using the method of least squares. The increased K_La value obtained: 788.1 h^{-1}, proved that Triton X-100 improved mass transfer of anthracene from the silicone oil to the aqueous phase. Enzyme inactivation occurred in two stages and could be modeled by using a three parameter biexponential model. Finally, the possibility of reusing the aqueous phase in several cycles until negligible oxidative potential was demonstrated. Also, the feasibility of reusing the silicone oil to dissolve more anthracene was proven in three consequent cycles with high percentages of anthracene removal.

Despite the promising results obtained in Chapter 2, the use of silicone oil as organic phase in the TPPB involves several drawbacks as high cost and limited availability. In addition, its non biodegradability may constitute a limitation for its application as extraction agent in polluted soils. The use of biodegradable vegetable oils as organic phase in TPPBs instead of mineral oils involves a number of advantages, such as lower cost, lower toxicity and potential high efficiency in extracting hydrophobic contaminants from soils. In fact, previous research demonstrated that vegetable oils can be applied to extract organic contaminants for remediation purposes. Hence, aiming to make the TPPB process a viable alternative closer to a real application, the feasibility of anthracene oxidation by laccase using either sunflower or pomace olive oils as organic phase in a surfactant-assisted TPPB was investigated in **Chapter 3.** The highest oxidation of anthracene was obtained with pomace olive oil. Furthermore, the influence of several factors such as oxygen level, mediator concentration and laccase addition were investigated to maximize the removal of anthracene. Both dissolved oxygen and HBT concentrations were found to limit the rate and extension of the process. The strategy of their addition in pulses led to total removal of anthracene after 48 h, with a conversion rate of 30.3 $\mu mol/L_R.h$. The results of anthracene degradation in the surfactant-assisted TPPB under the most favorable strategy were modeled by applying mass

balances in the organic and aqueous phases. For this system, the overall mass transfer coefficient K_La resulted two times higher than the obtained for the analogous TPPB with silicone oil. This fact was consequence of the lower interfacial tension of the systems pomace olive oil—1% Triton X-100 in comparison to the system silicone oil—1% Triton X-100. The first order kinetic constant was also enhanced respect to the previous work with silicone oil, likely due to the higher concentration of mediator in the reaction medium as well as the presence of oxy radicals resulted from lipid oxidation of pomace olive oil.

So far the results suggested the potential application of TPPB with oxidative enzymes as a suitable treatment for the recovery of vegetable oils contaminated with hydrophobic pollutants. However, there was still the need to couple the extraction process of the polluted soil with the regeneration of the solvent in the TPPB. Consequently, a novel process for removing anthracene from soil was investigated in **Chapter 4**. The process started with the extraction of anthracene from the soil present in a high concentration (1004 mg/kg) by using pomace olive oil as extracting agent. The organic fraction was thereafter treated in the surfactant-assisted TPPB operated with laccase-mediator system. The main outcomes of this study showed high extraction efficiency of anthracene for both fresh and regenerated pomace olive oil (higher than 84%) and almost complete removal of the target pollutant in the TPPB after 48 h. In addition, the possibility of reusing both the aqueous and organic phases in successive batches of anthracene degradation in the TPPB was indicative of the low requirements of the degradation system.

The second section of the Thesis focused on applying the catalytic potential of laccase to remove EOCs from aqueous systems. The term EOC encompass a wide range of chemicals used worldwide and their metabolites, commonly present in waters at trace concentrations, and still not covered by existing water-quality regulations or in process of regulation. Their presence in water bodies is an issue of concern since these are potential threats to environmental ecosystems and human health. The industrial chemical bisphenol A (BPA), which is known as endocrine disruptor, and pharmaceuticals active compounds (PhACs) are examples of EOCs frequently detected in wastewaters, considered as the main source of EOCs entering the environment. The application of laccases in tertiary wastewater treatment requires the retention of the biocatalyst in the

reactor and two main approaches could be considered to fulfill such requirement.

The first approach relies on the use of the free enzyme in an Enzymatic Membrane Reactor (EMR). This was investigated in **Chapter 5** to perform the laccase-catalyzed transformation of BPA. Firstly, the influence of key parameters, namely, type of laccase, pH and enzyme activity was evaluated in batch experiments. The high redox potential laccase from *Trametes versicolor* was more efficient in oxidizing BPA than laccase from *Myceliophthora thermophila,* a low redox potential but commercially available low-cost laccase. The pH significantly affected the enzymatic activity: at neutral pH, laccase was more stable than at acid pH, but the removal rate of BPA was lower. Laccase catalyzed transformation of BPA was also dependent on the initial activity of the enzyme and the higher transformation rates were obtained for the highest enzymatic activity, which was 1000 U/L. The best conditions determined from batch experiments (pH 6 and 1000 U/L of laccase from *T. versicolor*), were then applied in the continuous EMR. The reactor configuration consisted of a stirred tank reactor coupled to a ceramic membrane, which prevented the adsorption of the pollutant and allowed the recovery and recycling of laccase. Experiments were performed at a BPA concentration of 75 µg/L and different hydraulic retention times (HRTs): 2.5, 5, 7.5 and 10 h. Removal percentages of BPA were above 93% for all the HRT evaluated, and for the TRH of 10 h, complete BPA conversion was achieved while maintaining the enzymatic activity fairly constant during the 50-h operation. Finally, the EMR was operated with spiked real secondary effluent from a municipal wastewater treatment plant (WWTP) to assess the effect of a real wastewater matrix on the catalytic action of laccase. In this case, the removal of BPA remained high (above 94.5%) while the presence of colloids, certain ions and the formation of precipitates on the membrane potentially affected enzyme stability and made necessary the periodic addition of laccase. Polymerization and degradation were observed as probable mechanisms of BPA transformation by laccase.

A second approach to allow the enzyme reuse relies on the use of insolubilized or immobilized laccases to facilitate its retention in continuous systems. In addition, enzymes immobilization was proved as a useful tool to increase their stability. In **Chapter 6**, laccases from *Trametes versicolor* (*TvL*) and

Myceliophthora thermophila (*MtL*), were successfully immobilized and co-immobilized onto fumed silica nanoparticles (fsNP) using glutaraldehyde as cross linking agent. Enzyme loads of 1.78±0.07, 0.69±0.03 and 1.10±0.01 U/mg fsNP were attained for the optimal doses of *TvL*, *MtL* and co-immobilized laccases, respectively. In general, the laccase-fsNP conjugates showed higher resistance than that of free laccase against acidic pH and also higher storage stability, especially when incubated in the secondary effluent from a WWTP. The ability of the laccase-fsNP to remove a mixture of 14C-BPA and the PhAC 14C-sodium diclofenac (DCF) from spiked secondary effluent was assessed in batch experiments. The catalytic efficiency was highly dependent on both, the microbial source of the biocatalyst and state. The high redox potential *TvL* as free enzyme attained a percentage of BPA transformation 4-fold higher than free *MtL*. Compared to free laccases, immobilized enzymes led to much slower rates of BPA transformation. For instance, after 24 h, the percentages of BPA transformation by 1000 U/L of a mixture of free and co-immobilized enzymes were 67.8±5.2 and 27.0±3.9%, respectively. Nevertheless, the use of 8000 U/L of co-immobilized laccase led to nearly complete removal of BPA, despite the unfavorable conditions for laccase catalysis (pH~ 8.4). DCF transformation was not observed for any of the enzymatic systems showing that this compound is highly recalcitrant towards laccase oxidation under realistic conditions.

In **Chapter 7**, insolubilization of laccases as cross-linked enzyme aggregates (CLEAs) was investigated to increase stability and reusability of biocatalyst. Since conventional CLEAs usually present poor mechanical properties, the use of magnetic mesoporous silica microbeads (MMSMB) as support was proposed to produce mechanical resistant and magnetically-separable CLEAs (MCLEAs). The effect of several parameters such as cross linking time, addition of bovine serum albumin (BSA) as protein feeder, pH, glutaraldehyde concentration and ratio laccase:MMSMB was investigated. The best evaluated conditions: 2 h of cross linking time, absence of BSA, pH 7, 5 mM glutaraldehyde and ratio laccase: MMSMB of 1:2 (w:w), allowed the rapid preparation of MCLEAs with high enzyme load: 1.53 U laccase/mg MCLEAs. The stability of the MCLEAs was improved with regards to low pH, presence of chemical denaturants and real wastewater matrix, compared to free laccase. In addition, the novel biocatalyst exhibited good operational stability, maintaining up to 70% of its initial activity

after 10 successive batch reactions operated with magnetic separation. To evaluate the catalytic performance of the MCLEAs, these were applied for the elimination of a cocktail of 10 PhACs: acetaminophen, ketoprofen, cyclophosphamide, mefenamic acid, caffeine, indomethacin, naproxen, fenofibrate, trimethoprim and ibuprofen at concentrations in the range of 10-50 µg/L from real secondary effluent. The novel biocatalyst showed the ability to transform the phenolic compound acetaminophen and certain non-phenolic PhACs as mefenamic acid, fenofibrate and indomethacin, with similar or even higher efficiency than free laccase. The other PhACs did not suffer appreciable transformation by either free laccase or MCLEAs.

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Chapter 1
GENERAL INTRODUCTION

Summary

Laccases, the most widely distributed ligninolytic enzyme among white-rot fungi, are promising biocatalysts due to their oxidative versatility and low catalytic requirements: they use oxygen from air and release water as the sole by-product. Laccases are able to oxidatively detoxify and remove a great number of pollutants. The potential of laccase was explored in the current research aiming to develop continuous or semicontinuous technologies to remove pollutants from real environmental matrices. Polycyclic Aromatic Hydrocarbons (PAHs) and Emerging Organic Contaminants (EOCs) were the target contaminants selected.

PAHs are known to exert acute toxic effects and/or possess mutagenic, teratogenic or carcinogenic properties. Their natural biodegradation is restricted by two major factors: low water solubility and high hydrophobicity, which make PAHs persistent pollutants in soil. Extraction with solvents or surfactants is an alternative to remove PAHs from soils. However, this technique by itself does not destroy the pollutants, but only attains their transfer to another phase. Enzymatic remediation with laccase may be a feasible alternative, but requires the addition of surfactants or solvent to increase the pollutant bioavailability for the enzymatic action. Coupling solvent extraction and the technology based on Two Phase Partitioning Bioreactor may constitute a promising remediation process to remove and degrade hydrophobic pollutants present in soils.

On the other hand, the presence of EOCs in water bodies is an issue of concern since these are potential threats to environmental ecosystems and human health. Bisphenol A and pharmaceuticals active compounds are examples of EOCs frequently detected in wastewaters, considered as the main sources of EOCs entering the environment. When it comes to the application of laccase-based technologies to remove micropollutants, two main approaches are possible under the perspective of continuous operation: enzymatic membrane reactors with free enzyme, or immobilized laccase.

This Chapter establishes the framework of the research developed in this Thesis, which deals with the development of laccase-based technologies to remove specific environmental pollutants from soil and wastewaters.

1

INDEX

1.1 WHITE ROT FUNGI AND OXIDATIVE ENZYMES

White Rot Fungi (WRF) are a diverse ecophysiological group, including basidiomycetes and certain ascomycetes, which are characterized by their distinctive capability to efficiently depolymerize, degrade, and mineralize all components of plant cell walls including cellulose, hemicellulose and lignin (Kersten and Cullen 2007, Pointing 2001). The term "white-rot" has been traditionally used to describe forms of wood decay where lignin (as well as cellulose and hemicelluloses) is broken down, leaving a light, white, rather fibrous residue completely different from the brown powder left by brown rot fungi (Schwarze et al. 2000). WRF, which are common inhabitants of forest litter and fallen trees, are key regulators of the global C-cycle (Wesenberg et al. 2003). Generally, they are unable to use lignin as a sole carbon source, but they degrade it to gain access to cellulose and hemicellulose. Delignification is based on the capacity of this type of fungi to produce extracellular lignin-modifying enzymes (LMEs) (Pointing 2001). WRF produce LMEs during their secondary metabolism. Thereby, synthesis and secretion of these enzymes is often induced by limited nutrient levels (mostly C or N) (Wesenberg et al. 2003). In addition, WRF secrete low molecular weight mediators that enlarge the range of compounds they are capable to degrade (Cabana et al. 2007b).

The ability of WRF to degrade lignin, which is an extremely recalcitrant compound, with great structural complexity and heterogeneity, shows in fact that this enzymatic system is nonspecific and it can also be considered to remove different recalcitrant and xenobiotic substances. Indeed, various fungal species have been successfully applied for the degradation of a wide range of pollutants, such as polycyclic aromatic hydrocarbons (PAHs), chlorinated and phenolic compounds, textile dyes, and even pharmaceuticals and endocrine disrupting compounds (EDCs) (Cabana et al. 2007b, Pointing 2001).

The main LMEs associated with the lignin-degrading ability of WRF are peroxidases, which comprises lignin peroxidases (LiP, E.C. 1.11.1.14), manganese-dependent peroxidases (MnP, E.C. 1.11.1.13), versatile peroxidases (VP, E.C. 1.11.1.16) and laccases (Lac, E.C. 1.10.3.2). These enzymes act by generating highly reactive and nonspecific free radicals that affect lignin degradation (Kersten and Cullen 2007).

1.1.1 Peroxidases

Peroxidases are hemoproteins which require the presence of hydrogen peroxide as electron acceptor to oxidize lignin and lignin-related compounds (Mester and Tien 2000). The typical enzymatic cycle characteristic of peroxidases is depicted in Figure 1.1.

The native (ferric) enzyme is initially oxidized by H_2O_2, generating a two-electron oxidation state of the enzyme (compound I). During the oxidation of the ferric enzyme by hydrogen peroxide, one electron is withdrawn from Fe^{3+}, and one from the porphyrin ring, generating Fe^{4+} and porphyrin cation radical, respectively. Compound I is reduced back in two steps via a Fe^{4+} intermediate (compound II) in the presence of appropriate reducing substrates. Another reduction, involving a second molecule of specific substrate, takes place in the third step of the reaction. The enzyme gets back to its resting state and one more radical product is formed.

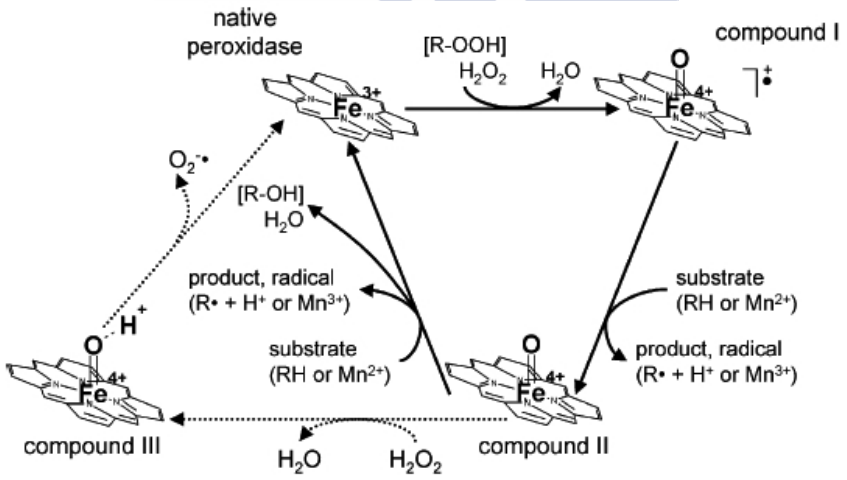


Figure 1.1. Generic scheme of the catalytic cycle of peroxidases as reported by Wesenberg et al. (2003).

High concentrations of H_2O_2 can cause reversible inactivation of the enzyme by forming compound III, a catalytically inactive intermediate that can be converted to the native state, spontaneously or by oxidation with a substrate, releasing a superoxide anion ($O_2^{\bullet-}$) (Martínez 2002, Mester and Tien

2000). Ligninolytic peroxidases differ in their reducing substrates that are one-electron oxidized by compounds I and II in the second and third step (Martínez 2002).

1.1.1.1 Lignin peroxidase

LiP, known as ligninase in early publications, was the first ligninolytic peroxidase isolated in 1980's decade from the fungus *Phanerochaete chrysosporium* (Tien and Kirk 1988). It is a glycoprotein able to catalyze the oxidation of phenolic and aromatic compounds of similar structure to lignin. LiP shows a classical peroxidase mechanism, but it is unique in its ability to oxidize high redox-potential aromatic compounds (including veratryl alcohol) with redox potentials up to 1.4 V (Martínez 2002). LiP has a distinctive property of an unusually low optimum pH, near pH 3 (Wong 2009).

1.1.1.2 Manganese peroxidase

MnP was first discovered in *P. chrysosporium* (Kuwahara et al. 1984) and is considered as the most common ligninolytic peroxidase produced by nearly all white-rot basidiomycetes (Wesenberg et al. 2003). This enzyme has a similar specificity and catalytic cycle to other peroxidases; however, MnP preferentially oxidizes Mn^{2+}, always present in wood and soils, into highly reactive Mn^{3+}, which is capable of penetrating the cell wall matrix and oxidizing phenolic substrates (Hofrichter 2002, Wong 2009). The Mn^{3+} formed is quite unstable in aqueous media but WRF secrete oxalic and other organic acids that form Mn^{3+} chelates acting as stable diffusing oxidizers of phenolic compounds and dyes (Hofrichter 2002, Martínez 2002, Wong 2009).

1.1.1.3 Versatile peroxidase

The enzyme VP was described first in *Pleurotus eryngii* (Martínez et al. 1996) and it seems to be produced by fungi from the genera *Pleurotus, Bjerkandera* and *Lepista*, and maybe also by *Panus* and *Trametes* species (Pérez-Boada et al. 2005). VP is considered a hybrid between MnP and LiP (Wesenberg et al. 2003), since it can oxidize not only Mn^{2+} but also veratryl alcohol and phenolic aromatic compounds with high molecular weight by manganese-independent reactions (Wong 2009).

1.1.2 Laccases

Laccases (p-diphenol:dioxygen oxidoreductases) are multicopper-containing oxidases with phenoloxidase activity, which catalyze the oxidation of substituted phenols, anilines and aromatic thiols, with the concomitant four-electron reduction of oxygen to water (Thurston 1994).

1.1.2.1 Occurrence and role

Yoshida first described laccase in 1883 present in the exudates of the Japanese lacquer tree, *Rhus vernicifera*. In 1896 laccase was demonstrated to be present in fungi for the first time by both Bertrand and Labord (Thurston 1994). Currently, it is known that laccase is widely distributed in higher plants and fungi, and also in some insects and bacteria (Kunamneni et al. 2007). For instance, plant laccases have been identified in cabbages, turnips, beets, apples, asparagus, potatoes, pears, lacquer, mango, peach, pine, prune, etc. Laccase was also found in several insects of genera that include *Bombyx*, *Calliphora*, *Diploptera*, *Drosophilia*, *Lucilia*, *Manduca*, *Musca*, *Oryctes*, *Papilio*, *Phormia*, *Rhodnius*, *Sarcophaga*, *Schistocerca* and *Tenebrio* (Madhavi and Lele 2009), and in a few bacteria species: *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Streptomyces griseus*, *Streptomyces cyaneus, Streptomyces lavendulae* and *Bacillus subtilis* (Viswanath et al. 2014).

The majority of laccases described in literature were isolated from higher fungi (Morozova et al. 2007). Most common laccase producers are the wood rotting fungi *Trametes versicolor*, *T. hirsuta*, *T. ochracea*, *T. villosa*, *T. gallica*, *Cerrena maxima*, *Coriolopsis polyzona*, *Lentinus tigrinus*, *P. eryngii*, etc (Madhavi and Lele 2009). Besides, laccases are also found in saprophytic compost inhabiting ascomycetes *Myceliophthora thermophila* and *Chaetomium thermophilum* (Morozova et al. 2007). Fungal laccases, which have higher redox potential than bacterial or plant laccases (up to +800 mV) (Kunamneni et al. 2007), are involved in the degradation of lignin, pathogenesis and detoxification, as well as in the development and morphogenesis of fungi (Morozova et al. 2007). The role of laccases during delignification is not clear in detail. It was suggested that the function of laccase is not only the degradation of lignin, but also the polymerization of their oxidation products (Mayer and Staples 2002). Enzymatic decomposition

of lignin results in some toxic products that are dangerous for the fungal mycelium. In this sense, laccase detoxifies these low-molecular-weight phenolic components arising from lignin degradation, converting them to polymers nontoxic for fungal hyphae (Kunamneni et al. 2007, Morozova et al. 2007). Fungal laccases from *T. versicolor* and *M. thermophila* were the enzymes assessed in the current research, as it will be presented and detailed in the following chapters.

1.1.2.2 Molecular structure and catalytic mechanism

Laccases are monomeric, dimeric or tetraedric glycoproteins with molecular weight of 50-130 kDa (Majeau et al. 2010, Morozova et al. 2007). These contain four copper atoms (belonging to three types: 1, 2 or 3) per monomer located at the catalytic site. Type-I (T1, one Cu atom) acts as the primary electron acceptor site where the enzyme catalyzes the oxidation of the substrate and provides blue color to the enzyme. Type-II (T2, one Cu atom) forms a trinuclear copper-cluster with the type-III copper (T3, two Cu atoms), where the reduction of O_2 takes place (Morozova et al. 2007, Wong 2009). The catalytic cyclic of laccase is represented in Figure 1.2.

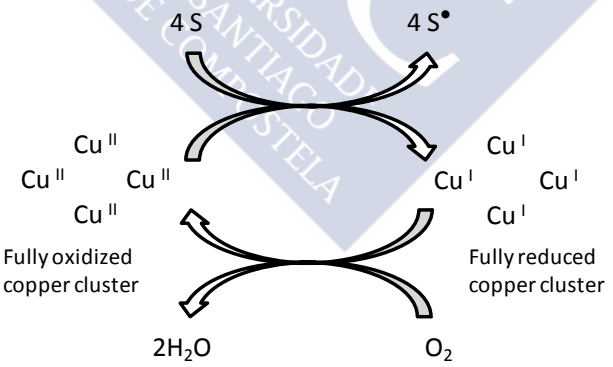


Figure 1.2. Schematic representation of the laccase catalytic cycle involving the reduction of molecular oxygen and the concomitant oxidation (at the T1 copper site) of substrate molecules to the corresponding radicals (adapted from Riva (2006)). S: substrate molecule; S•: oxidized substrate radicals.

The redox potential of the T1 copper site is directly responsible for the catalytic capacity of the enzyme and according to this value, copper-containing oxidases are classified in high- medium-, and low-potential enzymes

(Morozova et al. 2007). For instance, laccases from *Trametes* species, with potentials of the T1 site in the range 730-790 mV, have been characterized as high redox potential laccases (Fokina et al. 2015, Morozova et al. 2007), whereas laccase from *Coprinus cinereus* (560 mV) and *Rhus vernicifera* (430 mV) are medium and low redox potential laccases, respectively.

The first step of catalysis is the reduction of the reducing substrate by copper (Cu^{2+} to Cu^{+}) at the T1 site. The electrons extracted from the reducing substrate are transferred to the T2/T3 trinuclear site, resulting in the conversion of the resting form (fully oxidized) of the enzyme to a fully reduced state. A successive 4e– oxidation (from substrate molecules) is required to reduce the enzyme. Reduction of dioxygen occurs in two steps via the formation of bound oxygen intermediates. Firstly, the dioxygen molecule binds to the T2/T3 site, and two electrons are rapidly transferred from the T3 coppers, resulting in the formation of a peroxide intermediate. The peroxide bridges between the oxidized T3 and the reduced T2 copper sites, although the configuration of oxygen has not been entirely determined. The diffusion of dioxygen to the trinuclear site is rate limiting, followed by a rapid 1e– transfer from T1. The peroxide intermediate decays to an oxy radical and undergoes a 2e– reductive cleavage of the O-O bond with the release of a water molecule. The slow decay of the intermediate is facilitated by the final electron transfer from the T2 copper. In the last step, all four copper centers are oxidized, and O^{2-} is released as a second water molecule. The reoxidation of T2 copper correlates with the decay of the intermediate in which one molecule of water is released and the second one remains bound and slowly exchanged with the bulk solution (Wong 2009).

The stoichiometric ratio corresponding to the molar ratio of substrate/dioxygen transformation is generally 4/1, i.e., four electrons withdrawn from four substrate molecules per one dioxygen reduced. If substrate molecules donate more than one electron, a lower ratio (or decimal values) may be required (Majeau et al. 2010).

Summarizing, the overall outcome of the catalytic cycle is the reduction of one molecule of dioxygen into two molecules of water, coupled with the oxidation of the substrate molecules (generally four) into radicals that can form dimers, oligomers and polymers.

1.1.2.3 Laccase-mediator system

The broad substrate specificity and low catalytic requirements of laccases: they use oxygen from the air and generation of water as the sole reaction by-product; make these enzymes suitable for biotechnological applications (Camarero et al. 2012). However, laccases possess relative low redox potential (≤0.8 V) and their action would be restricted to the oxidation of the phenolic lignin moiety (less than 20% of lignin polymer) whereas non-phenolic substrates having redox potential above 1.3 V cannot be directly oxidized by laccases (Cañas and Camarero 2010). By mimicking nature, it is possible to overcome this limitation by adding mediators, typically defined as low-molecular weight compounds that act as 'electron shuttles' between the oxidized enzyme and the target compound; thereby expanding the catalytic activity of laccase towards more recalcitrant compounds such as non-phenolic lignin units (Barreca et al. 2003). Once oxidized by the enzyme and stabilized in more or less stable radicals, mediators diffuse far away from the enzymatic pocket. Mechanisms different from the enzymatic one allow the oxidation of compounds that in principle are not substrates of laccase because of their high size or high redox potential (Bourbonnais and Paice 1990) according the scheme depicted in Figure 1.3.

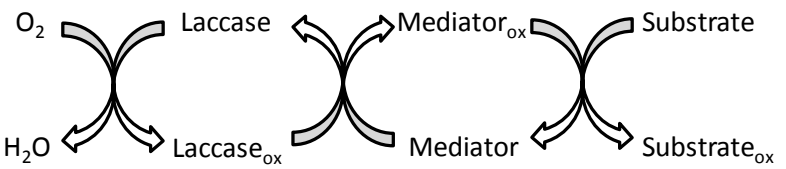


Figure 1.3. Scheme of substrate oxidation by the laccase-mediator system.

1.1.2.4 Applications

Laccases have been subject of intensive research in the last decades due to their high biotechnological applicability. Currently, the directed evolution techniques can be used to confer improved features to enzymes and expand their substrate range (Maté et al. 2011). Besides, nanotechnology offers a promising tool to increase enzyme stability. Several applications of laccases are detailed below (Demarche et al. 2012b, Kunamneni et al. 2008a, Kunamneni et

al. 2008c, Madhavi and Lele 2009, Majeau et al. 2010, Rodríguez Couto and Toca Herrera 2006, Xu 2005).

Laccases in the textile industry

The use of laccase in the textile industry is growing very fast. Apart from decolorizing textile effluents, this enzyme can be applied to bleach textiles and even to synthesize dyes. The rationale behind this interest is the potential benefits associated: reduction of chemicals, energy and water use. For instance, laccase may improve whiteness in conventional bleaching of cotton and recently biostoning (Maryan and Montazer 2013, Pereira et al. 2005). The enzyme can also be used to convert dye precursors for better, more efficient fabric dyeing (Kitamoto et al. 2004).

Medical and personal care applications

Many products generated by laccases are antimicrobial, detoxifying or active personal care agents. Due to their specificity and bio-based nature, potential applications of laccases in the field are being investigated. This type of enzymes can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, antibiotics, sedatives, etc (Kunamneni et al. 2008c). One potential application is the laccase-based *in situ* generation of iodine, chemical widely used as disinfectant (Xu 2005). A novel application field for laccases is in the cosmetics sector. For example, laccase-based hair dyes could be less irritant and easier to handle than the current ones, since laccases replace H_2O_2 as an oxidizing agent in the dye formulation (Rodríguez Couto and Toca Herrera 2006). Laccases may find use as deodorants for personal-hygiene products, including toothpaste, mouthwash, detergent, soap and diapers (Xu 2005).

Laccases in the food industry

Laccases may be used as O_2-scavengers for better food packing (Farneth et al. 2005). Wine stabilization is one of the main applications of laccase in the food industry as alternative to physical-chemical adsorbents (Minussi et al. 2007). Commercial laccase (Suberzyme®) is available for preparing cork stoppers for wine bottles (Conrad et al. 2000). The enzyme can also be used for fruit juices stabilization and brewery, to remove remaining polyphenols and excess of oxygen and thereby, enhance the storage life of beers. Other uses of

laccase include their use for odor control, taste enhancement, or reduction of undesired products in several food products. For instance, the flavor quality of oils can be improved by removing oxygen present in such ones and the use of laccase from *Pleurotus* can enhance the color of tea-based products (Osma et al. 2010).

Biosensors and diagnostic application of laccases

Laccase catalysis, coupled with various physical transducers, could be useful as biosensors for detecting O_2 and a wide variety of reducing substrates, especially phenols or anilines (Xu 2005). For instance, *T. versicolor* laccase was encapsulated in nanofibers by electrospinning to produce a biosensor for chlorophenol monitoring (Liu et al. 2011). Laccase can be used to assay other enzymes and this enzyme covalently conjugated to a biobinding molecule can be used as a reporter for immunochemical (ELISA, Western blotting), histochemical, cytochemical or nucleic acid-detection assays (Kunamneni et al. 2008c).

Biofuel cells

Enzymatic biofuel cells use enzymes to catalyze chemical reactions; thereby, replacing traditional electrocatalysts present in conventional fuel cells (Le Goff et al. 2015). Laccase can be adsorbed, entrapped or wired onto the cathode to catalyze O_2 reduction (Fokina et al. 2015, Xu 2005). In a recent work by Sané et al. (2013), the crude culture supernatant from *T. versicolor* was used to supply laccase to the cathode of a mediator-free biofuel cell. The oxygen reduction activity of such enzymatic cathode was sustained over a period of at least 120 days by the periodic resupply of crude culture supernatant, with no appreciable loss of potential.

Laccases for organic synthesis

As previously mentioned, direct laccase-mediated oxidation of phenols generates reactive radical intermediates that undergo coupling reactions, leading to the formation of dimers, oligomers and, eventually, polymers, which can be of synthetic relevance (Riva 2006). Laccase is of great interest in the enzyme-catalyzed production of new biologically active compounds via phenolic oxidation, phenolic oxidative coupling and oxidation coupled with nuclear amination (Jeon et al. 2012, Mikolasch and Schauer 2009).

Laccases in the pulp and paper industry

Conventional pulp delignification relies on either chlorine- or oxygen-based chemical oxidants. Although very effective, these agents can cause serious problems associated to by-products production or cellulose fiber-strength loss. The laccase-mediator system constitutes an alternative for bleaching high-quality pulps from flax and other non-wood fibers (Camarero et al. 2004, Virk et al. 2012). Laccases were also investigated for biografting of organic molecules onto lignin for improved properties of lignocellulosic biomass. This innovative process results in fiber materials with completely novel properties such as hydrophobicity, charge, antimicrobial properties, etc (Kalia et al. 2014). Other related application of laccases are deinking and decolorization of printed paper (Nyman and Hakala 2011).

Laccases for the removal of pollutants

Laccases as well as laccase-mediator systems have been successfully used to oxidatively detoxify and remove a great number of pollutants; some examples are summarized in Table 1.1. The proved ability of laccase to detoxify a wide range of pollutants will be evaluated in the current research aiming to develop continuous or semicontinuous technologies applicable to real environmental matrices. Two types of pollutants were selected as target contaminants. On the one hand, polycyclic aromatic hydrocarbons, and more specifically, anthracene, was proposed as model compound of hydrophobic and highly persistent contaminant in soils. On the other hand, bisphenol A and several pharmaceutical active compounds were proposed as model of emerging organic contaminants frequently detected in wastewaters.

Table 1.1. Examples of removal of recalcitrant compounds by laccases.

Pollutants		Laccase source	Mediator	Reference
Dyes	Basic green 4 and acid violet 17	*Cyathus bulleri*	-	(Chhabra et al. 2009)
	Reactive red 239, reactive yellow 15 and reactive blue 114	Commercial laccase formulation-DeniLite® IIS	-	(Tavares et al. 2009)
	Reactive black 5, acid blue 25, methyl orange, remazol brilliant blue R, methyl green and acid green 27	*Myceliophthora thermophila*	Without and with HBT	(Kunamneni et al. 2008b)
	Reactive Black 5	*Pycnoporus cinnabarinus* and *Trametes villosa*	Phenolic compounds	(Camarero et al. 2005)
PAHs	Red FN-2BL, red BWS, remazol blue RR and blue 4BL	*Trametes versicolor*	Syringaldehyde	(Mendoza et al. 2011)
	Acenaphthylene, acenaphthene, fluorene, anthracene, benzo[a]pyrene, perylene , etc	*Trametes versicolor*	Without and with HBT	(Majcherczyk et al. 1998)
	Anthracene, pyrene, fluorene, fluoranthene, phenanthrene and perylene	*Pleurotus ostreatus*	Without and with HBT or ABTS	(Pozdnyakova et al. 2006a)
	Benzopyrene	*Pycnoporus cinnabarinus*	p-hydroxycinnamic acids	(Camarero et al. 2008)

*HBT: 1-Hydroxybenzotriazole; ABTS: 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

Table 1.1. Examples of removal of recalcitrant compounds by laccases (continuation).

Pollutants		Laccase source	Mediator	Reference
Pesticides	Pentachlorophenol	*Trametes versicolor*	-	(Ullah et al. 2000)
	Halogenated pesticides	*Coriolopsis gallica*	Acetosyringone and syringaldehyde	(Torres-Duarte et al. 2009)
EDCs	Triclosan	*Ganoderma lucidum*	Without and with HBT, ABTS and natural mediators	(Murugesan et al. 2010)
	Estrone (E1),17b-estradiol (E2), estriol (E3) and 17a-ethinylestradiol (EE2)	*Trametes versicolor*	Without and with HBT	(Auriol et al. 2007)
	E1, E2, E3,EE2, bisphenol A, bisphenol F bis(4 hydroxyphenyl)propane, bisphenol S, nonylphenol 4-diethylhexylphthalate, etc	Commercial laccase formulation (Daiwa)	Without and with HBT or ABTS	(Sei et al. 2007)
Pharmaceuticals	Naproxen and diclofenac	*Myceliophthora thermophila*	Without and with HBT or natural mediators	(Lloret et al. 2010)
	Tetracycline antibiotics	*Trametes versicolor*	HBT	(Suda et al. 2012)
	Acetaminophen	Not specified	-	(Lu et al. 2009)

1.2 POLYCYCLIC AROMATIC HYDROCARBONS

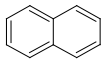
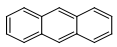
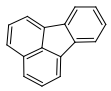
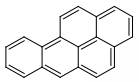
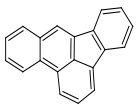
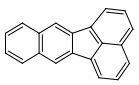
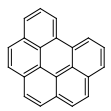
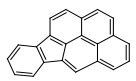
Polycyclic aromatic hydrocarbons (PAHs) are a class of hydrophobic organic contaminant, generic term applied to a range of chemicals characterized by being barely soluble in water as well as fairly resistant to biological, chemical and photolytic breakdown (Reid et al. 2000). These chemicals are of environmental concern because of their genotoxic and carcinogenic potential and high persistence in the environment (Cerniglia 1993).

1.2.1 Physical and chemical properties

PAHs consist of fused aromatic rings. The term “hydrocarbon” refers to their carbon and hydrogen composition, “polycyclic” indicates that two or more rings compose these molecules; whereas “aromatic” refers to the chemical bonds between carbon atoms. When any carbon atom in the ring is substituted by nitrogen, sulphur or oxygen, then they are called “heterocyclic aromatic hydrocarbons”. If there is any radical linked to the ring, they are known as “PAHs derivatives”.

Table 1.2 shows physical properties of the eight PAHs included in the List of priority substances in the field of water policy Directive 2013/39/EC (European Commission 2013). PAHs occur as colorless, white/pale yellow solids with low solubility in water, high melting and boiling points and low vapor pressure (Haritash and Kaushik 2009). In general, as the number of benzene rings in a PAH compound increases, solubility decreases (Wilson and Jones 1993), although there are exceptions to the rule. Symmetry, planarity, and the presence of substituents affect PAH solubility in organic solvents. PAH octanol-water coefficients: K_{OW}, are relatively high, indicative of their tendency to adsorb onto soil particles and bioaccumulate in organisms (Wick et al. 2011)

Table 1.2. Physico-chemical properties of PAHs.

Compound	Molecular weight	Log K_{ow}	Water solubility (mg/L)	Melting point (°C)	Vapor pressure (mPa)
 Naphthalene	128.16	3.37	31.7	80.5	11960
 Anthracene	178.24	4.54	0.07	216	2.3
 Fluoranthene	202.26	5.22	0.26	111	1.2
 Benzo(a)pyrene	252.32	5.91	0.0038	179	$7 \cdot 10^{-4}$
 Benzo(b)fluoranthene	252.32	5.80	0.0015	168	$6.7 \cdot 10^{-2}$
 Benzo(k)fluoranthene	252.32	6.06	0.0008	217	$5.2 \cdot 10^{-5}$
 Benzo(g,h,i)perylene	276.34	6.63	0.00026	273	$1.3 \cdot 10^{-5}$
 Indeno(1,2,3-cd) pyrene	276	6.50	0.00019	164	$1.3 \cdot 10^{-5}$

Sources: (Eibes 2007)

1.2.2 Environmental risks: occurrence and effects

The common sources of PAHs in environment include natural as well as anthropogenic. Natural sources are forest and brush fires, oil seeps, volcanic eruptions and exudates from trees. Anthropogenic sources of PAHs include industrial emissions from activities involving burning of fossil fuel, coal tar, wood, garbage, used lubricating oil, municipal solid waste incineration as well as petroleum spills and discharge. Domestic processes as tobacco smoke, automobile exhaust fumes or charbroiled food are also sources of PAHs (Haritash and Kaushik 2009, Samanta et al. 2002). As curiosity, PAHs are the most abundant organic molecules in space, making up to 20% of the total cosmic carbon (Samanta et al. 2002).

PAHs are present in air, water and soil. In air, they may occur in vapor phase (molecular weight, MW<200) or particulate phase (MW>200). PAHs released into atmosphere have a strong affinity for particles since, due to their low vapor pressure, they tend to condense onto particulate matter in air. Then, they move to distant places with the wind currents until the particles settle to earth surface and thereby, PAHs reach soil. The other sources of addition of PAHs to soil are wastewater discharge and spillage of oil. At the same time, disposal of effluents and municipal sewage, as well as surface runoff, constitute the main sources of contamination in water bodies and their sediments (Kaushik and Haritash 2006). Plants and animals can then uptake PAHs from the environment. Figure 1.4 illustrates the fate of PAHs in the environment.

Soils can be contaminated in levels in a wide range: 1 µg/kg - 300 g/kg PAHs, depending on the source of contamination (e.g., old coal gasification sites have the higher levels stated). Atmospheric levels of PAHs resulting from the incomplete combustion of materials such as coal and wood have been found to be between 60 $\mu g/m^3$ - 3 mg/m^3 air (Bamforth and Singleton 2005).

Sixteen PAHs, among them, anthracene, are recognized as priority pollutants by the US Environmental Protection Agency. In 18^{th} century, Sir Percival Pott, an English surgeon, was the first to report a connection between occupational exposure and cancer. He described an unusually high incidence of scrotal cancer among chimneysweepers and suggested that it was due to their

exposure to soot and ash (Kaushik and Haritash 2006). Since then, several studies demonstrated that various PAHs caused tumors in laboratory animals when they breathed these substances in the air, when they ate them, or when they had long periods of skin contact with them. In the same way, humans can also develop cancer after long-term exposition or skin contact to mixtures that contain PAHs (ATSDR 1995).

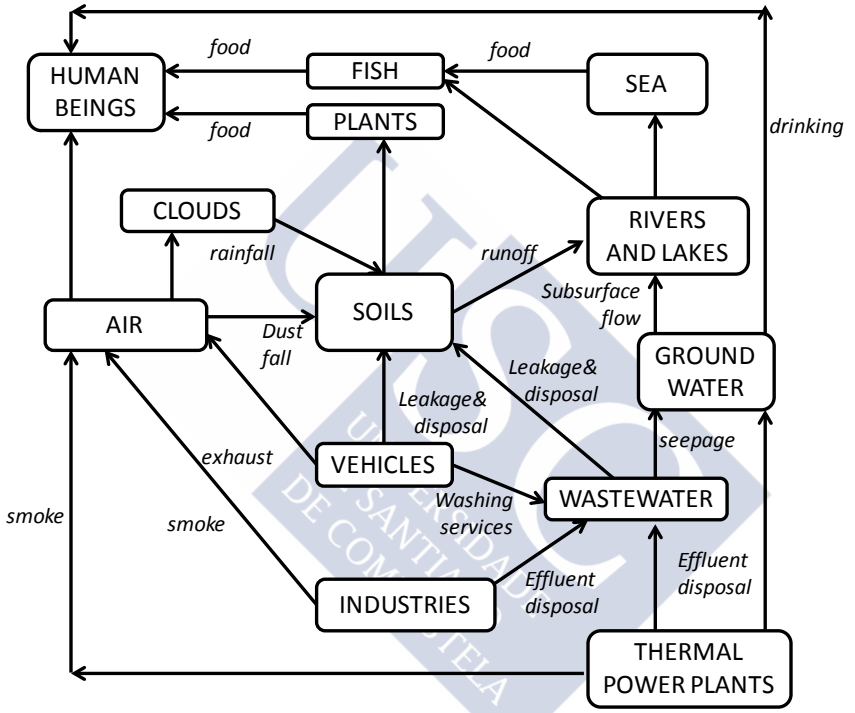


Figure 1.4. Fate of PAHs in the environment (adapted from Kaushik and Haritash (2006).

1.2.3 Conventional treatments for the removal of PAHs from soils

Although PAHs are important pollutants in air, soil acts as the ultimate depositary of these chemicals (Haritash and Kaushik 2009). In Europe, PAHs have been recognized as one of the top three main contaminants found in contaminated sites, contributing to 11% of the total pollutants affecting soils (Liedekerke et al. 2014). Their poor solubility in water and high hydrophobicity limit their availability to indigenous microorganisms. In addition, extractability

and bioavailability of PAHs decrease significantly with time in aging processes (Hatzinger and Alexander 1995, Luo et al. 2012). Therefore, the development and implementation of strategies to remove these persistent and hazardous pollutants constitute a priority. In the beginning, the excavation of contaminated soil and its disposal as landfill ("dig and dump") was the most common remediation technique applied. But growing regulatory control of landfill operations and associated increasing costs, along with the development of improved *ex-situ* and *in-situ* remediation techniques, modified the pattern of remediation (Liedekerke et al. 2014). Overall, to treat PAHs contaminated soils, physical, chemical or bioremediation processes can be considered.

1.2.3.1 Physical and chemical treatments

Extraction processes

Solvent extraction is a clean-up method that uses solvents to extract or remove harmful chemicals from polluted materials. The polluted soil is excavated and mixed with the solvent in an extractor. Such method can be efficient in removing hydrophobic organic contaminants from soils (USEPA 2001). However, the use of organic solvents such as propane, butane, acetone, methanol, n-hexane and dimethyl ether involves substantial risks as well as the necessity of implementing costly measures to minimize their environmental impact (Anderson 1995, Lau et al. 2014). Alternative non-toxic extracting agents, as vegetable oils have being recently investigated as extracting agents with promising results (Yap et al. 2010).

Soil washing is a technology based on the same concept, the use of water combined with chemical additives as surfactants or solvents, and mechanical processes to scrub soils (Khan et al. 2004, USEPA 1996). A potential drawback is that remaining surfactant in soil may inhibit microbial activity (Laha and Luthy 1992). The use of cyclodextrines presents several advantages over classical enhanced-solubility agents (e.g. solvents and surfactants) such as their non-toxicity and their biodegradability (Sánchez-Trujillo et al. 2013). Nevertheless, these techniques by themselves do not destroy PAHs, but only attain the transfer of the pollutants to other phase.

Chemical oxidation

In situ chemical oxidation is a process where oxidants (ozone, hydrogen peroxide, potassium permanganate, hypochlorites, chlorine and chlorine dioxide) are injected into the contaminated soil to convert PAHs to more stable and less mobile forms (Wick et al. 2011). Regarding ozone treatment and Fenton process, they can be applied *in situ*, *on site* and *off site* (Rivas 2006, Yin and Allen 1999). These chemicals form hydroxyl radicals ($^{\bullet}OH$), which are strong, non-specific oxidizing agents. Chemical oxidation with ozone, introduced either in a gaseous or aqueous form, occurs when ozone decomposes to form $^{\bullet}OH$ via a catalytic reaction at the reactive site on soils (Bavel 2006). Chemical oxidation using Fenton process is conducted under acidic conditions where $^{\bullet}OH$ are formed by using hydrogen peroxide with Fe^{2+} as a catalyst. A limitation related to Fenton oxidation is that low pH can adversely affect the natural soil systems and change its characteristics (Jorfi et al. 2013). Other drawbacks or considerations which must be taken into account are: i) oxidant introduction can negatively impact subsurface soils; ii) decreased soil permeability (colloid formation), iii) release of previously sorbed metals to groundwater resources, iv) toxic byproduct generation, v) heat and gas production, and vi) logistics of handling and storing oxidizing chemicals (Wick et al. 2011).

1.2.3.2 Bioremediation

Bioremediation, that involves the capabilities of biological agents (i.e. bacteria, fungi and plants) to degrade and detoxify environmental contaminants is a relatively efficient and cost-effective technology (Juwarkar et al. 2010, Megharaj et al. 2011). Bioremediation derives its scientific justification from the emerging concept of Green Engineering, defined as the design, commercialization, and use of processes and products that are feasible and economical while minimizing pollution at the source and risk to human health and the environment (Kirchhoff 2003). Polluted soils, sediments and groundwaters can decontaminate by *in situ* or *ex situ* methods. Table 1.3 shows different available technologies for bioremediation.

Table 1.3. Overview of in situ and ex situ available technologies in soil bioremediation.

In situ		*Ex situ*	
Technology	Description	**Technology**	Description
Bioattenuation	Natural process of degradation, by the microorganisms and naturally occurring chemicals	**Landfarming**	Excavation and placement of contaminated soils into line beds with aeration by periodic tilling
Biostimulation	Stimulation of indigenous microbial population by adding nutrients and carbon	**Composting**	Aerobic, thermophilic treatment in which contaminated soil is mixed with nonhazardous organic amendants, e.g.manure or agricultural wastes
Bioventing	Treatment of soil by drawing oxygen through the soil to stimulate microbial growth and activity	**Biopiles**	Hybrid of landfarming and composting. Engineered cells are constructed as aerated composted piles
Bioaugmentation	Inoculation of specific competent microorganisms to improve the metabolic capacity of the indigenous population of microbes.	**Bioreactors**	
		-Slurry	-Contaminated soil is treated in aqueous suspension (10-30% w/v) in bioreactors equipped with effective mixing
Phytoremediation	Use of plants to remove pollutants from soils	***-TPPB liquid-liquid***	-Extraction of pollutant into organic solvent, and delivery of substrate to microbes in a bioreactor
		-TPPB solid-liquid	-Extraction of pollutant into polymer beads and delivery of substrate to microbes in a bioreactor

In general, bioremediation presents some advantages over other remediation technologies. First, it is a natural process and thereby is perceived by the public as an acceptable waste treatment process for contaminated soils. Instead of transferring contaminants from one environmental medium to another, i.e from land to water or air, the complete destruction of target pollutants is possible. Many legally considered hazardous compounds can be transformed to harmless products, which eliminates the chance of future liability associated with treatment and disposal of contaminated material. Bioremediation can often be implemented *on site*, without causing a major disruption of normal activities. This also eliminates the need of transporting waste off site and the potential threats to human health and the environment that can arise during transportation (Vidali 2001).

However, when treating PAHs, mass transfer limitations of these hydrophobic compounds to the aqueous phase in the soil solution can be a major limiting factor for bioremediation (Volkering et al. 1992). Therefore, the pretreatment of the contaminated soil (Haritash and Kaushik 2009, Tomei and Daugulis 2012) with surfactants, biosurfactants or solvents (Christofi and Ivshina 2002, Kosaric 2001) must be considered aimed at increasing PAHs bioavailability.

In addition, the use of whole living cells presents some constraints (Megharaj et al. 2011, Singh and Walker 2006), including the need for continuous supply of fresh inocula, aeration, nutritional supplements and suitable thermal conditions (Rayu et al. 2012). Moreover, the microorganisms must be able to resist adverse conditions that are far from the optimal conditions found at lab experiments (Alcalde et al. 2006). Some classes of contaminants are difficult to biodegrade because of their toxicity to the microbial agents employed, requiring thereby acclimatized microbial populations and long reaction times to attain the desirable remediation degree (Tomei and Daugulis 2012).

1.2.4 Enzymatic bioremediation

The limitations related to the use of whole cells have boosted the use of formulated enzymes rather than living microorganisms as bioremediation agents, a process known as enzymatic bioremediation. Enzyme-based

technologies present several advantages over the use of microbial cells (Torres et al. 2003):

i. isolated enzymes act with higher specificity than parent organisms;
ii. enzymes can be active under a variety of environmental conditions (pH, temperature, ionic strength);
iii. their activity can be better standardized and they are easier to handle and store;
iv. higher pollutant concentrations can be treated with reduced inhibition problems;
v. shorter operational times with no lag period due to microbial growth;
vi. in case of needing organic co-solvents or surfactants to enhance bioavailability, their addition is more feasible when using enzymes than for whole cells.
vii. moreover, the potential of recombinant-DNA technology to produce more stable and active enzymes at higher scale and lower cost (Alcalde et al. 2006)

Nevertheless, prior to considering the use of isolated enzymes as biocatalysts to degrade pollutants, several requirements are essential:

i. a suitable enzyme with high substrate affinity supporting thousands of products turnovers, and the source of such enzyme must be identified;
ii. the cell-free approach is only valid for those enzymes which maintain activity when cells are broken (Scott et al. 2008);
iii. at the same time, the enzyme must be stable at the operational conditions and have no dependency of expensive coenzymes or cofactors as NAD(P)H, reduced flavin or glutathione (Sutherland et al. 2004)
iv. diffusible enzymes or related mediators are desirable, taking into account that the interaction of the enzymes and the substrate may be constrained to the large size of the enzymes
v. an efficient enzyme production system is also required. In fact, the high cost associated with enzyme production has been

identified as a constraint that must be overcome (Alcalde et al. 2006, Gianfreda and Rao 2004, Rayu et al. 2012).

The proposal of a type of enzyme able to fulfill all these requisites is not easy. Our hipothesis is that ligninolytic enzymes are good candidates, and more specifically, laccase (section 1.1.2).

1.2.4.1 *In vitro* degradation of PAHs by laccase

The feasibility of using laccase for the *in vitro* removal of PAHs has been demonstrated in several works since late 90s. The first studies consisted of batch experiments to test the ability of free laccase in presence of different mediators to remove model PAHs from aqueous media. In these works, small amounts of water miscible solvents or surfactants were used to increase PAHs solubility (Cañas et al. 2007, Collins et al. 1996, Johannes and Majcherczyk 2000, Johannes et al. 1996, Majcherczyk et al. 1998, Pozdnyakova et al. 2006b). In order to facilitate the reuse and stability of the biocatalyst, the use of immobilized laccase to remove PAHs pollutants was also subject of study. For instance, laccase from *T. versicolor* was immobilized in kaolinite and further applied to remove anthracene and benzopyrene from water containing 1% v:v of Tween 80 (Dodor et al. 2004). In subsequent studies, laccase was immobilized onto mesoporous silica nanoparticles (SBA-15) before its application to treat PAHs from aqueous medium (Fernando Bautista et al. 2010, Hu et al. 2009, Hu et al. 2007). Although these studies reveal the potential of free or immobilized laccase to remove PAHs, these do not address their elimination from polluted soils, only from aqueous solutions.

A more direct bioremediation strategy would consist in the direct application of enzymes over the PAHs-contaminated soil. A US patent by Stolzenburg and Duner (1999) describes a process where a multi-enzyme complex EZT-MZC™ from Enzyme Technologies, Inc, was applied for the removal of PAHs in polluted soils from a gas plant. In less than two weeks, the target organic contaminants were reduced by 80% or even higher levels. However, prior to the addition of the enzymatic complex, it was necessary to reduce the population of the indigenous organisms to a level below which they are able to interfere with the enzymes. The same approach was also assessed by Wu et al. (2008), who applied free laccase from *Trametes* sp. to transform priority PAHs from a liquefied petroleum gas station. Anthracene,

benzo(a)pyrene and dibenzo(a,h) anthracene were the most degradable PAHs, with percentages of transformation of 86%, 60% and 41%, respectively, after only 24 h of laccase treatment (10 U/g). Nonetheless, although enzymes are more mobile than microorganisms because of their smaller size (Rao et al. 2010), the low PAHs bioavailability can hamper enzymatic removal.

For this reason, the transfer of PAHs from soils to another medium where pollutants are more accessible to laccase may constitute a more efficient alternative. For instance, a novel system based on laccase-carrying electrospun fibrous membranes (LCEFM) was recently proposed to remove a mixture of PAHs from a soil-water suspension (Dai et al. 2011). LCEFMs were composed of core-shell structural nanofibers (for PAH adsorption), with laccase in the core (for PAH degradation) and pores on the shell (for mass transfer). In such system, to achieve equilibrium distribution between soil surface and aqueous phase, PAHs were released into water from soil particles. Due to the high hydrophobicity of the target pollutants, the process is slow. However, in the presence of the LCEFMs, mass transfer of PAHs was promoted since PAHs in the water could be adsorbed onto the membrane. Benefitting from the strong adsorption capacity of the membrane, low levels of PAHs remained in the aqueous phase while more PAH was released from the soil particles under the stress of equilibrium. The adsorbed PAHs migrate into the core-shell where laccase conducts the degradation. With the migration of PAHs from the surface into the interior of the fibers, there would be more sites for PAH adsorption, facilitating the phase transfer of PAHs from the soil to the membrane.

A second approach to decontaminate PAHs-contaminated soils would combine the extraction of the hydrophobic pollutants from the soil by organic solvents or surfactants and the subsequent treatment in an enzymatic reactor. The type of extracting agent will determine the nature of the mixture in the reactor vessel. In case of using a mixture of surfactant and water to extract the contaminated soil, the enzymatic treatment will be conducted in micellar systems. Micelles consist of organized aggregates of a large number of molecules of surfactant. These are formed when the concentration of surfactants exceeds a certain critical value called critical micelle concentration (CMC) (Paria 2008). The physical properties like surface tension, interfacial tension, adsorption, and detergency change below CMC with the

concentration but there is no change in these properties above CMC (Figure 1.5) (Paria 2008).

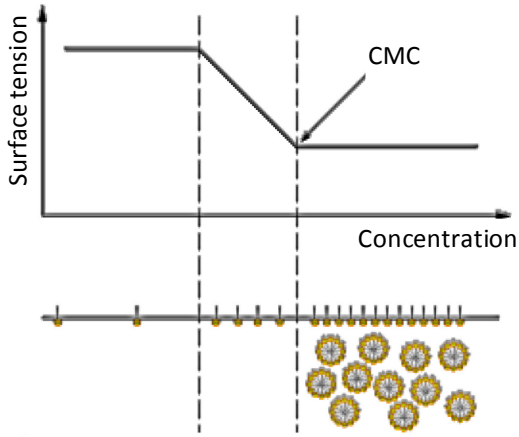


Figure 1.5. Dependency of surface tension with surfactant concentration and scheme of micelle formation when increasing concentration.

Micelles enhance the aqueous solubility of PAHs by the inclusion of these hydrophobic molecules into surfactant micelles (Edwards et al. 1991, Prak and Pritchard 2002).

If a miscible organic solvent is used to extract polluted soils, the enzymatic treatment of extracted PAHs can be performed in monophasic reactors. Environmental and safety concerns associated to the use of surfactant and water miscible organic solvents as extracting agents have been discussed in section 1.2.3.1. Another limitation is that after the enzymatic treatment, surfactant or solvents form a homogenous mixture and the separation by conventional physical or mechanical processes is not possible. Therefore, the reutilization of the solvents or surfactants is not feasible, neither the recovery of the enzyme.

Using a water immiscible organic solvent as extracting agent is an interesting alternative in order to facilitate the enzymatic treatment and further reuse of the solvent. In this case, the configuration of the enzymatic reactor is a Two Phase Partitioning Bioreactor (TPPB). In biphasic reactors, the substrate is specially present in the immiscible phase and diffuses to the aqueous phase, as the biocatalyst degrades the pollutant in the aqueous

phase, in order to re-establish equilibrium (Vrionis et al. 2002). Figure 1.6 illustrates the TPPB configuration.

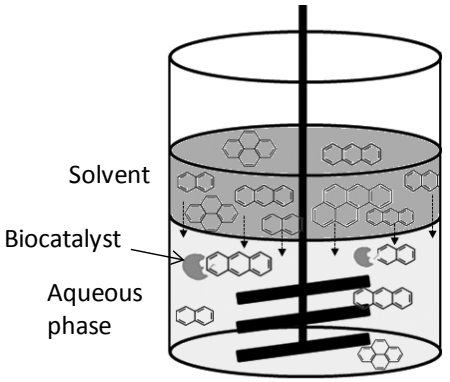


Figure 1.6. Scheme of a TPPB for the treatment of hydrophobic compounds.

In this system, the selection of a suitable solvent is crucial. This should fulfill several requisites such as non toxicity for the biocatalyst, immiscible in water, non-volatile, inexpensive and readily available (MacLeod and Daugulis 2003).

The TPPB technology was devised in the mid-seventies for the microbial and enzymatic bioconversion of hydrophobic/toxic substrates into products of commercial significance (Déziel et al. 1999, Quijano et al. 2009). This concept was later extended to the microbial degradation of highly toxic hydrophobic contaminants such as BTX, PAHs or phenolic compounds (Collins and Daugulis 1997, 1999, Daugulis 2001, Guieysse et al. 2001, Janikowski et al. 2002, Köhler et al. 1994, MacLeod and Daugulis 2003). The evaluation of a two-liquid-phase slurry bioreactor was also performed to improve the biodegradation of PAHs in polluted soils (Villemur et al. 2000). Although microbial TPPBs were proposed as an alternative technology to conventional slurry phase bioreactors (Tomei and Daugulis 2012) (Table 1.3), there are no studies of full scale application, only a pilot scale demonstration to remove model PAHs solubilized in dodecane (Daugulis and Janikowski 2002).

There is also one study of our group dealing with the application of the TPPB for the *in vitro* degradation of PAHs (Eibes et al. 2007). These authors used the ligninolytic enzyme MnP to remove anthracene from silicone oil

previously saturated with the pollutant. In a further study, the effect of the addition of surfactant at concentrations below the CMC value to the aqueous phase was also investigated (Eibes et al. 2010). However, to our knowledge, there is no previous research considering the global process combining solvent extraction with the enzymatic catalysis in a TPPB. This is, in fact, one of the main objectives of the present thesis, which will be discussed in more detail in section 1.4.

1.3 EMERGING ORGANIC CONTAMINANTS

Emerging organic contaminants (EOCs) encompass a wide range of chemicals used worldwide and their metabolites, which are considered potential threats to environmental ecosystems and human health (Bletsou et al. 2015, Gavrilescu et al. 2015). These are commonly present in waters at trace concentrations, ranging from a few ng/L to several µg/L (Luo et al. 2014) and are still not covered by existing water-quality regulations or are in process of regularization (Esplugas et al. 2007, Farré et al. 2008). Emerging pollutants are not necessarily new chemicals, but their presence and significance in the environment are only now being elucidated due to analytical developments (Lapworth et al. 2012, NORMAN).

A review of the literature showed that trace pollutants and emerging contaminants receiving the most attention by researchers and regulators generally belong to one of three broad groups: i) industrial; ii) pesticides; iii) pharmaceuticals and personal care products (PPCPs) (Murray et al. 2010), which are further subdivided as shown in Table 1.4. Within these, the occurrence and fate of those compounds with the ability to alter endocrine functions at trace concentrations is of particular concern. They are referred as endocrine disrupting compounds (EDCs). Essentially, the compounds which can cause endocrine disruption are both natural and synthetically-produced hormones, and a variety of man-made chemicals designed for uses in industry, pesticides, or in consumer goods (European Commission 1999).

Table 1.4. Classification of widely reported emerging substances in the aquatic environment.

Group	Sub-group	Common use
Industrial	Anti-oxidants	food additives
	Perfluorates	water proofing or protective coatings
	Phenols	surfactants, household cleaners, constituents of epoxy resins or plastics
	Phthalates	plasticizers
	Polybrominated diphenylethers (PBDEs)	plasticizers, flame retardant
	Triazoles	corrosion inhibitors, deicing
Pesticides	Carbamates	fungicide, insecticide
	Chloroacetanilides	herbicide
	Chlorophenoxyacids	herbicide
	Organochlorines	insecticide
	Organophosphates	insecticide
	Pyrethroids	insecticide
	Triazines	herbicide
PPCPs	Analgesics	antipyretic, pain reliever
	Anti-epileptic drugs (AEDs)	anticonvulsant
	Antihyperlipidemics	lipid regulator
	Antimicrobials	antibiotic, antiseptic
	Polycyclic musks	fragrances
	Non-steroidal and anti-inflammatory drugs (NSAIDs)	anti-inflammatory
	Hormones	hormone

1.3.1 Environmental risks: occurrence and effects

EOCs, derived from a variety of municipal, agricultural, and industrial sources and pathways, enter the environment in different ways depending on their pattern of usage and mode of application (Lapworth et al. 2012). Figure 1.7 illustrates the routes by which emerging contaminants enter various receptors (groundwater, surface water and even drinking water).

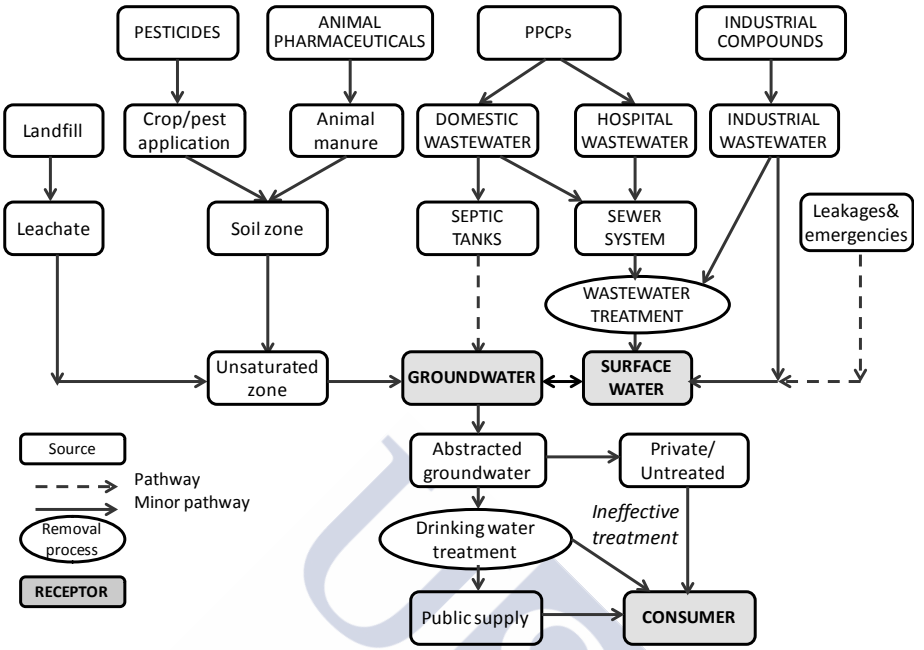


Figure 1.7. Schematic pathway of emerging contaminants from sources to receptors- adapted from Stuart et al. (2012).

Certain compounds as pesticides are intentionally released in measured applications. Veterinary pharmaceuticals may be released to the environment through livestock activities including waste lagoons and manure application to soil (Watanabe et al. 2010). Some industrial byproducts reach the environment through regulated or unregulated industrial discharges to water. Finally, a vast array of compounds, including household and industrial chemicals, PPCPs and biogenic hormones enter WWTPs as part of the influent. Since conventional treatments in WWTP are not designed to remove them, these pollutants may persist as part of the effluent and be released into receiving waters as trace pollutants (Halling-Sørensen et al. 1998, Kolpin et al. 2002, Murray et al. 2010). In fact, wastewaters are considered the main sources of EOCs in the environment (Auriol et al. 2006, Kosma et al. 2014, Lapworth et al. 2012).

In EU countries around 3000 different pharmaceutical active compounds (PhACs) are used in human medicine belonging to different therapeutic classes (Hernando et al. 2006). These compounds may be excreted both, as parent forms and as active metabolites, and not only after use, but also during manufacturing and disposal of unused or expired drugs; and thus, entering

municipal sewage treatment systems (Hofmann et al. 2007). These chemicals are of high concern because they may be active at extremely low concentrations. In addition, these are designed to be resistant to some forms of degradation in order to avoid inactivation of the substance that may hinder the desired healing effect (Halling-Sørensen et al. 1998). The literature shows that many of these compounds survive biodegradation and metabolic conjugates can even be converted back to their parent form. Many of these compounds are ubiquitous and display persistence in surface waters. Other PPCPs that might have low persistence, can display the same exposure potential as truly persistent pollutants due to their continuous release into the environment (Daughton and Ternes 1999, Hernando et al. 2006). This may lead to chronic low level exposure and accumulation with undesired effects on human health and environment as well as collateral generation of microbial resistances (Martínez et al. 2011).

On the other hand, a large number of industrial compounds have been reported in WWTP effluents. For instance, phthalates and bisphenol A used in plastic sector were found in relatively high concentrations (µg/L) in municipal wastewater due to urban runoff and discharges from industry and households (Oehlmann et al. 2008, Rubin 2011). Perfluorinated alkylated substances have been detected in various environmental matrices as surface water, rainwater, drinking water and wastewater (Clara et al. 2009). Alkylphenols, used in the manufacture of a wide variety of both household and industrial products, including detergents, degrade into 4-para-nonylphenol and para-tert-octylphenol, which are frequently detected in wastewater (Gasperi et al. 2008). Many of these anthropogenic compounds have been attributed as a cause of reproductive disturbance in humans and wildlife (Liu et al. 2009). It is believed that the effects of these EDCs are due to their ability to disturb synthesis, secretion, transport, binding or action of the endogenous hormones which are responsible for maintaining homeostasis, reproduction, development and integrity in living organisms and their progeny (Cabana et al. 2007a).

In the current research, two types of target pollutants were selected as representative of EOCs in wastewater. The first one was bisphenol A, an industrial chemical known as endocrine disruptor. The second type comprised

several pharmaceuticals, with different physicochemical properties, belonging to different therapeutic classes.

1.3.2 Target pollutants

1.3.2.1 Bisphenol A

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, is one of the highest volume chemicals, widely used in manufacturing polycarbonate plastics and epoxy resins. Final products include cans, metal jar lids, powder paints, automotive lenses, medical devices, protective coatings, coating for PVC pipes or adhesives, food packaging and plastic bottles (European Commission 2003, Kang et al. 2006, Staples et al. 1998). As the demand for these products has increased, so has BPA production. The global consumption of BPA in 2011 was predicted to exceed 5.5 million metric tons (Flint et al. 2012).

With its two benzene rings and two (4,4')-OH substituents (Figure 1.8), BPA was reported to fit in the estrogen receptor (ER) binding pocket (Vandenberg et al. 2009). However, the affinity of BPA for the ERs is approximately 10,000-fold weaker than that of estradiol (Witorsch 2002). For that reason, it was considered a weak environmental estrogen. Nevertheless, results from recent studies have revealed a variety of pathways through which BPA can stimulate cellular responses at very low concentrations, below the levels expected (Vandenberg et al. 2009).

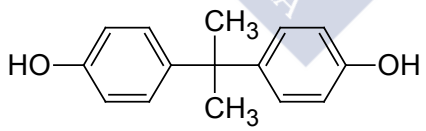


Figure 1.8. Chemical structure of BPA.

For many years, there has been an intensive controversy among researchers and federal agencies over the safe levels of human exposure to BPA. Whereas the National Toxicology Program (NTP) has identified low-dose BPA as 0.05 mg/kg per body weight, the Food and Drug Administration (FDA) has determined the appropriate non-observed adverse effect level of BPA for systemic toxicity to be 5 mg/kg per body weight per day. In March 2012, FDA

concluded that “the scientific evidence at this time does not suggest that the very low levels of human exposure to BPA through the diet are unsafe”. However, the FDA recognized potential uncertainties in the overall interpretation of these studies including the route of exposure used in the studies and the relevance of animal models to human health. On 17th July 2012, the FDA banned the use of BPA in baby bottles and sippy cups.

The first government in the world in concluding that BPA is hazardous to human health and in imposing a limited ban on BPA in baby bottles was Canada (2008 Health Risk Assessment). In October 2010, the Canadian government formally declared BPA to be toxic, setting the stage for further restrictions on the chemical throughout the country. In 2011, the European Union banned the manufacture and sale of baby bottles containing BPA (Directive 2011/8/EU).

BPA emissions to the environment can occur during chemical manufacture, transport and processing. Effluents from municipal and mixed municipal-industrial wastewater treatment plants are a major source of environmental BPA (Deblonde et al. 2011). Other important sources comprise leaching from landfills and the natural breakdown of plastics (Asakura et al. 2004, Sajiki and Yonekubo 2004).

There are a myriad of studies evaluating the negative effects of this xenoestrogen on different target organisms, especially invertebrates, fish, reptiles and amphibians, recently reviewed by Flint et al. (2012). These revealed that BPA may be harmful even at environmentally relevant concentrations (12 µg/L) or lower. For instance, Lahnsteiner et al. (2005) observed reduced sperm quality and delayed ovulation in brown trout (*Salmo trutta f. fario*) following BPA exposure (1.75 µg/L) and complete inhibition of ovulation at 5 µg/L BPA.

Recently, BPA was listed as a possible priority substance subject to review for identification in the field of water policy (European Commission 2013).

1.3.2.2 Pharmaceutical active compounds

The PhACs used in the current research belong to different therapeutic classes: analgesics or antiinflammatories (acetaminophen, diclofenac, ibuprofen, indomethacin, ketoprofen, mefenamic acid, naproxen); antibiotics (trimethoprim); lipid regulators (fenofibrate); antineoplasic agent (cyclophosphamide) and the stimulant drug caffeine (Figures 1.9-1.14). These drugs were selected based on their different physicochemical properties that lead to different behaviors in WWTPs. In fact, whereas ibuprofen can be generally removed by conventional activated sludge processes, others PhACs like diclofenac show a remarkably high persistence and they can pass unchanged or poorly transformed in the aquatic environment (Oulton et al. 2010).

Anti-inflammatories

Non-steroidal anti-inflammatory drugs (NSAIDs) have analgesic, anti-inflammatory and antipyretic effects and they are usually used to mitigate symptoms of arthritis, bursitis, gout, swelling, stiffness and joint pain (Esplugas et al. 2007). There are many different types of NSAIDs widely used without prescription with an estimated annual consumption of several hundred tons in developed countries (Ziylan and Ince 2011). Diclofenac, ibuprofen, indomethacin, ketoprofen, mefenamic acid and naproxen, belong to this group (Ziylan and Ince 2011). In Directive 2013/39/EU, diclofenac was proposed for inclusion in the 1st Watch List, for the purpose of facilitating the determination of appropriate measures to address the risk posed by those substances (European Commission 2013).

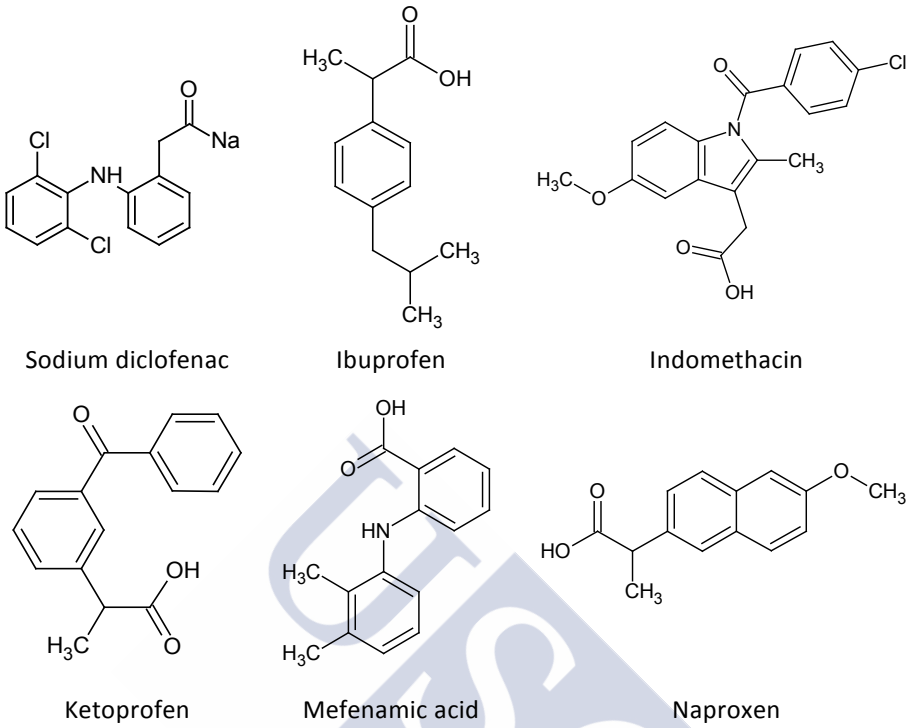
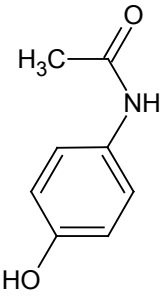


Figure 1.9. Chemical structure of the target NSAIDs.

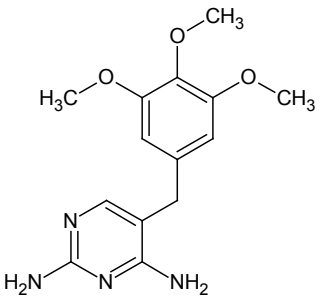
Acetaminophen



Acetaminophen, 4-acetamidophenol, or paracetamol is a very common analgesic and antipyretic agent used for the relief of fever as well as aches and pains associated with many conditions (headache, muscle aches, arthritis, backache, toothaches).

Figure 1.10. Chemical structure of acetaminophen.

Trimethoprim



Trimethoprim is an antibiotic, used to treat infections caused by bacteria, such as some chest and urine infections. It works by killing the bacteria responsible for the infection. it is more effective when combined with another antibiotic, sulfamethoxazole, and is rarely used alone.

Figure 1.11. Chemical structure of trimethoprim.

Caffeine

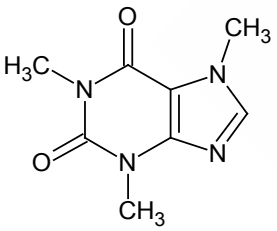


Figure 1.12. Chemical structure of caffeine.

This central nervous system stimulant is an ingredient in a variety of beverages (coffee, tea, and certain soft drinks) and food products (chocolate and dairy desserts). Caffeine is a key active ingredient in many headache medications and certain dietary supplements for weight loss. Although the human body metabolizes this stimulant efficiently, between 0.5 and 10.0% is excreted, mostly in the urine (Ferreira 2005).

Cyclophosphamide

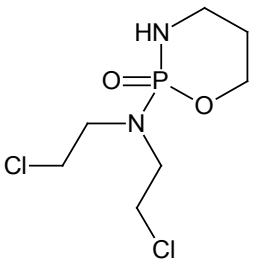
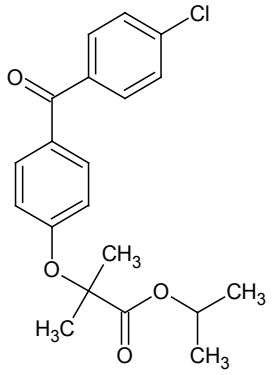


Figure 1.13. Chemical structure of cyclophosphamide.

Cyclophosphamide is an anti-cancer ("antineoplastic" or "cytotoxic") chemotherapy drug. It is used to treat bronchial-, brest-, ovarian- cancer, lymphomas, leukemia, etc; and also autoimmune disorders. Its annual consumption in Switzerland (2002) was 55 kg (Buerge et al. 2006).

Fenofibrate



Fenofibrate is a fibric acid derivative with lipid-modifying effects. It is used to treat high cholesterol and high triglyceride levels.

Figure 1.14. Chemical structure of fenofibrate.

1.3.2.3 Physicochemical properties of the target compounds

The knowledge of physicochemical properties of EOCs is a helpful tool for the rationalization of the complex processes occurring throughout the WWTPs. The main physicochemical properties of BPA and pharmaceuticals used in this investigation are shown in Table 1.5.

The K_{OW} value is indicative of the lipophilic character of each compound. In general, compounds with log K_{OW} lower than 2.5 may be considered relatively hydrophilic, and those with values higher than 4 are very hydrophobic. The dissociation constant (Ka) is indicative of the tendency of a substance to be ionized or dissociated in aqueous phase. The lower the pKa of an acidic drug, the stronger the acid. This parameter governs the electrostatic interactions of chemicals with the negatively charged surfaces of the microorganisms. In general, negatively charged molecules, such as the anionic species of acidic compounds will not adsorb, whereas cationic species of other EOCs (e.g. trimethoprim) will be more favored due to Van der Waals-type interactions (Suárez et al. 2008).Finally, the target EOCs in this study have low vapor pressure (data not shown), which suggests that negligible volatilization should be expected (Luo et al. 2014).

Table 1.5. Physico-chemical properties of the target compounds.

Compound	Molecular weight	Water solubility (mg/L) (25ºC)	Log K_{ow}	pKa	Charge at pH 7
BPA	228.3	300	3.40	9.6	neutral
Diclofenac	318.13	50	0.7	4.2	negative
Ibuprofen	206.3	21	4.0	4.9-5.2	negative
Indomethacin	357.8	0.94	4.2	4.5	negative
Ketoprofen	254.3	51	3.1	4.4	negative
Mefenamic acid	241.3	20	5.1	4.2	negative
Naproxen	230.3	16	3.2	4.2	negative
Acetaminophen	151.16	14000	0.46	9.4	neutral
Trimethoprim	290.3	400	0.91	7.1	neutral/positive
Caffeine	194.2	21600	-0.07	14	neutral
Cyclophosphamide	261.1	40000	0.63	-	neutral
Fenofibrate	360.8	0.42	5.2	-	neutral

References: (Verlicchi et al. 2012), (Staples et al. 1998), (Margot 2015), (Ziylan and Ince 2011), https://pubchem.ncbi.nlm.nih.gov/

1.3.3 Removal of the selected EOCs during conventional wastewater treatments

Conventional processes in WWTP include:

i) Pre-treatment, consisting of several physical and mechanical operations such as screening, sieving, blast cleaning and grease separation.

ii) Primary treatment, constituted by physical (sedimentation and flotation) and chemical (coagulation and flocculation) processes used to remove matter in suspension (solids, oils, grease and foam).

iii) Secondary treatment, where biological reactors perform the decomposition of organic matter and removal of nutrients (as for instance, lagooning, aerobic and anaerobic treatments and biofiltration processes).

iv) Tertiary treatment, designed for the removal of remaining unwanted nutrients (mainly nitrogen and phosphorous) through high performance biological or chemical processes as well as for disinfection by means of chlorination or UV treatment.

The fate of micropollutants in WWTPs depends on internal factors, which are micropollutant-related (characteristics of micropollutants, as hydrophobicity, biodegradability and volatility, resulted from their physico-chemical properties); and external factors, linked to treatment conditions of wastewater treatment processes, the mixture of micropollutants that can act as competitors, and wastewater characteristics (pH and temperature) (Luo et al. 2014). During these processes, the main mechanisms of micropollutants removal include volatilization, sorption to solids and chemical and/or biological transformation (Suárez et al. 2008). Nevertheless, losses due to stripping are not expected for BPA and PhACs, due to their low volatility. The sorption potential of micropollutants is a function of both their lipophilic character (K_{OW}) and acidity (pKa) (Suárez et al. 2008). Very hydrophobic compounds enter WWTPs associated to the particulate phase and are mainly removed with the removal of suspended solids. In that case, removal by sorption can be significant (10-80%) for hydrophobic drugs such as mefenamic acid (log K_{OW} = 5.1) or fenofibrate (log K_{OW} = 5.2) (Table 1.5) (Jelic et al. 2011). On the contrary, EOCs with high solubility, low hydrophobicity and negative charge at neutral pH (low sorption affinity on biological sludge which is negatively charged as well) are mostly found in the “dissolved” phase with negligible removal by sorption (<5%) (Margot 2015). Therefore, biodegradation or biotransformation would be the main removal mechanism for most pharmaceuticals. Biodegradability is linked to chemical structure and persistent micropollutants are usually: i) compounds with long, highly branched side chains, ii) saturated or polycyclic compounds, and iii) compounds possessing sulfate, halogen or electron withdrawing functional groups (Luo et al. 2014).

A number of researchers have reported the removal efficiencies attained by conventional processes in WWTPs. Table 1.6 summarizes the data for the target compounds, as well as the predicted non-effect concentration values (PNEC) reported for each compound.

Table 1.6. Occurrence and fate of BPA and the target PhACs in wastewater treatment plants and PNEC values (μg/L).

Compound	Influent		Effluent		Removal (RSD) (%)	PNEC
	Average	Max	Average	Max		
BPA	2.1	11.8	0.60	4.1	82.0(12.1)	0.18
Diclofenac	15	3.1	0.8	11	35.8(23.0)	9.7
Ibuprofen	373	373	3.6	48	91.4(8.1)	1.65
Indomethacin	1	1	0.21	<1	23.4(22.3)	3.9
Ketoprofen	8.5	8.5	0.36	<1	51.7(23.5)	15.6
Mefenamic acid	3	3	0.63	3.5	36.0 (34.0)	0.43
Naproxen	53	53	1	6.5	75.5(18.5)	2.62
Acetaminophen	246	246	0.89	10	99.8 (0.5)	1
Trimethoprim	10.5	10.5	0.36	6.7	46.8(26.3)	2.6
Caffeine	118	118	1.8	12	88.7(15.9)	5.2
Cyclophosphamide	na	na	0.012	0.02	23.8(18.7)	11
Fenofibrate	na	na	0.11	0.2	100*	0.1

*Removal exclusively by sorption onto sludge
na: not available
References: Luo et al. (2014), Česen et al. (2015), Verlicchi et al. (2012), Deblonde et al. (2011), Radjenovic et al. (2007), Jelic et al. (2011), U.S. Environmental Protection Agency (2010)

Table 1.6 suggests that these EOCs may be found at concentrations in the effluents of WWTPs higher than PNEC value. Ying et al. (2009) determined that in the worst case scenario, with the effluent accounting for 100% of the river flow during dry seasons in Australia, certain PhACs present in WWTP effluents, as diclofenac, could pose potential risk to the aquatic organisms.

The occurrence of these ones, and many other EOCs, at potentially harmful concentrations calls for their removal during WWTPs. Therefore, tertiary treatment processes to remove compounds at very low concentrations are of interest.

1.3.4 Tertiary treatments to remove the target EOCs

Due to the ineffectiveness of the conventional treatments to completely remove these compounds, alternative post-treatments methods are being investigated: i) physical methods such as adsorption or membrane separation; (ii) chemical treatments, such as those based on advanced oxidation processes (AOPs); (iii) microbial degradation with bacteria and fungi and iv) enzymatic treatments with oxidative enzymes.

1.3.4.1 Physical processes

Adsorption on activated carbon

This technique has a great potential for the treatment of micropollutants from secondary effluents. Both powdered activated carbon (PAC) and granular activated carbon (GAC) have been widely used in adsorption processes. Moreover, PAC dose, GAC regeneration and contact time play important roles in efficient removal of micropollutants (Luo et al. 2014). For instance, PAC and GAC attained removal percentages of diclofenac higher than 96% (Grover et al. 2011, Kovalova et al. 2013) whereas 66% BPA removal was reported with GAC (Hernández-Leal et al. 2011). Some important considerations are that AC efficacy may significantly be reduced by the presence of natural organic matter, which attaches to GAC.

Membrane filtration processes

In general, the retention of EOCs in membrane processes can be achieved by size exclusion, adsorption onto membrane and charge repulsion. These removal mechanisms depend on several factors as membrane process type, membrane characteristics, operating conditions, specific micropollutant characteristics and membrane fouling (Schäfer et al. 2011). Whereas microfiltration (MF) and ultrafiltration (UF) poorly remove EOCs, nanofiltration (NF) achieves variable removal of BPA, depending on the membrane material. For instance, polyamide based dense and loose NF membranes exhibited rejections of BPA of 98% and 80%, respectively, whereas cellulose acetate based membranes offered a low and variable (10-40%) rejection for BPA (Yüksel et al. 2013). Reverse osmosis (RO) generally shows great potential to remove micropollutants. Sahar et al. (2011) assessed the efficiency of RO in eliminating micropollutants present in treated effluents. Although high

removal rates were attained (99% for macrolide antibiotics, cholesterol, and BPA, 95% for diclofenac and 93% for sulfonamides), residual concentrations in the range 28‑223 ng/L of ibuprofen, diclofenac, salicylic acid, cholesterol and BPA were detected in the permeate. These results showed that although RO is an efficient removal solution, it could not serve as an absolute barrier to micropollutants.

1.3.4.2 Advanced oxidation processes

Advanced oxidation processes (AOPs) have emerged as promising technologies for the oxidation and destruction of a wide range of organic pollutants in water and wastewater. The most common AOPs and examples of their application on the removal of the target pollutants evaluated in the current research are detailed below. Some of these processes are commercially available, e.g. UV photolysis, which has more than 3000 applications in Europe and a large number in USA (Ziylan and Ince 2011).

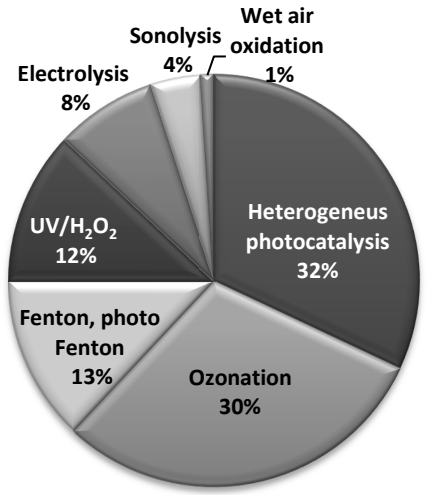


Figure 1.15. Distribution of AOPs tested for pharmaceutical degradation- adapted from Klavarioti et al. (2009).

Combined process UV/H_2O_2

The combined process UV/H_2O_2 is one of the most viable AOP techniques by its potential for photolytic cleavage of all H_2O_2 to $^{\bullet}OH$ at a stoichiometric ratio of 1:2, provided that the light source has sufficient emission at 190–200

nm. Sánchez-Polo et al. (2013) evaluated BPA degradation by the UV/H_2O_2 system under different conditions such as initial concentrations of BPA and H_2O_2, pH and wastewater composition. Although the system was adequate for removing BPA from wastewater, showing an efficacy of 100% after 60 min, the toxicity of byproducts was higher than that of BPA. On the other hand, Kim et al. (2009) reported removal of 90% of a large number of PhACs, including acetaminophen, diclofenac, naproxen, ketoprofen, indomethacin and mefenamic acid from real secondary effluent in lab-scale operations with a drug concentration range of 3 to 1359 ng/L. Nevertheless, the antineoplastic agent cyclophosphamide exhibited a high resistance to the treatment because, in spite of H_2O_2 addition, considerable UV dose of 1695 mJ·cm^{-2} was required for its 90% degradation

Ozonation

Ozone is a strong oxidizing agent and its application is becoming common in WWTPs as a clarifying and disinfecting agent. Organic contaminants are oxidized through direct reaction with molecular ozone or through indirect reactions with the produced $^{\bullet}OH$ (Umar et al. 2013). In a recent paper of Umar et al. (2013), the use of ozone to remove BPA from water and wastewater was reviewed. Whereas in water BPA removal was close to 100%, these authors concluded that BPA degradation in wastewater requires a longer contact time and higher ozone concentration than in water, due to the need to satisfy the ozone demand of the organic components in the more complex matrix, and may result in lower degradation efficiency. The elimination of PhACs by ozonation was also object of several studies (Rivera-Utrilla et al. 2013). For instance, the removal of diclofenac by this AOP was over 99% (Gerrity et al. 2011) whereas the percentages of mineralization ranged 22 and 99% depending on the time and dose of O_3. The removal of mefenamic acid by O_3 was highly dependent on pH and the degree of mineralization was limited: only 25% of the pollutant was mineralized at pH 9 (Chang et al. 2012). Moreover, ozonation may be not effective for toxicity reduction; it was suggested that it should not be applied without subsequent post-treatment for oxidation byproducts (Stalter et al. 2010).

Heterogeneous photocatalysis

This technique relies on the capacity of semiconducting materials to act as sensitizers for light-reduced redox processes due to their electronic structure. Among the possible photocatalysts, the most widely applied is titanium dioxide (TiO_2). Martínez et al. (2011) demonstrated its effectiveness under UV and NUV/Vis irradiation to remove 8 mg/L of diclofenac before 30 min. However, the most important reaction pathway was photocyclization to the corresponding monohalogenated carbazol, which presents higher phototoxicity than the parent molecule of diclofenac. On the other hand, Tsai et al. (2009) performed the photocatalytic degradation of BPA in a batch reactor containing TiO_2, attaining 99% degradation after 1 h of UV irradiation.

Fenton and UV/Fenton

Combined Fenton oxidation with UV irradiation, namely photo-Fenton process, inherently produces more $^{\bullet}OH$ and is therefore more effective than the dark method. A pilot scale facility using a compound parabolic collector reactor was applied for the removal of diclofenac by photo-Fenton reaction. The obtained results showed rapid and complete oxidation of diclofenac after 60 min, and total mineralization (disappearance of dissolved organic carbon, DOC) after 100 min of sunlight exposure (Pérez-Estrada et al. 2005). A photo-Fenton like treatment was also applied to treat 50 mg/L of aqueous BPA solution (Molkenthin et al. 2013). Both complete BPA degradation and significant DOC removal were achieved. Nevertheless, the oxidation reaction was significantly delayed when using freshwater, probably because the significant concentrations of bicarbonate alkalinity and DOC in such matrix.

Electrolysis

Electrochemical oxidation over anodes made of graphite, Pt, TiO_2, IrO_2, PbO_2, several Ti-based alloys and, more recently, boron-doped diamond (BDD) electrodes in the presence of a suitable electrolyte (typically NaCl) may be employed for the treatment of organic-containing effluents. Two mechanisms are responsible for the electrochemical degradation of organic matter: the direct anodic oxidation where the pollutants are adsorbed on the anode surface and destroyed by the anodic electron transfer reaction; and the indirect oxidation in the liquid bulk, mediated by the oxidants that are formed

electrochemically, such as chlorine, hypochlorite, hydroxyl radicals, ozone and hydrogen peroxide (Klavarioti et al. 2009). Xue et al. (2011) studied the electrochemical oxidation of 0.1 mM of BPA on Ti/SnO_2-Sb_2O_5/PbO_2 anode, with Na_2SO_4 0.02 M as support electrolyte, pH 4 and current density of 40 mA/cm^2. They observed that the slope of COD removal was linear with the electrolysis time during the first 80 min, and thereafter the COD removal rate was gradually reduced, with a percentage of removal of 80% after 350 min. Murugananthan et al. (2010) studied the mineralization of ketoprofen (5 μM) from distilled water by anodic oxidation with BDD and Pt electrodes. Whereas complete mineralization was achieved after 12 h using Na_2SO_4 (0.1 M) as supporting electrolyte; poor removal was attained with NaCl as supporting electrolyte due to the formation of refractory chlorinated organic compounds.

Sonolysis

Generation of hydroxyl radicals in water by ultrasonic pressure waves is based on the formation, growth and violent implosion of cavitation bubbles to release very extreme local conditions (5000 K, 2000 atm) that lead to high energy chemistry. Hartmann et al. (2008) found that irradiation of a synthetic diclofenac solution (50 mg/L) successively by 216, 617 and 850 kHz at 90 W for 1 h provided at least 87%, 90% and 24% degradation of the drug, respectively. Inoue et al. (2008) attained complete degradation of 114 mg/L of BPA after 10, 3 and 2 h of ultrasonic irradiation at a frequency of 404 kHz, and intensities of 3.5, 9.0 and 12.9 kW/m^2, respectively.

1.3.4.3 Biological treatment

An environmentally friendly alternative for the removal of the selected compounds may be the use of microorganisms as bacteria, algae or fungi. For instance, Gattullo et al. (2012) tested freshwater green alga *Monoraphidium braunii* to remove BPA. After 4-day growth, the removal of such pollutant was 39%, 48% and 35% for initial concentrations of BPA of 2, 4 and 10 mg/L, respectively. However, freshwater algae are usually inhibited by high concentrations of BPA (Ji et al. 2014). A facultative anaerobic bacterial strain, *Bacillus* sp. GZB, was isolated and identified to effectively degrade BPA under anaerobic and aerobic conditions (Li et al. 2012). The use of biofilters was also proposed for the tertiary treatment of wastewater. In this sense, Rattier et al. (2014) developed systems consisting of anthracite media filters colonized by a

bacterial biofilm to treat a broad range of micropollutants, with initial concentrations of 2 µg/L. Whereas acetaminophen, ibuprofen and caffeine attained percentages of removal above 80%, other as indomethacin, trimethoprim or diclofenac were not effectively degraded.

The use of white rot fungi constitutes other alternative since they have been reported to degrade a wide range of organic pollutants (Pointing 2001). Several authors have demonstrated the ability of different WRF to remove BPA and several pharmaceuticals (Table 1.7).

However, a major drawback of using WRF as biocatalysts is the necessity of working in aseptic conditions so that the development of this alternative is costly and problematical. For this reason, the use of their ligninolytic enzymes instead of whole cells could represent a more viable technology, with several advantages previously discussed in section 1.2.4.

1.3.4.4 Enzymatic removal by laccase

Since many EOCs possess a structure prone to oxidation by oxidative enzymes, one treatment option may rely on the use of such enzymes, i.e., laccases, aiming at oxidizing EOCs before their release into the environment. Laccases seem to be the most promising biocatalysts because their broad substrate range and use of oxygen instead of hydrogen peroxide as electron acceptor.

Batch studies with free laccase

Extensive batch experiments were performed to prove the ability of laccases to remove a vast number of EOCs, including BPA and several pharmaceuticals. First trials performed in batch mode to remove BPA by laccase from *T. villosa* demonstrated the capability of this enzyme to remove EDCs as well as the estrogenic activity associated (Fukuda et al. 2004, Fukuda et al. 2001, Uchida et al. 2001). However, the applied BPA concentrations and temperatures in these reactions were considerably higher than the expected in WWTP effluents; whereas lower pH values (ranging from 4.5 to 6) were more favorable for laccase catalysis. In a recent work, the influence of treatment conditions (namely pH, temperature, enzyme concentration and reaction times) on the transformation of BPA, diclofenac and mefenamic acid by *T. versicolor* laccase was investigated (Margot et al. 2013). The authors reported

that pH had the greatest influence, and the optimal pH values for the removal of BPA, diclofenac and mefenamic acid were around 5.5, 4.5 and 6.3, respectively.

Table 1.7. Elimination of BPA and pharmaceuticals by WRF.

Compound	WRF	Concentration	Removal (%)	Reference
BPA	*Pleurotus ostreatus*	0.4 mM	80 (12 d)	(Hirano et al. 2000)
	Stereum hirsutum *Heterobasidium insulare*	200 mg/L	100 (14 d)	(Lee et al. 2005)
	Irpex lacteus	10 mg/L	100 (3 d)	(Cajthaml et al. 2009)
	Bjerkandera adusta		75 (14 d)	
	P. chrysosporium		0 (14 d)	
	Phanerochaete magnolia		100 (14 d)	
	P. ostreatus		100 (3 d)	
	T. versicolor		100 (7 d)	
	Pycnoporus cinnabarinus		100 (14 d)	
	Dichomitus squalens		100 (7 d)	
	I. lacteus	100 mg/L	50 (3 d)	(Kum et al. 2009)
Diclofenac	*T. versicolor*	10 mg/L 45 µg/L	94 (1 h)	(Marco-Urrea et al. 2010b)
	T. versicolor	10 µg/L	100 (2 d)	(Tran et al. 2010)
	P. sordida	0.1 mM	100 (6 d)	(Hata et al. 2010)
	Bjerkandera sp R1	1 mg/L	>99 (4 d)	(Rodarte-Morales et al. 2011)
	B. adusta		>40 (4 d)	
	P. chrysosporium		>70 (4 d)	
Ibuprofen	*T. versicolor*	10 mg/L	100 (7 d)	
	I. lacteus		100 (7 d)	
	Ganoderma lucidum		100 (7 d)	(Marco-Urrea et al. 2009)
	P. chrysosporium		88 (7 d)	
	Bjerkandera sp R1	1 mg/L	>70 (4 d)	(Rodarte-Morales et al. 2011)
	B. adusta		>90 (4 d)	
	P. chrysosporium		>95(4 d)	
Indomethacin	*T. versicolor*	10 µg/L	100 (2 d)	(Tran et al. 2010)

Table 1.7. Elimination of BPA and pharmaceuticals by WRF (continuation).

Compound	WRF	Concentration	Removal (%)	Reference
Ketoprofen	*T. versicolor*	100 µg/L	100 (2 d)	(Tran et al. 2010)
		11 mg/L	100 (24 h)	(Marco-Urrea et al. 2010c)
		40 µg/L	95 (5 h)	
Mefenamic acid	*Phanerochaete sordida*	0.1 mM	90 (6 d)	(Hata et al. 2010)
Naproxen	*T. versicolor*	10 mg/L	100 (6 h)	(Marco-Urrea et al. 2010a)
		55 µg/L	95 (5 h)	
	T. versicolor	10 µg/L	100 (2 d)	(Tran et al. 2010)
	B. sp R1	1 mg/L	>90 (7 d)	(Rodarte-Morales et al. 2011)
	B. adusta		>90 (7 d)	
	P. chrysosporium		>99 (4 d)	
Acetaminophen	*T. hirsuta*	100 ng/L	>70 (5 d)	(Haroune et al. 2014)
Caffeine	*T. hirsuta*	100 ng/L	>20 (5 d)	(Haroune et al. 2014)
Cyclophosphamide	*T. versicolor*	10 mg/L	0 (9 d)	(Ferrando-Climent et al. 2015)

The low redox potential laccase from *M. thermophila* was also reported to transform diclofenac at acid pH (65% and 40% of transformation after 8 h at pH 4 and pH 5 respectively), but not at neutral pH (Lloret et al. 2010). Laccase-mediated acetaminophen removal was also investigated by Lu et al. (2009) in batch experiments at pH 7. The use of 1000 U/L of laccase allowed the transformation of 73% of acetaminophen (50 µM) via polymerization through radical-radical coupling mechanism. Tran et al. (2010) evaluated the ability of laccase to transform 10 ionic PhAcs (10 µg/L) from a mixture including diclofenac, ibuprofen, indomethacin, ketoprofen and naproxen, in acetate buffer at pH 4.5. Whereas diclofenac, naproxen and indomethacin were completely removed by 6000 U/L of *T. versicolor* laccase before 2-h treatment, the percentages of elimination of ketoprofen and ibuprofen after 3 h were 50% and 37%, respectively.

Recently, crude laccase from *Trametes pubescens* (E_0= 750 mV) was applied as biocatalyst in batch experiments to remove a mixture of micropollutants including BPA, diclofenac, naproxen and ketoprofen from

model solutions (ultrapure water at pH 6.9 spiked with 1 µg/L of 18 analytes) and also real wastewater (sample collected after primary sedimentation, pH 6.7) (Spina et al. 2015). After 24-h treatment with 100 U/L laccase, removal percentages of 79.4, 62.2, 56.0 and 17.5% were attained for BPA, diclofenac, naproxen and ketoprofen, respectively. Interestingly, in real wastewater the removal was slightly higher for the PhACs despite a pronounced inactivation of laccase (54% in 24 h) whereas for BPA the percentage of elimination decreased considerably, 35%. The estrogenic activity of the real effluent was strongly reduced after laccase treatment.

Hence, while results of these studies suggest that EOC transformation by laccases is in principle possible and leads to detoxification, they seem not directly compatible under WWTP effluent conditions. On the other hand, although the addition of mediators as HBT, ABTS, syringaldehyde or violuric acid to improve the removal rates of BPA (Cabana et al. 2007a, Kim and Nicell 2006) or diclofenac (Lloret et al. 2013a, Sathishkumar et al. 2014) was considered in several studies, their use would increase treatment costs considerably. Moreover, the presence of mediators might even increase the toxicity of WWTP effluents, leading to an ineffective or even harmful treatment. Therefore, the use of laccase-mediator systems for wastewater treatment seems unattractive.

Towards continuous application

The application of laccases in tertiary wastewater treatment requires the retention of the biocatalyst in the reactor since continuous additions would lead to unaffordable treatment costs. Two main approaches have been considered in view of continuous treatments with laccase.

Enzymatic membrane reactors with dissolved laccase

The first approach relies on the use of the free enzyme in an Enzymatic Membrane Reactor (EMR). Such configuration is based on the use of membranes with pore size smaller than the enzyme aiming at preventing their washout (López et al. 2004). The semi-permeable membrane facilitates the separation of the enzyme from products and substrates. The enzyme remains within the system (reactor) allowing continuous operation, with feeding and treated effluent withdrawal without significant loss of the enzyme. Two

possible configurations can be considered (Figure 1.16) i) separated reactor associated to a membrane filtration unit; (b) bundle of hollow-fibers or assembly of flat membranes submerged into the bioreactor (de Cazes et al. 2014a).

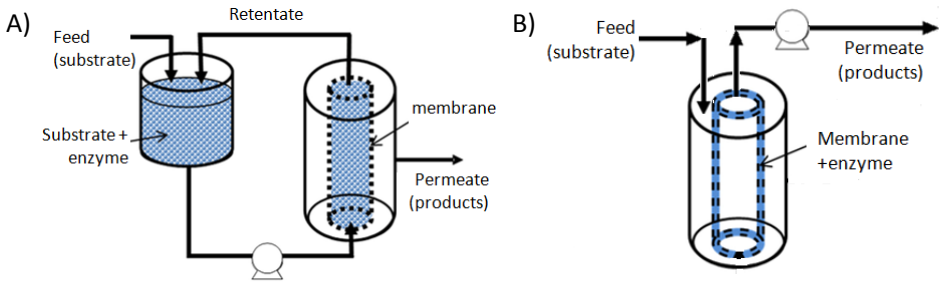


Figure 1.16. Possible configurations of EMRs: (A) Enzymatic reactor coupled with filtration unit; (B) enzymatic reactor with submerged membrane.

An EMR using an external UF polymeric membrane module to retain laccase from *M. thermophila* was successfully applied for the continuous removal of estrogens from real wastewater (Lloret et al. 2013b). Recently, Escalona et al. (2014) operated a reactor coupled to an external nanofiltration polymeric membrane to continuously remove very high concentrations of BPA (20 mg/L) from a buffer solution (pH 6) by laccase from *T. versicolor*. However, the detected concentrations of BPA on the permeate were high, in the range from 1 to 3 mg/L. In parallel, Nguyen et al. (2014) investigated the removal of BPA and diclofenac at much lower concentrations in an EMR using a submerged UF polymeric membrane. During the continuous operation, the percentages of degradation for BPA and diclofenac were 85% and 60%, respectively, although addition of laccase every 12 h was necessary to maintain the desired activity in the system. Moreover, it should be noted that the experiment was performed using Milli-Q water at pH 7 as matrix, instead of real wastewater.

Therefore, although two studies have proposed the use of free laccase onto enzymatic reactors with polymeric membranes to treat the target EOCs, there is still the need of assessing the laccase-catalyzed removal of these compounds at low concentrations and in real wastewaters matrices.

Insolubilized laccases

A second approach would rely on the use of insolubilized laccase to facilitate its retention in continuous systems. One possibility, linked to the previous approach, consists of the use of EMRs where the enzyme is immobilized onto the membrane. In such system, the membrane acts as a selective barrier, and at the same time, as the support for the immobilization. Georgieva et al. (2010) immobilized laccase onto a polypropylene membrane, which was subsequently used to perform the continuous transformation of phenolic compounds, such as BPA and acetaminophen (50 µM), from an aqueous solution at pH 5.5. The reactor consisted of two half cells separated by the catalytic membrane. The working solution was circulated through both cells, which were thermostated separately to allow a temperature gradient, but always with an average temperature of 25°C. The substrate removal rate increased with increasing temperature gradient. For instance, applying a temperature difference of 30°C allowed the complete removal of BPA and nonylphenol within 30 min, and the elimination of 44% of acetaminophen. However, these authors did not perform a control lacking enzyme to analyze to which extent other processes than laccase-catalyzed transformation contributed to the substrate removal.

De Cazes et al. (2014b) evaluated the degradation of high concentrations of the antibiotic tetracycline (20 mg/L) from model solutions during 24 h by laccase covalently linked onto a ceramic membrane previously coated with a polymer layer (i.e. gelatin). The system proved to be more efficient for tetracycline degradation than free enzymes, with a degradation yield of 56%, versus 30% attained by dissolved laccase. In parallel, Hou et al. (2014) immobilized laccase from *T. versicolor* on nanostructured TiO_2 coated polyvinylidene fluoride (PVDF) microfiltration membranes. Thereafter, the obtained catalytic system was applied for the removal of high concentration of BPA (150 µM) in 0.1 M acetate buffer (pH 5.5) solution.

A few studies focused on the immobilization of laccase onto different supports, with different retention devices depending on the type of support. For instance, laccase from *C. polyzona* was immobilized on diatomaceous earth support Celite® R-633 and then applied in a packed bed reactor to transform nonylphenol (23 µM), BPA (22 µM), and triclosan (18 µM). Apart from the

consideration of high concentrations of the pollutants, the treatment was conducted at pH 5 (Cabana et al. 2009a). In a further study, Cabana et al. (2009b) performed the continuous transformation of the same EDCs, separately, in a perfusion basket reactor using insolubilized *C. polyzona* laccase as cross-linked enzyme aggregates (CLEAs). Optimal values of pH and temperature for the removal of BPA were 4.7 and 48°C, respectively. Operating the reactor at pH 5 and room temperature allowed the removal of at least 85 % of BPA (22 μM) with a hydraulic retention time of 5.4 h.

Additional studies investigated the immobilization of laccases onto silica support materials and some of them, evaluating the transformation of the target micropollutants in real wastewater matrices (Table 1.8). In the last years, the use of nanomaterials as supports for enzyme immobilization has attracted much attention because they offer the ideal characteristics for balancing the key factors that determine biocatalysts efficiency, including specific surface area, mass transfer resistance, and effective enzyme loading. The group of professor Corvini published several studies focusing on the immobilization of laccases from genus *Thielavia* and *C. polyzona* onto silica nanoparticles and their application for the removal of micropollutants, and more specifically, BPA from wastewater (Table 1.8).

Finally, it is worthy to mention recent studies focused on applying enzyme combinations instead of laccase alone to expand the range of compounds and conditions that can be targeted by laccase treatments. In this line, Ba et al. (2014) produced combi-CLEAs of laccase and mushroom tyrosinase. The novel biocatalyst achieved removals between 80 and up to 100% of the acetaminophen from municipal and hospital wastewaters. Touahar et al. (2014) produced combi-CLEAs of VP, glucose oxidase and laccase and tested its catalytic potential in acidified wastewater (pH 5) spiked with a cocktail of 14 PhACs (1 mg/L), including acetaminophen, naproxen, mefenamic acid, indomethacin, diclofenac, ketoprofen, caffeine, trimethoprim and fenofibrate. High removal was achieved (more than 80%) for the five first compounds. However, in real wastewater, only 25% of acetaminophen was removed.

Table 1.8. Studies reporting removal of BPA and PhACs by immobilized laccase in WWTP effluent matrices.

Laccase source	Support	Compound	Reactor type	pH	Reaction time/ Hydraulic residence time	Feed concentration	EOC removal	Reference
Thielavia genus	Fumed silica nanoparticles	BPA	Batch	7.5	3 d	0.73 µM	99% after 1 d	(Hommes et al. 2012)
Coriolopsis polyzona	Fumed silica nanoparticles	BPA	Batch	7.5	3 d	0.73 µM	93% after 1 d	(Hommes et al. 2012)
Coriolopsis polyzona	Mesoporous silica particles	BPA	CSTMR	5	1.85 h	50 µM	90% for 50 h	(Demarche et al. 2012a)
Coriolopsis gallica	Mesoporous silica particles	BPA, diclofenac	CSTMR	7.8	1.25 h	10 µM	>85% and 30% for 80 h	(Nair et al. 2013)
Thielavia genus	Fumed silica nanoparticles	BPA	Continuous membrane reactor	8-10	7 h	1.27 nM	66% for 12 d	(Gasser et al. 2014)

CSTMR: Continuous stirred tank membrane reactor

1.4 OBJECTIVES

In the present Thesis, the development of enzymatic technologies to treat environmental pollutants from real matrices was considered. The ligninolytic enzyme laccase was proposed as the biocatalyst.

On the one hand, the PAH anthracene was selected as model compound of hydrophobic, low bioavailable and high persistent pollutant in soils. The Two Phase Partitioning Reactor concept was considered to conduct the laccase-catalyzed degradation of anthracene (Chapters 2-4)

The specific objectives of this first part of this Thesis can be described as follows:

i. Study of different reactor configurations (micellar, biphasic and the combination of both) to perform the laccase-catalyzed degradation of anthracene. Determine the main parameters affecting the laccase stability and establish the process model for a controlled operation of the TPPB (Chapter 2).
ii. Selection of a suitable water immiscible solvent in view of a real application and investigate the influence of operational parameters, such as oxygen and mediator concentration, and laccase addition to maximize the removal of anthracene in the TPPB (Chapter 3).
iii. Development of a combined process of soil extraction and enzymatic catalysis to remove poorly soluble pollutants from soils (Chapter 4).

In the second section of this Thesis, the industrial chemical BPA and several PhACs were selected as models of EOCs in wastewaters. The enzymatic treatment of these micropollutants from real biologically treated wastewater was considered. The specific objectives of the second section are:

iv. Operation of an Enzymatic Membrane Reactor with a ceramic membrane to perform the continuous removal of BPA by laccase from real wastewater (Chapter 5)
v. Assessment of the use of laccases immobilized onto fumed silica nanoparticles for their application to remove BPA and diclofenac from secondary effluents (Chapter 6).
vi. Production of robust magnetically-separable cross-linked laccase aggregates to remove PhACs from secondary effluents (Chapter 7).

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Chapter 2

SURFACTANT-ASSISTED TWO PHASE PARTITIONING BIOREACTORS FOR LACCASE-CATALYZED DEGRADATION OF ANTHRACENE[1,2]

Summary

The degradation of anthracene by laccase from *Trametes versicolor* in enzymatic reactors was evaluated in this chapter. The use of a surfactant (Triton X-100) at concentration above critical micelle concentration (CMC) enhanced anthracene solubility and facilitated its degradation. Moreover, Triton exerted a beneficial effect on the laccase stability and protected it from the oxidative action of the mediator 1-hydroxybenzotriazole (HBT). In a further stage, the combined configuration of a two phase partitioning bioreactor (TPPB) operating with silicone oil as an immiscible solvent and the surfactant achieved the degradation of anthracene at high conversion rate: 16 $\mu mol/L_R \cdot h$. Furthermore, a model for anthracene degradation by laccase-mediator system was developed. Both the first order kinetic constant (k) and the overall mass transfer coefficient (K_La) were estimated by using the method of least squares. The increased K_La value obtained: 788.1 h^{-1}, proved that Triton X-100 also improved mass transfer. Anthracene concentration in aqueous phase was close to that corresponding to equilibrium state suggesting that mass transfer mechanism did not limit the global process. The kinetic constant, which is expected to depend on the initial concentration of enzyme, resulted in 52.2 h^{-1}. Enzyme inactivation occurred in two stages and could be modeled by using a three parameter biexponential model. The possibility of reusing the aqueous phase in several cycles until negligible oxidative potential was demonstrated. Also, the feasibility of reusing the silicone oil to dissolve more anthracene was proven in three consequent cycles with high percentages of anthracene removal.

[1] **A. Arca-Ramos, G. Eibes, M.T. Moreira, G. Feijoo and J.M. Lema (2012).** Surfactant-assisted Two Phase Partitioning Bioreactors for laccase-catalyzed degradation of anthracene. Process Biochemistry 47 (1115-1121)

[2] **A. Arca-Ramos, G. Eibes, M.T. Moreira, G. Feijoo and J.M. Lema (2012).** Mass transfer enhancement by the addition of surfactant in a Two Phase Partitioning Bioreactor for the degradation of anthracene by laccase. Chemical Engineering Transactions 27 (187-192)

OUTLINE

2.1 INTRODUCTION

The low water solubility and high hydrophobicity of PAHs restrict their natural biodegradation (Eibes et al. 2007) so that the increased solubilization of polyaromatics in aqueous media would have beneficial effects on the potential degradation of these persistent pollutants (Mihelcic et al. 1993). The use of organic solvents and surfactants has been proposed to desorb hydrophobic compounds from soil matrices and facilitate their transfer to aqueous environments where microbes can attack these molecules (Costa et al. 2010, Lee et al. 2001, Paria 2008, Robles-González et al. 2012). The two-phase partitioning bioreactors (TPPB) concept is based on the use of a water immiscible and biocompatible organic solvent in contact with an aqueous phase that contains the biocatalyst. The solvent is used to dissolve high concentrations of the target compound, which then partitions into the aqueous phase at levels determined by its partition coefficient. As the microorganisms or enzymes are only able to degrade the pollutant in the aqueous phase, this one diffuses from the organic phase to re-establish the equilibrium (Vrionis et al. 2002). Subsequently, substrate delivery is maintained until the organic phase becomes completely depleted. The demand and supply of substrate are driven by cellular or enzymatic processes. Moreover, the organic solvent, once depleted, can be separated from the aqueous phase, reused for the solubilization of the hydrophobic pollutant and returned to the aqueous phase in a further operation (Eibes et al. 2007). This bioreactor configuration has been successfully applied for the biological treatment of phenanthrene and pyrene (Guieysse et al. 2001), and anthracene (Janikowski et al. 2002) by *Pseudomonas* sp. and *Sphingomonas* sp.

Another alternative to increase the solubilization of PAH relies on the use of surfactants (Edwards et al. 1991). When using this type of compounds over the critical micelle concentration (CMC), the surfactant acts as an emulsifier and the insoluble species in water can be transferred to the micelle core, which is itself solubilized in the bulk solvent. Thereafter, the aqueous solubility of organic compounds is enhanced by the inclusion of hydrophobic molecules into surfactant micelles. The surfactant can also exist as a monomer at concentrations below the CMC with only minimal effects in the aqueous solubility of organic compounds.

The use of a TPPB in association with suitable surfactant may be an option to overcome problems related to the transfer of the PAH from the organic phase to the aqueous phase (Eibes et al. 2010). In addition, surfactants may increase the apparent solubility of PAHs by micellar solubilization.

The selection of a suitable biocatalyst for the degradation of PAHs has to account for the high recalcitrance of this type of compounds. Extracellular lignin-modifying enzymes from white rot fungi (WRF) are suitable candidates since they are known to provide WRF the ability to degrade PAHs (Bezalel et al. 1996). The oxidation of PAHs by these enzymes generates more polar and water soluble metabolites, such as quinones, which could be easier biodegraded by indigenous bacteria present in soils and sediments (Meulenberg et al. 1997). In addition, the use of enzymes against microorganisms presents numerous advantages as previously described in Chapter 1, such as higher tolerance to high pollutant concentrations, shorter periods of treatment, higher specificity and no biomass production.

Laccase from the white rot basidiomycete *Trametes versicolor* was selected as the biocatalyst. This is a commercially available high-redox potential laccase (0.78 V) whose capability to degrade anthracene in the presence of 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT) (Johannes et al. 1996) and natural phenolic mediators has been demonstrated in batch small-scale experiments (Cañas et al. 2007). A drawback concerning laccase-mediator system is the inactivation of laccase due to its liability to the free radical attack of redox mediators (Li et al. 1999) and particularly to HBT free radicals (Khlifi et al. 2009). In this sense, it has been reported that the addition of the surfactant Triton X-100 may partially protect laccase from this type of inactivation (Ji et al. 2009).

The aim of the present work was to study the feasibility of a laccase-based reactor for the degradation of anthracene, a poorly soluble model compound. Three different configurations were considered: micellar, biphasic and the combination of both. These alternatives will allow operating with concentrations of anthracene considerably higher than that restricted by the solubility in aqueous phase. The effect of media composition on laccase inactivation was investigated and a mathematical model was proposed. In

addition, the possibility of exploiting the oxidative potential of the aqueous phase in several cycles was assessed. The reuse of the organic solvent, silicone oil, was also object of study.

2.2 MATERIALS AND METHODS

2.2.1 Chemical reagents and enzyme

Anthracene (99%), Triton X-100 (≥98%) and HBT (99%) were purchased from Janssen Chimica, Merck and Fluka, respectively. Anthraquinone (97%), silicone oil 50 cSt, ABTS (≥98%), organic solvents (HPLC grade) and the commercial laccase from *T. versicolor* were purchased from Sigma-Aldrich.

2.2.2 Preparation of silicone oil spiked with anthracene

The organic phase consisted of silicone oil previously spiked with anthracene in excess. The silicone oil was continuously agitated with a Teflon-coated magnetic stir bar in a glass bottle for 24 h to assure the saturation of anthracene in the organic phase. Thereafter, the spiked silicone oil was filtered through a glass fiber prefilter to remove the solid particles of anthracene that had not been dissolved.

2.2.3 Determination of enzyme activity

Laccase activity was determined by monitoring the oxidation rate of 5 mM ABTS to its cation radical ($ABTS^{\bullet+}$) at 436 nm (ε_{436} = 29,300 $M^{-1}cm^{-1}$) in 0.1 M sodium acetate buffer (pH 5) at 30ºC. One unit (U) of activity was defined as the amount of enzyme forming 1 µmol of $ABTS^{\bullet+}$ per min. All spectrophotometric measurements were carried out on a Shimadzu UV-1603 (Kyoto, Japan).

2.2.4 Stability of the biocatalyst

The experiments were carried out at 30ºC and 250 rpm for 24 h in a stirred tank reactor (BIOSTAT®Q reactor, B. Braun-Biotech International, Melsungen, Germany). It was equipped with pH, temperature and pO_2 sensor and a magnetic stirrer. The medium consisted of 400 U/L laccase, 0.1 M sodium acetate at pH 5, 1 mM HBT, 1% Triton X-100 (v:v) and 10% silicone oil

(v:v) in 225 mL water. Aliquots of the reaction medium were taken periodically for enzymatic measurements. Residual activity was expressed as the ratio of the activity at any instant (A in U/L) divided by the initial total activity of the mixture (A_0 in U/L).

2.2.5 Anthracene degradation by laccase in a monophasic system

The experiments were carried out at room temperature (i.e. 26±2 ºC) and magnetic stirring for 24 h in 100 mL Erlenmeyer flasks containing 3500 U/L laccase, 0.1 M sodium acetate at pH 5, 1 mM HBT, 0.5, 1 or 2% Triton X-100 (v:v) and anthracene (22 µM) in a final volume of 100 mL water.

All the experiments were performed in triplicate. Homogeneous samples of 1 mL were withdrawn at fixed time intervals and the reaction was stopped with 32 µL HCl (2.5 M) and frozen for further analysis.

2.2.6 Enzymatic elimination of anthracene by laccase in a two phase system without and with Triton X-100

The degradation of anthracene by laccase in two phase systems were performed at room temperature (i.e. 26±2 ºC), with magnetic stirring for 48 h in 50 mL Pyrex glass bottles (experiment without surfactant) and 100 mL Erlenmeyer flasks (experiment with surfactant). The distribution of the anthracene between the organic and the aqueous phase was facilitated by the agitation applied during the experiment.

The two phase system comprised 10% silicone oil (v:v) saturated with anthracene as organic phase whereas aqueous phase consisted of 2000 U/L or 1600 U/L laccase (experiments without and with Triton X-100 respectively), 0.1 M sodium acetate at pH 5, 1 mM HBT, 1% Triton X-100 (v:v).

After 48 h of incubation, a sample from the organic phase was withdrawn and centrifuged for 15 min at 3000 rpm to separate the two phases. An aliquot of 100 µL from the organic phase was added to a final volume of 10 mL of acetonitrile and mixed in a vortex for 5 min. A sample of 1 mL was withdrawn for HPLC analysis. To verify that degradation took place only due to enzyme oxidation, controls were run in parallel without laccase.

2.2.7 Anthracene degradation by laccase in a TPPB

The oxidation of anthracene was carried out at 30ºC and 250 rpm for 24 h in a two-phase partitioning bioreactor considering a conventional configuration of a stirred tank reactor (BIOSTAT®Q reactor, B. Braun-Biotech International, Melsungen, Germany), equipped with pH, temperature and pO_2 sensor and magnetic stirrer. The total reaction volume was 250 mL with 10% silicone oil (v:v) previously saturated in anthracene. The aqueous phase consisted of 1200 U/L laccase, 0.1 M sodium acetate buffer at pH 5, 1 mM HBT, 1% Triton X-100 (v:v). An experiment lacking laccase was carried out in parallel as the control. Before sampling, agitation was stopped for 5 min in order to allow the separation of both phases (Figure 2.1A). Samples from both organic and aqueous phases were withdrawn periodically to determine anthracene and anthraquinone concentrations. An aliquot of the organic sample (2 mL) were centrifuged for 20 min at 3000 rpm in order to separate tiny aqueous drops and 50 µL of the supernatant were added to a final volume of 5 mL acetonitrile. After 5 min vortexing, 1 mL of the sample in acetonitrile was analyzed by HPLC for anthracene and anthraquinone determination.

An aliquot of the aqueous phase (1 mL) was taken and laccase activity was determined. Then the sample was frozen after adding 32 µL of 2.5 M HCl to stop the enzymatic reaction. Prior to HPLC analysis, the sample was defrosted and centrifuged for 20 min in order to separate small organic drops.

2.2.8 Recovery and reuse of the aqueous phase in the surfactant-assisted TPPB

The reusability of the aqueous phase was investigated in two additional batch experiments of 24 h with no extra addition of enzyme or mediator. At the end of each oxidative cycle, the aqueous phase was separated from the exhausted organic phase by decantation and a silicone oil solution freshly spiked with anthracene was added so that the proportion of organic phase was maintained constant: 10% (v:v). The control assay was subjected to the same procedure.

2.2.9 Recovery and reuse of the organic phase in the surfactant-assisted TPPB

With the aim of investigating the reusability of the organic phase once depleted in anthracene, an experiment consisting of three consecutive cycles of operation (24 h each) in the surfactant-assisted TPPB in the BIOSTAT®Q reactor at 30ºC and 250 rpm was performed. The medium composition consisted of 1200 U/L laccase, 0.1 M sodium acetate buffer at pH 5, 1 mM HBT, 1% Triton X-100 (v:v) and 10% silicone oil.

At the end of each cycle, the organic phase was separated from the aqueous phase by decantation (Figure 2.1B) and the silicone oil solution underwent the saturation process previously described.

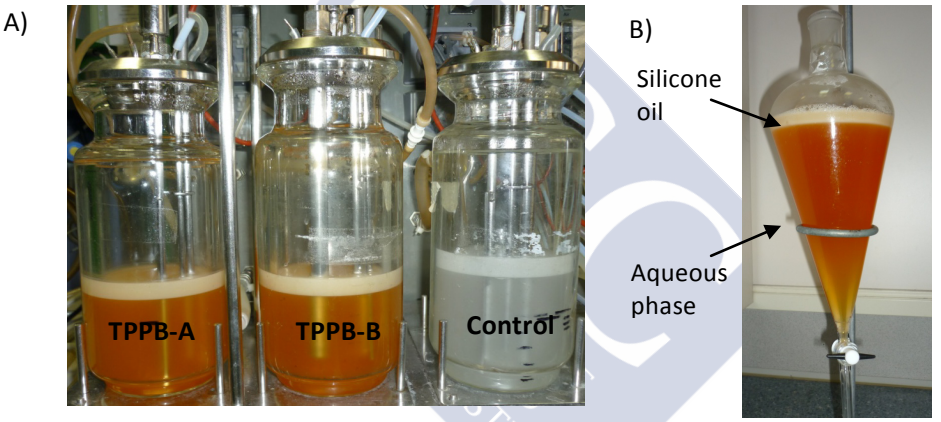

Figure 2.1. (A) Experimental set-up for parallel assays of anthracene degradation in the TPPB (and control without laccase) and (B) separation of both phases after a cycle.

Therefore, the organic solvent spiked with anthracene was added to a freshly aqueous phase. A control assay without laccase was performed in parallel. Both the organic and aqueous phases were periodically withdrawn for anthracene, anthraquinone and enzymatic activity determination.

2.2.10 High Performance Liquid Chromatography

Anthracene and anthraquinone in the absence of surfactant were analyzed by a Jasco XLC HPLC (Jasco Analitica, Madrid, Spain) equipped with a diode array detector monitoring the absorbance at 254 nm in a 4.6·150 mm Kinetex reverse phase column (2.6 µm C18 100Å) and a ChromNav data

processor. The injection volume was set at 25 µL and the isocratic eluent (80% acetonitrile and 20% water) was pumped at a rate of 1.3 mL/min.

In the presence of Triton X-100, anthraquinone was determined using a 4.6·200 mm Spherisorb ODS2 reverse phase column (5 µm; Waters). The injection volume was 100 µL and the isocratic eluent (80% acetonitrile and 20% water) was pumped at a rate of 1 mL/min.

2.3 RESULTS AND DISCUSSION

2.3.1 Degradation of anthracene by laccase

Organic solvents and surfactants represent valid alternatives to enhance the solubilization of organic compounds such as anthracene, whose aqueous solubility is only 0.08 mg/L at 30ºC (Eibes et al. 2010). Based on this fact, several configurations to carry out the degradation of anthracene by laccase were proposed.

First, the widely used non-ionic surfactant, Triton X-100, was evaluated for the degradation of anthracene by laccase from *T. versicolor*. The effect of the concentration of this surfactant on the oxidation of anthracene was studied by testing three different concentrations, higher than CMC. Since anthracene is not a substrate for laccase (Johannes et al. 1996), the synthetic mediator HBT was used to increase the oxidation potential of the enzyme.

In all experiments more than 95% of anthracene was degraded after 2 h and complete oxidation was achieved after 4 h. A concentration of 1% Triton X-100 was observed to provide the highest percentage of removal, corresponding to an oxidation rate of 10.6 µM/h.

This oxidation rate is in the same order of magnitude as the value obtained by Johannes et al. (1996) who evaluated the influence of Tween 20 on anthracene oxidation by laccase. They achieved nearly complete degradation of anthracene after 4 h with a degradation rate of approximately 20 µM/h. As far as the degradation of anthracene is concerned, the use of the surfactant increased the solubility of the anthracene. Nevertheless, the concentration to be degraded is limited by the apparent solubility of the

compound for a given concentration of surfactant higher than CMC. This could be calculated from several properties of surfactant and anthracene such as the molar solubilization ratio (MSR), defined as the number of moles of organic compound solubilized per mole of surfactant added to the solution; the micelle phase/aqueous phase partition coefficient (K_{mc}), which is the ratio of the mole fraction of the compound in the micellar pseudophase to the mole fraction of the compound in the aqueous pseudophase; the CMC and the surfactant concentration at which the apparent solubility is evaluated (Paria 2008, Volkering et al. 1995). In the case of anthracene in a solution of 1% (v:v) Triton-X 100 the apparent solubility was 9.4 mg/L.

With the aim of improving the efficiency of the process, the use of a two-phase partitioning reactor was considered. In this configuration, the organic phase consisted of 10% silicone oil saturated in anthracene (1900 µM), while the aqueous phase comprised the laccase mediator system. Silicone oil was chosen as the solvent due to reported successful implementation of silicone-based TPPBs (Bouchez et al. 1997, Mahanty et al. 2008, Marcoux et al. 2000, Muñoz et al. 2006) and remarkable good features in terms of hydrophobicity. This solvent was also used for the *in vitro* degradation of anthracene by the ligninolytic enzyme MnP in a TPPB (Eibes et al. 2007), where maximum anthracene oxidation after optimization was reached after 56 h with an oxidation rate of 9.9 µmol/L_R·h. Our experiments showed that the degradation rate of anthracene in a TPPB was unsatisfactorily low because only 38% of pollutant was removed after 48 h. This implied an oxidation rate of 1.6 µmol/L_R·h, which was comparatively slower than in the micellar system with Triton X-100. Two facts may be behind this result: the low solubility of anthracene in the aqueous phase and the limited mass transfer of anthracene from the organic to the aqueous phase, influenced by the high anthracene partition coefficient between silicone oil and water. Moreover, silicone oil may sequester anthracene and limit its transfer into the aqueous phase. In order to overcome these limitations, the combination of a TPPB with the addition of the surfactant Triton X-100 (1% v:v) in the aqueous phase was proposed. Contrarily to the results from the previous TPPB configuration, complete anthracene oxidation was obtained after 48 h. In view of these results, this system was

studied in more detail, monitoring laccase activity and anthracene and anthraquinone concentrations during the process.

2.3.2 Effect of the components of the reaction medium on laccase activity

The effectiveness of the application of laccase in many industrial applications will depend on the stability of the enzyme. For this purpose, laccase was incubated for 24 h at pH 5 and 30ºC in the presence of the chemicals required in the catalytic cycle of the enzyme. The results of these incubation experiments are shown in Figure 2.2.

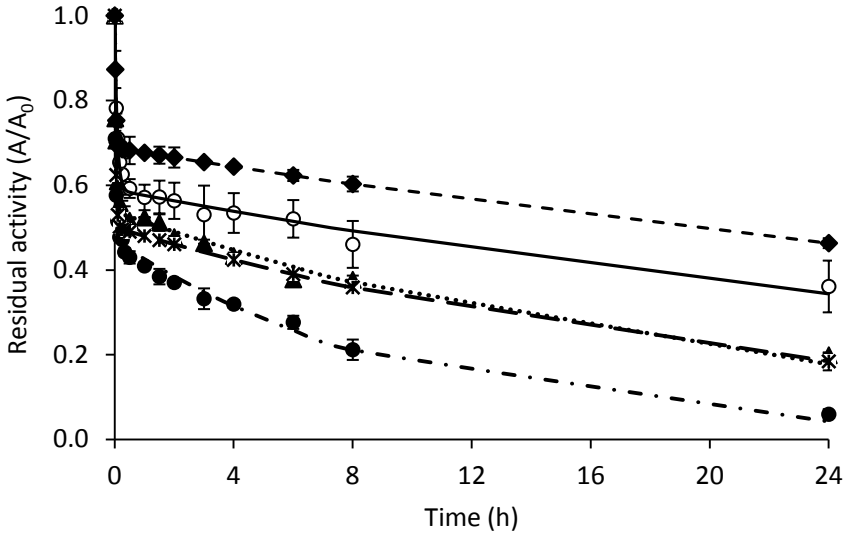

Figure 2.2. Stability of laccase in 0.1 M buffer sodium acetate (NaOAc) pH 5 at 30ºC, (○, —) in NaOAc-HBT (●,– · –), in NaOAc-Triton (◆, - - -), in NaOAc-HBT-Triton (▲,····) and in NaOAc-HBT-Triton-Silicone oil (✳,– –). The lines represent the inactivation kinetics modelled with the exponential model proposed by Aymard and Belarbi (2000).

An interesting observation is that the inactivation of the enzyme occurred in a two-stage period with different deactivation rates. Initially, the enzyme presented a rapid decline in activity followed by a period of slow decay. Such behavior can be modeled using the 3-parameter model of Aymard and Belarbi (2000) (equation (2.1)).

$$\frac{A}{A_0} = x \cdot \exp(-\alpha \cdot t) + (1-x) \cdot \exp(-\beta \cdot t) \qquad (2.1)$$

The ratio A/A_0 represents the residual enzyme activity after time *t*. The physical meaning of the parameters and their expression as a function of the individual rate constants differ depending on the considered mechanism (Cabana et al. 2007). The value of *x* (dimensionless) can be interpreted as an enzyme fraction and α and β are first order inactivation constants (h^{-1}). The values of the different parameters of this model were obtained by plotting the residual enzyme activity versus time using the method of least squares (Table 2.1). Using these parameters the half-life of the laccase was found to vary depending on the reaction medium.

Table 2.1 Kinetic decay parameters for laccase biphasic inactivation described in equation (2.1) under different incubation media at 30ºC and pH 5.

	x	α (h^{-1})	β (h^{-1})	R^2	Half-life (h)
NaOAc	0.41	12.48	0.02	0.99	8.28
NaOAc – HBT	0.53	39.07	0.10	0.99	0.07
NaOAc – HBT - Triton X-100	0.46	25.57	0.05	0.98	1.54
NaOAc - Triton X-100	0.31	31.20	0.02	0.95	16.10
NaOAc – HBT - Triton X-100-Silicone oil	0.50	27.80	0.04	0.99	0.18

Laccase was particularly unstable when incubated in a medium containing sodium acetate and mediator (HBT), which strongly reduced enzyme activity (up to 32% after 4 h) leading to a lower half-life of the enzyme. The significant loss of activity in the presence of the mediator can be linked to the formation of nitroxy radicals from the mediator, which may inactive the enzyme by the oxidation of aromatic aminoacids on the protein surface (Aracri et al. 2009, Li et al. 1999). The effect of HBT on laccase was previously described on laccase isoenzymes from crude extracts obtained from *Fomes sclerodermeus* and *Trametes troggi* (Khlifi et al. 2009, Papinutti et al. 2008).

Figure 2.2 also shows that the addition of Triton X-100 exerted a stabilizing effect on laccase. It is generally accepted that the enzyme inactivation is mainly induced by heating, the attack of intermediate free

radicals and the adsorption of oxidative polymerization products on the protein structure (Ji et al. 2009). The presence of this non ionic surfactant in the medium is believed to prevent the laccase inactivation induced by the intermediate free radical attack. Moreover, Triton X-100 had a positive effect on enzyme stability in the absence of HBT. Thus, laccase inactivation in the system sodium acetate-Triton was lower than that of sodium acetate exclusively. A possible explanation may be that Triton X-100 helps the enzyme to be in the optimal folding state. Consequently, the hydrophobic aminoacid residues of laccase in the presence of surfactant would be exposed to a less polar environment or restricted to the inner part of the conformation of the enzyme, which is beneficial for its folding and stabilization (Ji et al. 2009). Finally, the addition of silicone oil (10% v:v) had no significant effect on laccase residual activity after 24 h; whereas it increased the rate of deactivation in the first stage, resulting in a half-life of 0.18 h versus 1.54 h in the absence of the immiscible solvent.

2.3.3 Triton-assisted anthracene degradation by laccase in a TPPB

Anthracene oxidation in the TPPB was significantly enhanced by the use of the surfactant as shown in Figure 2.3A. Thereby, during the first 10 h, 90% of total anthracene was removed and complete anthracene oxidation took place before 24 h. The rate of oxidation was calculated from the slope of the first 10 h, resulting in 16 $\mu mol/L_R \cdot h$. This rate was 1.5 times higher than that of monophasic systems with only Triton X-100.

In the initial period, the anthracene concentration in the aqueous phase reached its maximum values by the beneficial action of the surfactant, which increased the solubilization of the anthracene in the aqueous phase. Accordingly, mass transfer and degradation kinetics were likely to be favored. When the concentration of anthracene decreased both in the organic and aqueous phases, the oxidation rates were lower.

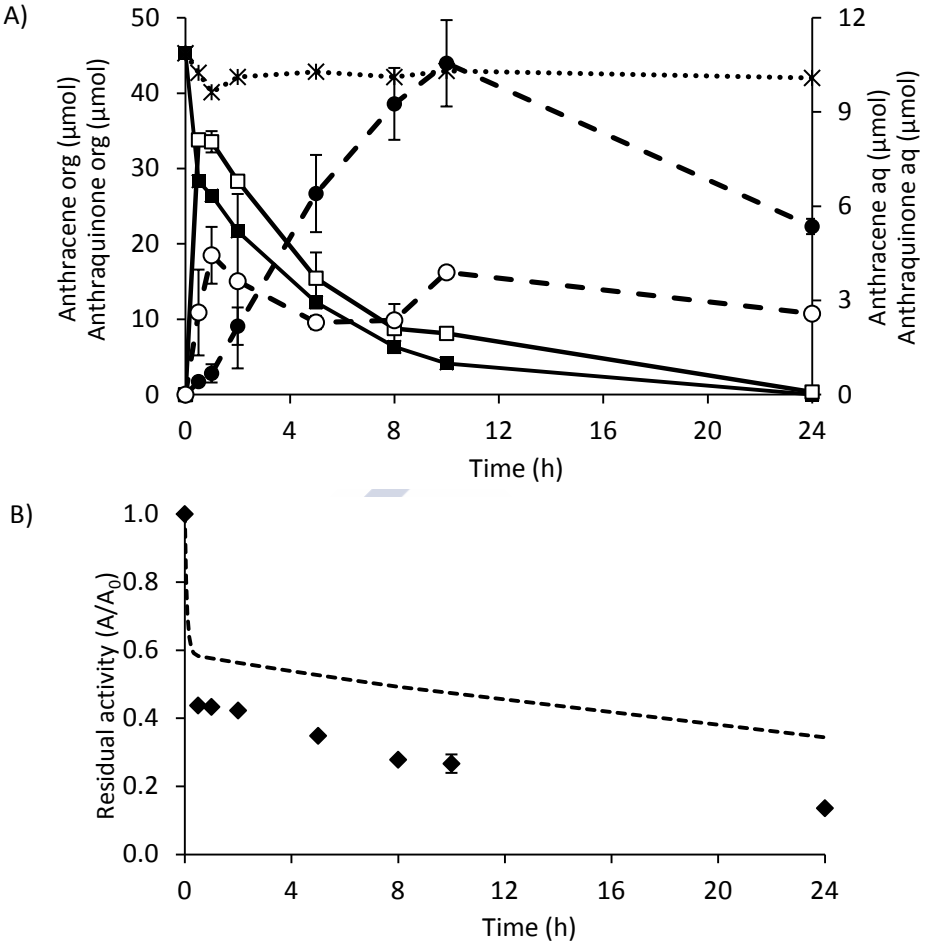

Figure 2.3. (A) Anthracene and anthraquinone partition in each phase, anthracene in organic solvent (■) anthracene in aqueous phase (□), anthraquinone in organic phase (●), anthraquinone in aqueous phase (○) and overall anthracene in control assay (✳); (B) residual activity profile of laccase during anthracene oxidation assay (◆) comparison with predicted deactivation in 0.1 M sodium acetate buffer pH 5 (- - - -).

Anthraquinone was obtained as the main product, but in a lower concentration than that predicted by stoichiometry. This is in agreement with Collins et al. (1996) who detected that anthraquinone was the major end product of anthracene oxidation by laccase in presence of ABTS as mediator. On the other hand, Johannes et al. (1996) observed that 9,10-anthraquinone was obtained stoichiometrically by reactions with laccase and HBT at very low concentrations, but at higher concentrations of the mediator, as those used in our study, anthraquinone formation did not strictly correspond to

disappearance of anthracene. Moreover, after 10 h of enzymatic treatment, the removal of anthraquinone was observed (Figure 2.3A). In an attempt to identify the degradation products of anthraquinone, standards of phthalic acid, the likely oxidation product of the quinone, were monitored during the experiments (Cajthaml et al. 2002). However, no conclusive results on their presence derived from anthraquinone were obtained.

The application of the TPPB in combination with the surfactant was the basis for the successful degradation of anthracene in the current configuration. Below the critical micellar concentration (CMC), surfactants exist as monomers and have only minimal effects on the solubilization of polyaromatics. Micellar solubilization occurs when the surfactant concentration exceeds the CMC, where the aqueous solubility of organic is enhanced by the incorporation of hydrophobic molecules into surfactant micelles (Edwards et al. 1991). Increasing the solubility of anthracene in the aqueous phase resulted in a large enhancement of the bioavailability of this compound for laccase. Thus, the anthracene concentration in the aqueous phase was approximately 8.5 mg/L, more than 100-fold the saturation value. As a result, the oxidation rate was much higher than in the biphasic system without surfactant. On the contrary, Kim et al. (2007) analyzed the effect of Tween 80 at concentrations below and above the CMC on the oxidation of pentachlorophenol (PCP) by horseradish peroxidase. They concluded that lower concentrations of Tween favoured the removal of PCP due to a positive effect on enzymatic activity, whereas when micelles were present, they sequestered PCP molecules impeding their oxidation by the enzyme.

In a previous study of our group, Eibes et al. (2010) used Triton X-100 in a lower dose than the CMC to degrade anthracene by the enzyme versatile peroxidase (VP) in a TPPB. It showed that the presence of Triton X-100 (0.25 CMC) promoted the degradation of anthracene with degradation rates of 3.7 $\mu mol/L_R \cdot h$ and 5.3 $\mu mol/L_R \cdot h$ in the absence and presence of Triton X-100 (0.25 CMC), respectively.

The highest percentage of degradation was 88% with Triton X-100 after 38 h. In the present work, the use of this surfactant at concentrations higher than the CMC largely improved the degradation rate with respect to the TPPB

without Triton X-100. In particular, the degradation of anthracene occurred in a shorter period of time (24 h), with considerably higher oxidation rate: 16 $\mu mol/L_R \cdot h$ versus 5.3 $\mu mol/L_R \cdot h$, even though laccase redox potential is lower than that of VP.

Another positive effect that arises from the use of the surfactant consists of the increased mass transfer coefficient respect to an analogous system without Triton X-100. The dispersion of non aqueous phase promoting a higher contact area and the facilitated transport of the pollutant, probably due to the reduction of surface tension, may explain the enhancement in mass transfer (Volkering et al. 1995).

The effect of Triton X-100 on laccase activity, previously discussed, is the third advantage resulting from its use. This positive effect had been detected in a BPA-conversion system by laccase (Ji et al. 2009). Moreover, this surfactant at 0.25 CMC has been demonstrated to reduce VP inactivation with decay coefficients about 1.5 times lower than in its absence (Eibes et al. 2010).

2.3.4 Modelling of the degradation and laccase inactivation processes in the surfactant-assisted TPPB

In order to provide information about the mechanism for anthracene oxidation by laccase in the TPPB, the kinetics of enzyme decay and anthracene oxidation were modeled. Regarding enzyme inactivation, similar behaviour to that observed in absence of anthracene could be observed (Figure 2.3B), consisting of an initial period of rapid deactivation followed by a period of slow decay. The 3-parameter model of Aymard and Belarbi (2000) was used to adjust experimental data (Figure 2.4A). The three kinetic decay parameters *x*, α, β for laccase biphasic inactivation resulted in 0.54, 40.09 h^{-1} and 0.05 h^{-1}, respectively (R^2= 0.998).

Concerning anthracene degradation, a model based on the assumption that PAH must be first dissolved in the aqueous phase to be available for the catalytic oxidation of the enzyme was developed. This model coupled two differential equations (equations (2.2) and (2.3)) to consider the mass balance in both organic and aqueous phases. Mass balance in the organic phase consists of the output rate of anthracene due to partition into the aqueous

phase, while mass balance in the aqueous phase takes into account the input rate of anthracene due to its solubilization, as well as the rate of removal due to enzyme oxidation.

$$\frac{dS_{org}}{dt} = -K_L a \left(\frac{S_{org}}{K_{SW}} - S_{aq} \right) \frac{V_{aq}}{V_{org}} \tag{2.2}$$

$$\frac{dS_{aq}}{dt} = K_L a \left(\frac{S_{org}}{K_{SW}} - S_{aq} \right) - k \cdot S_{aq} \tag{2.3}$$

S_{aq} and S_{org} (μM) are the anthracene concentrations in the aqueous and organic phases, respectively; V_{aq} and V_{org} (L) are the volumes of aqueous and organic phases, respectively; K_{SW} (dimensionless) is the anthracene partitioning coefficient; k (h^{-1}) is a pseudo first order kinetic constant and $K_L a$ (h^{-1}) is the overall mass transfer coefficient.

The partitioning coefficient K_{SW} was obtained from the equilibrium concentration of anthracene in the organic and in the pseudo-aqueous phase in the control, where no anthracene removal was observed (data not shown). The value of K_{SW} resulted in 28. The kinetic constant k and the overall mass transfer coefficient $K_L a$ were estimated by using the method of least squares. A first order kinetic was proposed for enzymatic anthracene degradation rate. Laccase activity values were not taken into account in the proposed model since the variation of activity throughout the experiment was considered not to cause an appreciable effect on the oxidation rate. In order to obtain the profile of anthracene in the organic phase, equation (2.2) was solved by using the finite differences numerical method, whereas the profile of anthracene in the aqueous phase was obtained by integrating the equation (2.3):

$$S_{aq} = \frac{K_L a\, S_{org} - e^{\left(-(K_L a + k)t\right)} \left(K_L a\, S_{org} - S_{aq0} K_{SW} K_L a - S_{aq0} K_{SW} k \right)}{K_{SW} \left(K_L a + k \right)} \tag{2.4}$$

S_{aq} depends on several parameters, among them, on the anthracene concentration in the organic phase (S_{org}). In the first step of the solving process, the values of S_{org} used to solve the equation (2.4) were the experimental data. Then, the pseudo first order kinetic constant k and the

overall mass transfer coefficient, K_La, were estimated by using the method of least squares. The solving method consisted of an iterative process where the value of the K_La was used to calculate dS_{org}/dt by means of the equation (2.2) and subsequently S_{org}. The calculated value of S_{org} was then applied along with the estimated value of k to recalculate S_{aq} by using the equation (2.4).

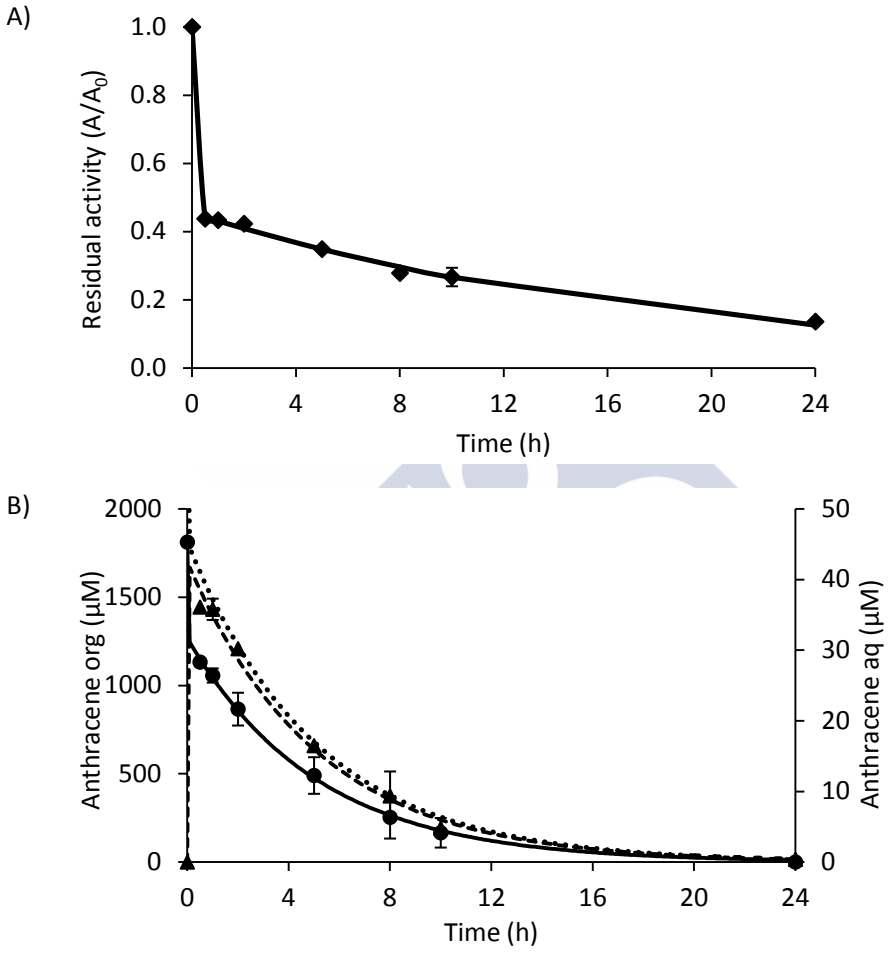

Figure 2.4. (A) Residual activity profile of laccase during anthracene oxidation assay. The solid line represents the inactivation kinetics modelled with the exponential model proposed by Aymard and Belarbi (2000); (B) experimental anthracene concentration during oxidation assay in organic phase (●) and model prediction for organic phase (—) R_2=0.999; experimental concentration of anthracene in aqueous phase (▲), model prediction (- - -), R_2=0.994; and anthracene concentration in aqueous phase corresponding to equilibrium state (·····).

The results relating to the comparisons of the experimental data of anthracene removal in the TPPB with the model predictions are shown in Figure 2.4B, where a good fitting of the model is observed (R^2=0.998). The kinetic constant *k* resulted in 52.2 h^{-1} and it is expected to be dependent on the initial laccase concentration in the system. The overall mass transfer coefficient K_La was 788.1 h^{-1}. Values of similar magnitude were reported by Srivastava et al. (2000) for the distribution of heptanoic acid between a dispersed limonene phase and an aqueous phase. Similarly in Melgarejo-Torres et al. (2011), mass transfer coefficient values ranged between 162 h^{-1} and 2404 h^{-1} from a dispersed phase based on the ionic liquid [MeBuPyrr][BTA] to the aqueous phase. However, the K_La estimated in the present study was much higher than the one reported by Eibes et al. (2010), who considered a much lower concentration of Triton X-100: 0.25 CMC (K_La of 16.2 h^{-1}), which confirms again the beneficial effect of Triton X-100 at concentrations above the CMC.

Moreover, anthracene concentration in aqueous phase throughout time was really close to that corresponding to equilibrium state, which suggests that mass transfer was not the limiting factor.

2.3.5 Anthracene degradation in successive cycles of reusing aqueous phase

The reuse of the aqueous phase containing the laccase-HBT system to degrade anthracene for two additional cycles with no extra addition of enzyme or mediator was demonstrated. The degrees of elimination were 68% and 40% for initial laccase activities of 150 and 90 U/L in each cycle respectively (Figure 2.5). The rate of anthracene oxidation resulted in 9.7 and 6.2 $\mu mol/L_R \cdot h$, lower than in the first batch. The decrease in the reaction rate could be consequence of several reasons, including the lower concentration of enzyme and mediator, the presence of intermediate products that may be more prone than anthracene to be oxidized by laccase-HBT system, or which compete with the mediator to reduce laccase.

Accumulation of anthracene was observed in the reactor used as control throughout the consecutive batchs (Figure 2.5). Anthraquinone profiles during second and third cycle were similar and characterized by an increase of concentration up to a maximum followed of a slightly decrease as shown in

Figure 2.6. However, the maximum concentration detected was lower than in the first batch. Regarding laccase activity, whereas in the first batch the inactivation occurred in two stages, in the subsequent cycles a slow and uniform decay took place (Figure 2.5).

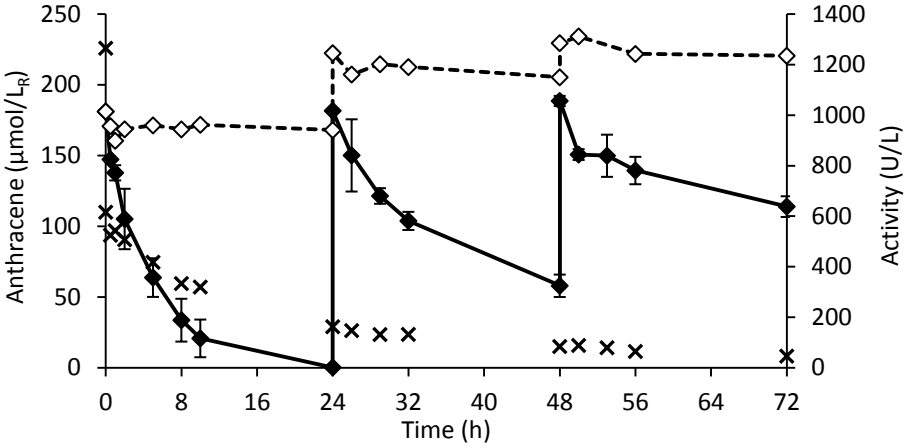

Figure 2.5. Anthracene concentration (◆) and laccase activity (✕) during the three consecutive cycles of the TPPB with the laccase-mediator system, and anthracene concentration in the control assay (◇).

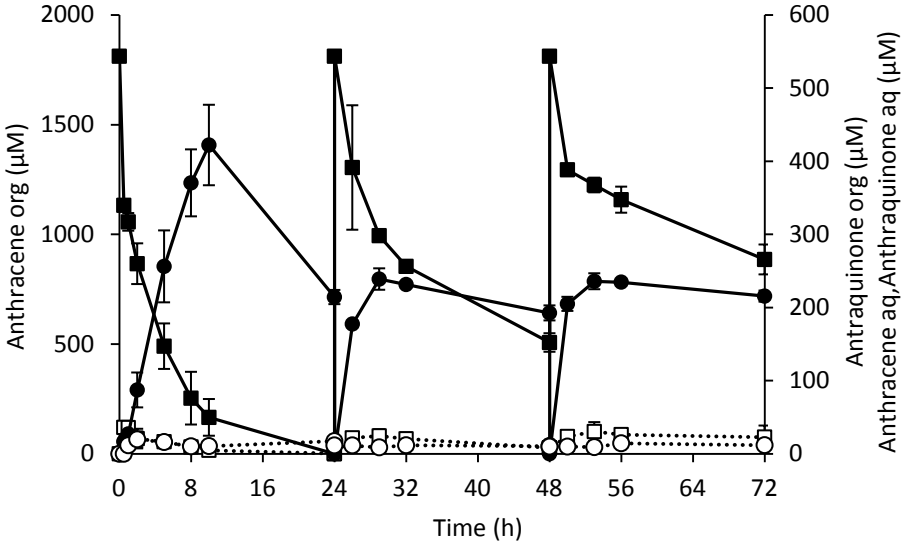

Figure 2.6. Anthracene and anthraquinone concentrations in each phase, anthracene in the organic solvent (■) anthracene in the aqueous phase (□), anthraquinone in the organic solvent (●), anthraquinone in the aqueous phase (○) during the three consecutive cycles of the TPPB.

2.3.6 Feasibility of silicone oil reuse

The reusability of the anthracene depleted silicone oil was evaluated through several cycles of degradation in the surfactant assisted TPPB. The profiles of anthracene removal, anthraquinone concentration and laccase residual activity through time in the three consecutives cycles are shown in Figure 2.7.

As expected, complete anthracene degradation was achieved in the first batch experiment. At the beginning of the second cycle, the organic solvent contained anthracene and a low concentration of anthraquinone, the main product of anthracene oxidation, which remained from the first batch. It could be observed that the percentage of degradation after 8 h was comparable to that of the first batch and anthracene resulted to be completely depleted after 24 h. Anthraquinone profile showed the trend observed in the original experiment, with an initial increase followed by a decrease.

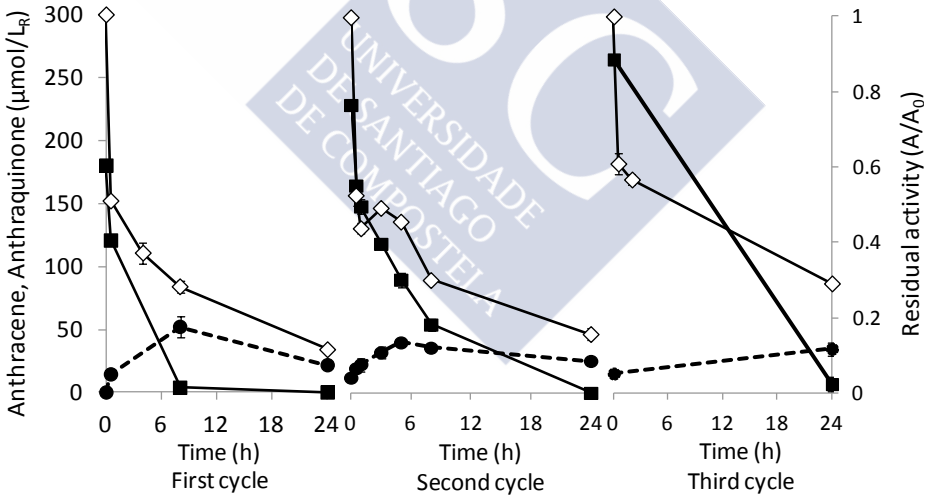

Figure 2.7. Anthracene (■) and anthraquinone (●) concentrations referred to the total reaction volume and residual laccase activity (◇) in an experiment with three consecutive cycles with reused silicone oil.

Regarding enzyme activity, a similar behavior was observed in both cycles, characterized by an initial rapid decline followed by a period of slow decay. Unfortunately, no intermediate samples of the third cycle could be taken,

however, the percentage of anthracene degraded after 24 h was very high: 97%. These results demonstrate the feasibility of reusing the silicone oil, which leads to the reduction of the treatment costs.

2.4 CONCLUSIONS

Several strategies for *in vitro* degradation of anthracene, a poorly soluble compound, based on the use of surfactant at higher concentration that the CMC and a water immiscible organic solvent were evaluated. A two-phase partitioning bioreactor (TPPB) with Triton X-100 was proved to be the best configuration to remove anthracene by laccase from *T. versicolor* and HBT. The addition of Triton X-100 improved mass transfer of anthracene from organic to aqueous phase and enhanced the solubility of pollutant in the aqueous phase. Additionally, this surfactant increased laccase stability, protecting it against deactivation, which was caused mainly by the mediator radicals. The combination of these effects led to the removal of higher loads of anthracene at faster oxidation rates. The aqueous phase, containing the laccase-mediator system, could be reused in two additional oxidative cycles with no extra addition of laccase or HBT. Moreover, the feasibility of reusing the silicone oil to dissolve anthracene and carry out subsequent degradation cycles was demonstrated. In conclusion, the results showed that the application of an enzymatic TPPB with surfactant constitutes a powerful technology for the treatment of highly hydrophobic recalcitrant compounds.

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Chapter 3

VEGETABLE OILS AS NAPLs IN TWO PHASE PARTITIONING BIOREACTORS FOR THE DEGRADATION OF ANTHRACENE BY LACCASE [1]

Summary

The enzymatic degradation of anthracene by the laccase-mediator system in Two Phase Partitioning Bioreactors (TPPB) with sunflower oil and pomace olive oil used as non-aqueous phase liquid (NAPL) was proposed. Triton X-100 (1% v:v) was added as surfactant to increase interfacial area and thus, to improve mass transfer. The highest oxidation of anthracene was obtained with pomace olive oil as NAPL. Furthermore, the influence of several factors such as oxygen level, mediator concentration and laccase addition were investigated to maximize the removal of anthracene. Both dissolved oxygen and 1-hydroxybenzotriazole (HBT) concentrations were found to limit the rate and extension of the process. The strategy of their addition in pulses led to total removal after 48 h with a high conversion rate of anthracene: 30.3 μmol/L_R·h. Experimental data were modeled according to pseudo-first order kinetics with a rate constant (k) of 170.7 h^{-1} and the overall mass transfer coefficient (K_La) was estimated in 1573.1 h^{-1}. This study suggests the potential application of TPPB with oxidative enzymes as a suitable treatment alternative for the recovery of vegetable oils contaminated with hydrophobic pollutants.

[1] **A. Arca-Ramos, G. Eibes, M.T. Moreira, G. Feijoo and J.M. Lema (2014).** Vegetable oils as NAPLs in Two Phase Partitioning Bioreactors for the degradation of anthracene by laccase. Chemical Engineering Journal 240 (281-289)

OUTLINE

3.1 INTRODUCTION

Solvent extraction has been reported as an effective method for removing hydrophobic contaminants from soils. However, the use of conventional solvents, as for instance, alkanes, alcohols, ketones or alkylamines involves substantial risks, as well as the necessity of implementing costly measures to minimize their environmental impact (Anderson 1995, Lau et al. 2012). As an alternative, less hazardous solvents have been proposed (Pannu et al. 2004). In this sense, several studies have demonstrated that vegetable oils can be applied to extract organic contaminants for remediation purposes (Berg Schuur and Mattiasson 2003, Chi et al. 2010, Gong et al. 2005, Lau et al. 2012, Pannu et al. 2004). The regeneration of the solvent after the extraction of PAH-contaminated soils is desirable, especially when large amounts are required. Activated carbon can be used as an adsorbent to remove PAHs from organic solvents and oils (Gong et al. 2007). However, the requirement of an additional thermal treatment to recover the contaminated activated carbon is a major drawback (Pannu et al. 2004). Another possibility for recycling the solvent consists of the physical separation of PAHs by using polystyrene. Nevertheless, the feasibility of the subsequent recovery of the pollutant from polystyrene should be considered (Berg Schuur and Mattiasson 2003, Pannu et al. 2004).

The regeneration of the solvent by means of enzymatic treatments is based on the biodegradation of the pollutants. For this purpose, the two-phase partitioning bioreactor (TPPB) configuration is proposed. In Chapter 2, a surfactant-assisted TPPB using silicone oil as organic phase was successfully applied for the oxidation of anthracene by the laccase-1-hydroxybenzotriazole (HBT) system. The addition of the surfactant Triton X-100 resulted in an effective strategy to overcome problems related to transfer of anthracene from the organic to the aqueous phase (Eibes et al. 2010) and to protect laccase against the inactivation caused by HBT free radicals (Ji et al. 2009). However, major drawbacks related to the use of silicone oil in the TPPB are its high cost and limited availability. In addition, although silicone oil has been one of the most investigated non aqueous phase liquid (NAPL) due to its non biodegradability (Quijano et al. 2009); such property may be troublesome for its application as extraction agent in polluted soils, since the degradability of the remaining oil in soil by indigenous microorganisms is desirable. As an

alternative, the use of vegetable oils as NAPL in TPPBs has a number of advantages, such as lower cost, lower toxicity and potential high efficiency in extracting hydrophobic contaminants from soils. Aiming to make the TPPB process a viable alternative closer to a real application, the feasibility of anthracene oxidation by laccase using either sunflower oil or pomace olive oil as organic phase in a surfactant-assisted TPPB was investigated in the present work.

Furthermore, the influence of several factors such as oxygen level, mediator concentration and laccase addition were investigated to maximize the removal of anthracene. Experimental data were modeled and kinetic parameters and mass transfer coefficient estimated to assess the influence of the oil type as NAPL in the degradation of anthracene.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and enzyme

Anthracene (99%), Triton X-100 (≥98%) and HBT (99%) were purchased from Janssen Chimica, Merck and Fluka, respectively. Anthraquinone (97%), 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (≥98%), organic solvents (HPLC grade) and the commercial laccase from *Trametes versicolor* were purchased from Sigma-Aldrich. The sunflower and the pomace olive oils were edible commercial oils. The Standard Fatty Acid Methyl Esters (FAME) mix C_4-C_{24} was purchased from Supelco Analytical (USA).

3.2.2 Spiking vegetable oils with anthracene

Vegetable oils were spiked with anthracene according to the following procedure. A certain amount of anthracene was added to the oil, which was agitated with Teflon-coated magnetic stir bars for 24 h. The oil saturated with anthracene was subsequently filtered through a glass fiber prefilter to remove the non-dissolved solid particles of anthracene.

3.2.3 Enzyme activity and anthracene analysis

Laccase activity was determined by a colorimetric assay using ABTS as substrate. The concentration of anthracene was determined by high

performance liquid chromatography (HPLC). The equipment and methodologies used were described in Chapter 2.

3.2.4 Anthracene degradation by laccase in a TPPB

The oxidation of anthracene was conducted at 30ºC and 250 rpm for at least 24 h in a TPPB consisting of a conventional stirred tank reactor (BIOSTAT® Q reactor, B. Braun-Biotech International), described in Chapter 2. The total reaction volume was 250 mL containing 10% (v:v) of the vegetable oil spiked with anthracene as the organic phase, while the aqueous phase comprised 1200 U/L laccase, 0.1 M sodium acetate (pH 5), 1 mM HBT and 1% Triton X-100 (v:v). To verify that degradation took place only due to enzyme oxidation, controls lacking laccase were performed in parallel.

The influence of several factors such as oxygen level, mediator concentration and laccase addition were investigated. The effect of gas supply was evaluated in experiments performed with air or oxygen. Initially, the supply of 0.1 L/min of air was applied whenever the concentration of dissolved oxygen was below 10% of saturation concentration (0.74 mg/L). Continuous aeration was not possible due to the foaming caused by the presence of the surfactant in the aqueous phase. In a subsequent experiment, oxygen was supplied by periodic pulses (30 s every 20 min at 1 bar) for the first 8 h, every hour until 24 h and thereafter stopped. For this purpose, an electrovalve located at the end of a flexible membrane tube controlled by a cyclic timer was used.

In a further experiment, the combination of aeration (periodic pulses 0.2 L/min of air for 1 min every 15 min) and the addition of mediator (pulses of 1 mM HBT added at 8 and 24 h) and enzyme (addition at the start of the experiment and after 32 h) was evaluated with the aim of maximizing the removal of anthracene.

Samples from both organic and aqueous phases were periodically withdrawn to determine anthracene concentration according to the methodology described in Chapter 2. The overall anthracene in the reactor was expressed in μmol/L_R calculated from a mass balance taking into account the volumes of both phases: organic and aqueous in the TPPB.

3.2.5 Effect of the laccase-HBT system on the stability of vegetable oils

Aiming to elucidate the potential oxidation of the vegetable oils by the laccase-HBT system, experiments lacking anthracene were conducted in the TPPB at 30ºC and 250 rpm for 72 h. The composition of the aqueous phase was identical to the one indicated for the anthracene degradation experiments in section 3.2.4; whereas the organic phase (10% v:v) comprised the vegetable oil lacking anthracene. Oil samples were periodically withdrawn and analyzed by gas chromatography as described by Johnson et al. (2009) to monitor the potential changes in the oil composition.

3.2.6 Interfacial tension measurements

The interfacial tension of the combinations between pomace olive oil and Triton X-100; and silicone oil and Triton X-100 were measured at 30ºC according to the Wilhelmy plate method in a Krüss K11 tensiometer as described by Rodríguez et al. (2010).

3.3 RESULTS AND DISCUSSION

3.3.1 Evaluation of sunflower oil and pomace olive oil as NAPL

The possibility of using sunflower oil and pomace olive oil as reservoirs of anthracene in surfactant-assisted TPPBs was evaluated. Firstly, the density of the pomace olive oil (0.914 kg/L) and sunflower oil (0.918 kg/L) were lower than that of the silicone oil (0.956 kg/L) which is an advantage to facilitate the separation of the aqueous and the depleted organic phase after the batch degradation of the pollutant. Solubility of anthracene in the sunflower oil (~15000 µM) was considerably lower than in the pomace olive oil (~27350 µM), so the latter solvent implied higher initial concentrations of anthracene in the TPPB. Figure 3.1 shows the time-course experiment, depicting profiles of anthracene concentration referred to the overall reaction volume, dissolved oxygen and laccase activity in experiments with the vegetable oils as well as with silicone oil, evaluated in Chapter 2 and considered for comparison purposes.

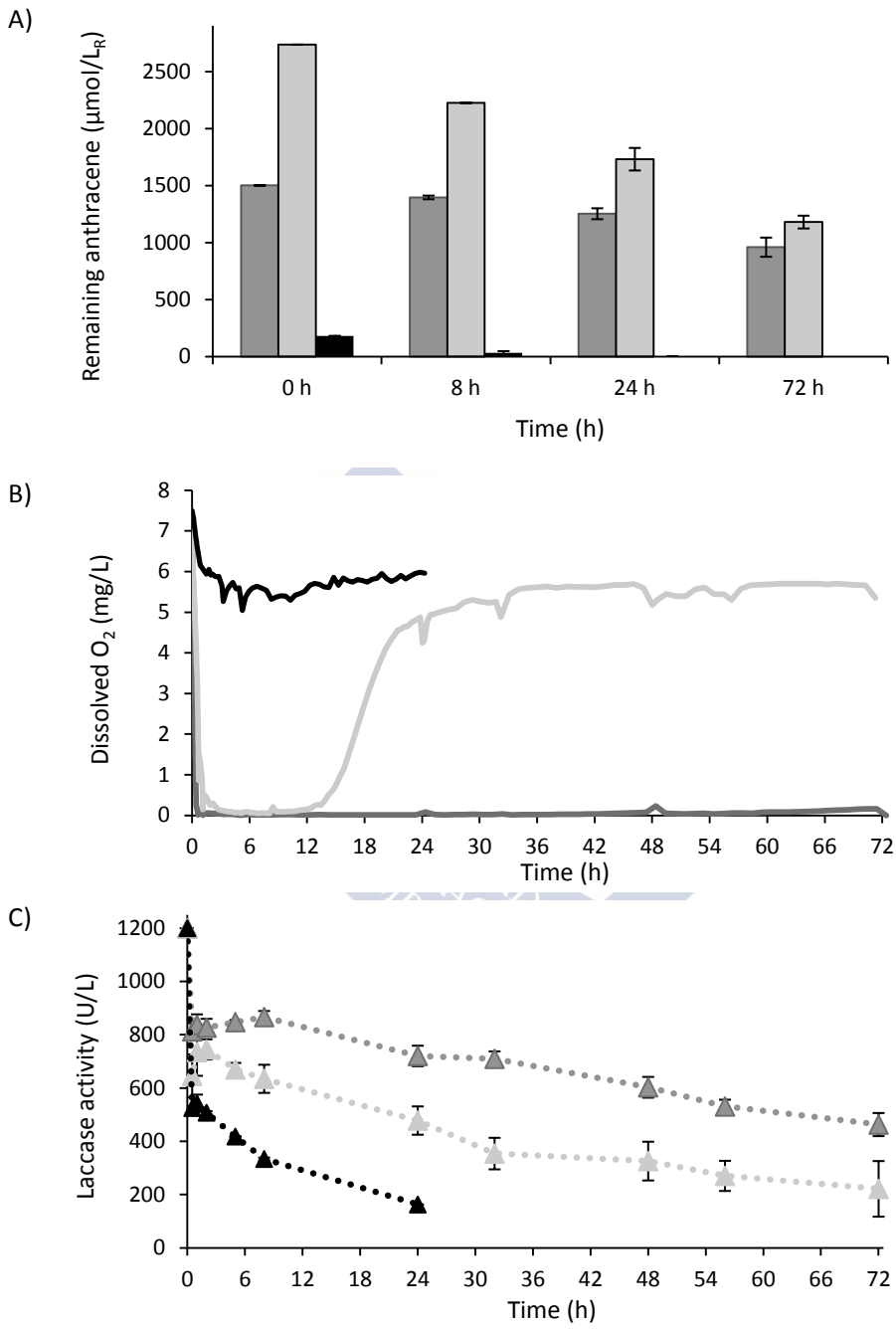

Figure 3.1. (A) Anthracene concentration in the reactor at 0, 8, 24 and 72 h, (B) dissolved oxygen concentration profile and (C) laccase activity in saturated solvents: sunflower oil (■), pomace olive oil (■) and silicone oil (■).

In all the biphasic systems evaluated, the principal oxidation product of anthracene was anthraquinone, but it was detected at lower concentrations than those predicted by the stoichiometry. This is in agreement with previous studies, which also identified anthraquinone as the main product of anthracene oxidation by several laccase-mediator systems (Cañas et al. 2007, Johannes et al. 1996).

The yield of anthracene oxidation in the TPPB was dependent on the oil used. The use of pomace olive oil as NAPL resulted in the oxidation of 251 µmol of anthracene after 24 h versus 62 µmol in the case of sunflower oil. The different outcomes are potentially linked to the singular composition of the oils. It is well known that vegetable oils are susceptible to oxidation in a process initiated by the formation of free radicals (Fox and Stachowiak 2007), generally from the abstraction of a hydrogen atom from the methylene group next to a double bond. Once this oxidation process is initiated, it can continue independently through propagation, provided that the oxygen concentration is sufficient. The nitroxy radical formed when HBT is oxidized by laccase is a potent electrophile that easily abstracts allylic hydrogens and, consequently, allows the oxidation of fatty acids (Böhmer et al. 1998, Molina et al. 2008).

Vegetable oils containing high percentage of polyunsaturated fatty acids, such as linoleic and linolenic acids, both present in sunflower oil, are expected to react more readily than those containing higher percentages of monounsaturated fatty acids such as oleic acid (Fox and Stachowiak 2007), which is predominant in pomace olive oil. The analysis of sunflower and pomace olive oil fatty acid content after 72 h of treatment with laccase-HBT in the absence of anthracene showed the oxidation of linoleic acid, with a peak area reduction of 21.3% and 28.1% compared to the control with no laccase for sunflower and pomace olive oil, respectively; and to a lesser extent, the oxidation of oleic acid with a peak area reduction of approximately 10.5% for both oils (Figure 3.2).

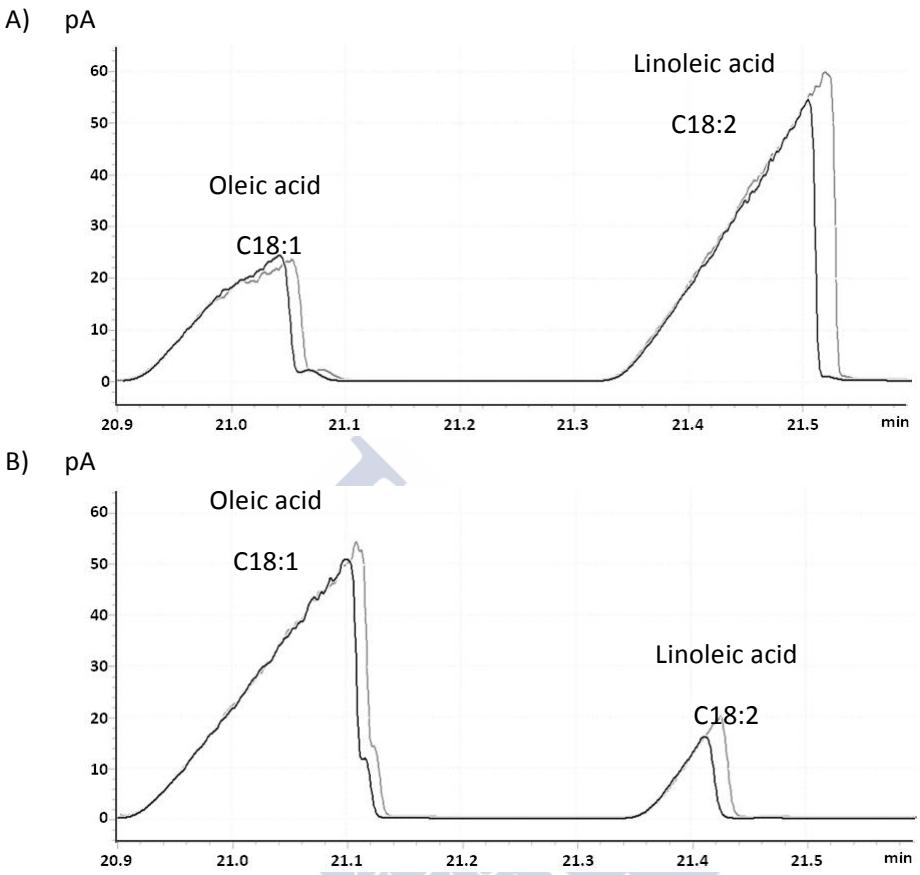

Figure 3.2. Chromatograms of oleic and linoleic acid degradation after 72 h in experiments with laccase (black line) and control without laccase (grey line) in sunflower oil (A) and in pomace olive oil (B).

Therefore, the polyunsaturated fatty acids present at high proportions in the sunflower oil could be oxidized by the nitroxy radicals, hindering the oxidation of anthracene.

The large demand of oxygen in the oxidation of the polyunsaturated acids present in the vegetable oils is likely to cause the rapid depletion of oxygen after few minutes (Figure 3.1B). On the other hand, oxy radicals generated during the peroxidation of fatty acids are known to cooxidize PAHs (Böhmer et al. 1998), which would favor the degradation of anthracene in the system. This could be the reason behind the much higher oxidation of anthracene in the reactor with pomace olive oil, in comparison with the TPPB with silicone oil (mineral oil).

Figure 3.3 shows the proposed mechanisms and the key species involved in the degradation of anthracene.

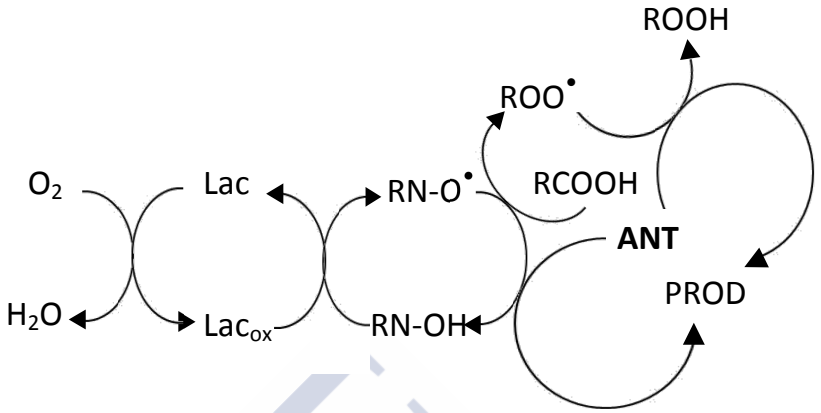

Figure 3.3. Proposed simplified scheme for the oxidation of anthracene to products by laccase-HBT system and by peroxyl radical generated from unsaturated fatty acids of vegetable oils. Abbreviations: native laccase (Lac); oxidized laccase (Lac_{ox}); HBT (RN-OH); HBT radical (RN-O$^{\bullet}$); unsaturated fatty acids (RH); peroxyl radicals (ROO$^{\bullet}$); hydroperoxides (ROOH); anthracene (ANT); products (PROD).

Firstly, the mediator HBT (RN-OH) is oxidized by laccase to render a nitroxy radical, which can oxidize anthracene and peroxidize unsaturated fatty acids (RH). At the same time, anthracene can be oxidized by these oxy radicals generated from the peroxidation of oleic and linoleic acids. Oxygen would also participate in the propagation of peroxyl radicals by a free radical chain mechanism (Frankel 1991). Conversely, this positive effect on the degradation of anthracene was not observed in the case of sunflower oil. The possible hypothesis would be related to the tocopherol content in the sunflower oil, a natural antioxidant that may hinder the free radical oxidation of lipids (Frankel 1991). It is worthwhile highlighting the significantly different oxygen profile when the mineral oil was used in the TPPB: a slight decrease in the first minutes to a steady concentration of O_2 above 6 mg/L was observed (Figure 3.1B)

With regard to laccase stability (Figure 3.1C), enzyme inactivation occurred in two clearly differentiated steps, as observed in Chapter 2: a first period with a rapid drop of activity followed by a period of slow decay. Laccase

stability in vegetable oils, especially in sunflower oil, was considerably higher than in silicone oil.

In term of effectiveness, defined as the ratio of the amount of anthracene degraded and the loss of enzyme activity, better results were attained with pomace olive oil with an efficiency of 1.0 µmol/U calculated at 8 h. The values obtained for sunflower and silicone oils at 8 h were considerably lower: 0.35 and 0.19 µmol/U, respectively. As the pomace olive oil provided better results in terms of anthracene oxidation and effectiveness, its use as NAPL in TPPB was studied in more detail aiming to maximize anthracene removal.

3.3.2 Anthracene oxidation in TPPBs with pomace olive oil

The degradation of anthracene dissolved in pomace olive oil (1000 mg/L) was evaluated in a TPPB under different strategies focusing on the following major parameters:

I. oxygen level due to its role as electron acceptor in the catalytic cycle of laccase
II. mediator concentration, which may increase the oxidation potential of the enzyme
III. laccase addition to counteract the potential inactivation of the enzyme throughout the operation.

3.3.2.1 Effect of aeration/oxygenation

Firstly, the degradation of anthracene was evaluated in a non-aerated experiment (Figure 3.4). The oxidation rate was 5.8 µmol/L_R·h referred to the first 8 h of reaction (Table 3.1). A rapid decline of dissolved oxygen in the first hour was observed and then, a moderate recovery after 24 h to values around 70% of saturation was detected (Figure 3.4). In the control run in parallel, neither anthracene nor oxygen decreased during the experiment.

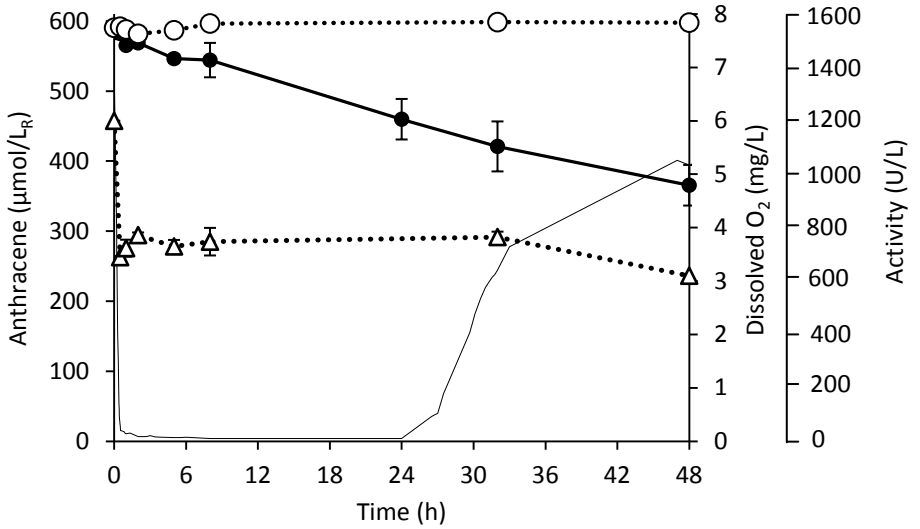

Figure 3.4. Anthracene concentration in the reactor (●) anthracene concentration in the control lacking laccase (○), dissolved oxygen concentration (——) and laccase activity profile (△) during anthracene oxidation experiment in the TPPB with non-saturated pomace olive oil.

Table 3.1. Results for the anthracene (ANT) degradation under the different evaluated strategies using pomace olive oil as NAPL.

Experiment	Initial ANT (µmol)	Time (h)	Oxidized ANT (µmol)	Activity loss (U/L)	Efficiency (µmol/U)	Oxidation rate of ANT (µmol/L_R·h)	ANT degradation (%)
Pomace olive oil (~1000 mg/L ANT)	150.4	8	11.5	453	0.11	5.8	7.6
		32	42.4	435	0.43	5.3	28.2
Pomace olive oil (~1000 mg/L ANT) Manual aeration	146.2	8	54.1	595	0.40	27.1	37.0
Pomace olive oil (~1000 mg/L ANT) Oxygenation	134.0	8	64.8	594	0.48	32.4	48.4
		32	82.1	762	0.48	10.3	61.2
Pomace olive oil (~1000 mg/L ANT) Aeration and HBT	143.7	8	60.7	647	0.42	30.3	42.2
		32	130.4	880	0.66	16.3	90.7

The above results showed that in the presence of laccase and vegetable oils, oxygen is rapidly consumed until nearly complete depletion. Oxygen transfer from the headspace of the reactor in the experiments with passive aeration was limited, and would not potentially fulfill the requirements for the catalytic action of the enzyme. In order to increase the concentration of O_2 in the reaction medium, periodical pulses of air or oxygen were considered.

During the first 8 hours of operation, air was supplied in pulses whenever the dissolved oxygen was below 10% saturation (Figure 3.5). In such strategy, O_2 concentration was always below the saturation value. The duration of air pulses was restricted by foaming with the subsequent limitation of oxygen, whose concentration was far from saturation. As foreseen, anthracene oxidation in the TPPB was significantly enhanced by aeration as shown in Table 3.1, with an oxidation rate five times higher than in the absence of aeration (27.1 µmol/L_R·h). Moreover, the decrease of the reaction rate in the period without air supply, between 9-24 h, was evident as observed in Figure 3.5.

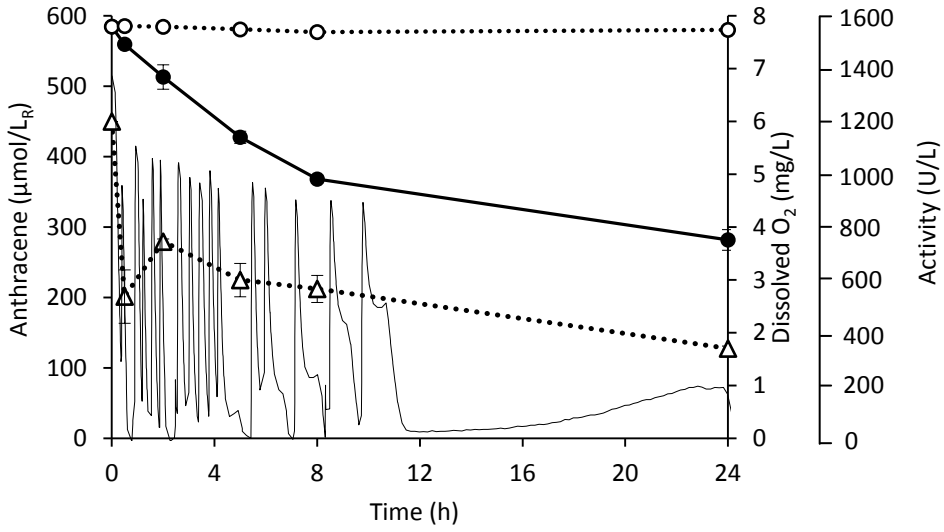

Figure 3.5. Anthracene concentration in the reactor (●) anthracene concentration in the control lacking laccase (○), dissolved oxygen concentration (—) and laccase activity profile (△) during anthracene oxidation experiment in the TPPB with non-saturated pomace olive oil and intermittent pulses of air.

In other research works, the improvement of laccase action by the supply of air or oxygen has also been reported (Fillat and Roncero 2009, Ghosh et al. 2008, Moldes and Vidal 2008, Oudia et al. 2008, Ravalason et al. 2012). Lloret et al. (2012) investigated the effect of aeration and oxygenation on the removal of estrogens by laccase. Although a significant effect was not observed under air supply, oxygenation by pulses considerably improved the removal of estrogens.

Therefore, with the aim of further improving the process under study, pulses of oxygen were applied according to the strategy described in section 3.2.4. During the period of frequent oxygenation, dissolved O_2 concentration increased after every pulse up to a maximum of 26 mg/L (Figure 3.6). In the second period of oxygenation, it ranged between 6-22 mg/L. Finally, when oxygenation was suspended, this level decreased to a steady value around 4 mg/L. Equilibrium was reached at this point so that the rate of O_2 consumption and the rate of O_2 transfer were equal. Although the current strategy led to a higher anthracene oxidation rate (32.4 1 µmol/L_R·h versus 27.1 µmol/L_R·h with pulses of air; Table 3.1), the degradation yield was expected to improve to a large extent with the increased concentration of dissolved oxygen. Moreover, the oxidation rate suffered a sharp decline after 8 h, as observed in Figure 3.6. A similar behavior had been previously observed in the experiment with aeration pulses (Figure 3.5), but then it was attributed to the low concentration of O_2 after suspending aeration. However, in the present experiment, it is likely that dissolved oxygen was not the limiting factor between 8 and 24 h, since its concentration was always above 5.7 mg/L.

Therefore, another parameter suspected to limit the oxidation process could be the mediator. The hypothesis was that the mediator is oxidized by laccase (Fillat and Roncero 2009, Li et al. 1999) and it was confirmed after a thorough examination of the HPLC chromatograms where the peak corresponding to HBT was identified in samples from the aqueous phase. A reduction of the peak area was determined in the experiments with laccase but not in the controls without enzyme (data not shown). It is noteworthy that no HBT was detected in the organic phase, which revealed that the presence and action of the mediator was limited to the aqueous phase. With the objective of finding out whether the depletion of HBT during the process might

be responsible for the decrease in the oxidation rate after 8 h, fed-batch addition of HBT was considered.

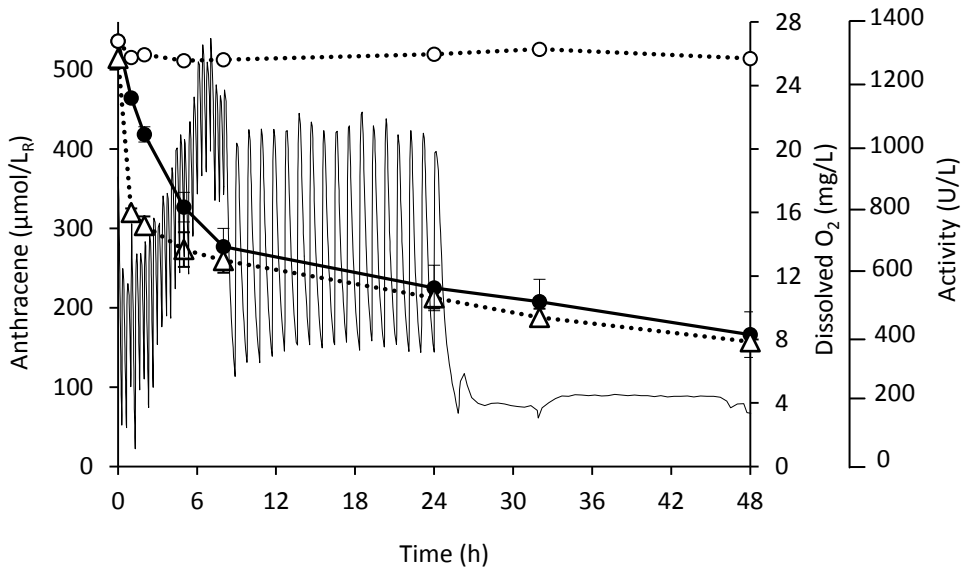

Figure 3.6. Anthracene concentration in the reactor (●) anthracene concentration in the control lacking laccase (○), dissolved oxygen concentration (—) and laccase activity profile (△) during anthracene oxidation experiment in the TPPB with non-saturated pomace olive oil and pulses of oxygen.

3.3.2.2 Combined effect of aeration and additions of mediator and laccase

The consumption of mediator has been identified as an issue of concern in the laccase-based process (Li et al. 1999, Okazaki et al. 2000). Aiming to demonstrate that the depletion of HBT in the enzymatic process was responsible for the slowing of the oxidation rate, a further experiment with 1000 mg/L of anthracene, pomace olive oil and pulses of HBT at 8 and 24 h was conducted. Moreover, air was supplied to the reactor to guarantee non-limiting concentrations of O_2. The rationale behind the use of air instead of oxygen was based on the high cost associated to oxygen, which could not justify the slight improvement in the oxidation rate. A pulse of laccase was also performed at 32 h to determine if the increase in the enzyme activity had a noticeable effect on the removal rate.

In this experiment, a gradual removal of anthracene was observed during the first 32 h (Figure 3.7). Furthermore, no remarkable decrease in the oxidation rate occurred. Conversely, anthracene degradation was maintained at a steady rate: 16.3 µmol/L_R·h, whereas in the experiment with oxygenation was considerably lower: 10.3 µmol/L_R·h.

The efficiency at 32 h also improved as consequence of the higher amount of anthracene oxidized: 90.7% versus 61.2% in the experiment with pulses of oxygen and no HBT addition (Table 3.1). Therefore, it can be stated that the mediator consumption throughout the process had a negative effect on the oxidation rate and its periodical addition is required to enhance the oxidation of anthracene. In Figure 3.7, sharp decline in the concentration of dissolved oxygen was observed immediately after the addition of HBT at 8 and 24 h. This fact also evidenced that the oxidation of HBT was the immediate cause of oxygen consumption. The pulse of enzyme at 32 h also promoted a reduction in the concentration of O_2, but the improvement of oxidation rate was negligible, probably due to the low concentration of anthracene at that moment.

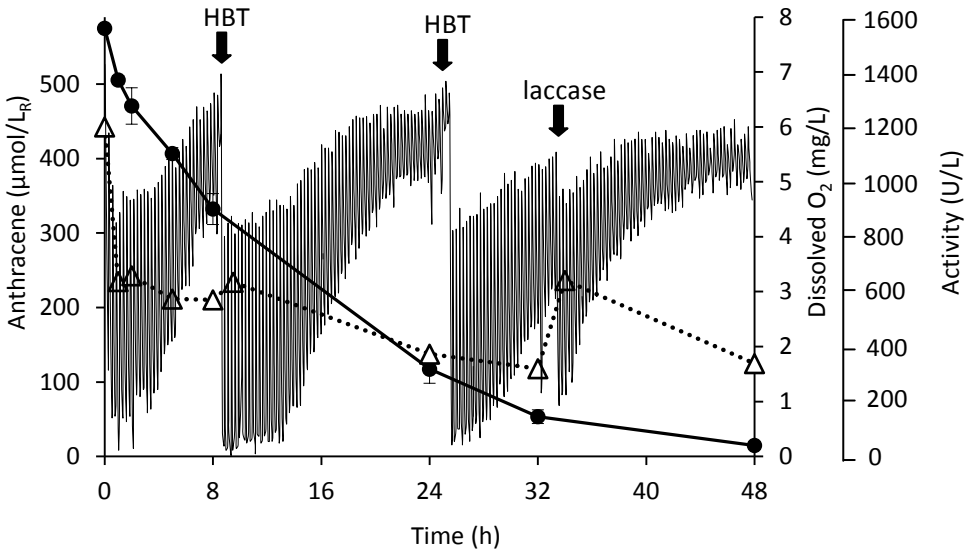

Figure 3.7. Anthracene concentration in the reactor (●), dissolved oxygen concentration (——) and laccase activity profile (△) during anthracene oxidation experiment in the TPPB with non-saturated pomace olive oil, periodic pulses of air, HBT addition at 8 h and 24 h and laccase addition at 32 h.

3.3.2.3 Analysis of anthracene partition during degradation experiments

In TPPB the target compound, initially in the vegetable oil, partitions between organic and aqueous phase at levels determined by its partition coefficient (K_{SW}) and it is considered that only the anthracene dissolved in the aqueous phase is available for the catalytic oxidation of laccase. At the same time as anthracene is oxidized in the aqueous phase, it diffuses from the organic phase to re-establish the equilibrium. Therefore, anthracene partition is the result of two main mechanisms: mass transfer and kinetics of enzymatic degradation. Aiming to a better knowledge of the mechanisms and factors involved in the oxidation of the PAH in the TPPB, time-course data for anthracene concentrations in both organic and aqueous phases for the evaluated strategies were compared.

In Figure 3.8A, B, C and D, experimental concentrations of anthracene in the organic and aqueous phases, as well as the anthracene concentration in the aqueous phase corresponding to equilibrium are shown. The equilibrium concentration values in the aqueous phase (dashed lines) were determined from the ratio of the experimental concentration in the organic phase and the partition coefficient (K_{SW}). The K_{SW} value of 260 was obtained from the equilibrium concentration of anthracene in the organic and in the aqueous phases in the control, where no anthracene removal occurred (data not shown). Initially, there was no anthracene in the aqueous phase but the concentration increased due to the transfer from the oil to restore equilibrium. At the same time, oxidation occurred in the aqueous phase.

In the experiment with no aeration (Figure 3.8A), anthracene concentration in the aqueous phase was really close to that corresponding to equilibrium. This fact suggests that the kinetics of the enzymatic oxidation in the aqueous phase was controlling the rate of the global process.

In the experiment with aeration (Figure 3.8B), the global rate of anthracene removal increased with respect to the previous experiment. Despite the enhancement of the oxidation rate in the aqueous phase due to the higher concentration of O_2, anthracene concentration in the aqueous phase was almost coincident with that of the equilibrium. Therefore, kinetics is still limiting the global process.

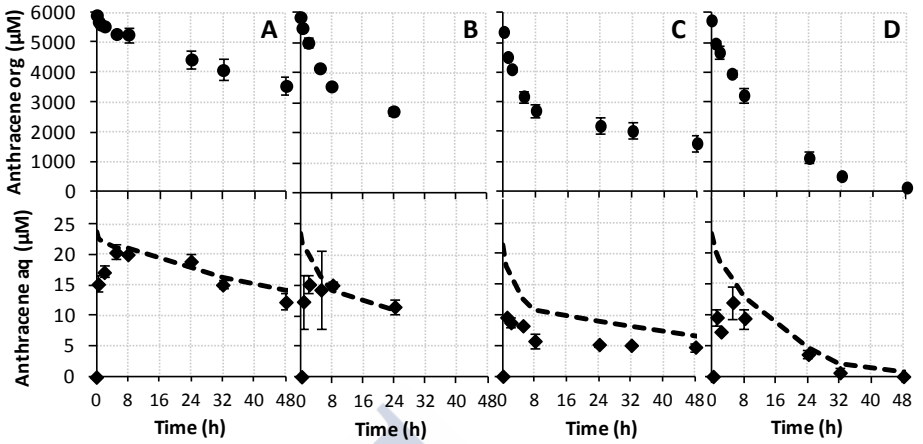

Figure 3.8. Anthracene concentration profiles in the organic phase (●), in the aqueous phase (◆) and anthracene concentration in aqueous phase corresponding to equilibrium state (- - -) in the experiments using pomace olive oil initially spiked with 1000 mg/L of anthracene and: no aeration (A); pulses of air (B); pulses of oxygen (C); aeration and addition of HBT and laccase in pulses (D).

When oxygen was used, the concentration of dissolved oxygen in the aqueous phase was higher. Consequently, the degradation kinetics was probably enhanced under oxygen and the anthracene concentration in the aqueous phase was slightly lower than the calculated concentration at equilibrium (Figure 3.8C). This fact suggests that when oxygen was supplied, mass transfer appeared to be the slowest stage in the overall process.

In order to enhance mass transfer from the organic to the aqueous phase, several strategies could be implemented in a further study. A possible alternative would be based on adding a higher proportion of surfactant to increase the apparent solubility of anthracene in the aqueous phase, which would also favor kinetics. Other options, aiming at increasing the interfacial surface, could consider a higher agitation rate or larger proportion of organic phase.

From Figure 3.8C, it can also be observed that after 8 h, the difference between the experimental and equilibrium concentrations in the aqueous phase diminishes. A possible reason could be the consumption of HBT, which would be causing the reduction in the enzymatic oxidation rate.

Finally, the last strategy consisting of aeration and addition of mediator and laccase in pulses (Figure 3.8D) implied that the anthracene concentration in the aqueous phase was close to that corresponding to equilibrium. It can be assumed that kinetics controls the overall process again, probably because of using air instead of pure oxygen.

3.3.2.4 Modeling of the degradation process in the TPPB

The results of anthracene degradation in the surfactant-assisted TPPB under the most favorable strategy were modeled by applying mass balances in the organic and aqueous phases as described in Chapter 2. Mass balance in the vegetable oil consists of the output rate of the pollutant due to its partitioning into the aqueous phase (equation (3.1)), while mass balance in the aqueous phase considers the input rate of anthracene due to its solubilization along with the rate of elimination by enzymatic oxidation (equation (3.2)).

$$\frac{dS_{org}}{dt} = -K_L a \left(\frac{S_{org}}{K_{SW}} - S_{aq} \right) \frac{V_{aq}}{V_{org}} \quad (3.1)$$

$$\frac{dS_{aq}}{dt} = K_L a \left(\frac{S_{org}}{K_{SW}} - S_{aq} \right) - k \cdot S_{aq} \quad (3.2)$$

S_{aq} and S_{org} (μM) are the anthracene concentrations in the aqueous and organic phases, respectively; V_{aq} and V_{org} (L) are the volumes of aqueous and organic phases, respectively; K_{SW} (dimensionless) is the anthracene partitioning coefficient previously defined; k (h^{-1}) is a pseudo first order kinetic constant and $K_L a$ (h^{-1}), the overall mass transfer coefficient. The profiles of anthracene in the organic and aqueous phases were obtained by solving the system of differential equations (equation (3.1) and (3.2)) as described in the previous Chapter (section 2.3.4).

Figure 3.9 shows the comparison of the experimental data of anthracene removal in the TPPB and the prediction of the model, where the goodness of the fit is presented (R^2=0.998). The pseudo first order kinetic constant, *k,* resulted in 170.7 h^{-1} and it is expected to involve several parameters such as laccase activity, mediator and oxygen concentration as well as the possible

presence of oxy radicals generated during lipid oxidation. The higher value of the kinetic constant in this study respect to the previous work with silicone oil (52.2 h^{-1}) could be just attributed to the higher concentration of mediator in the reaction medium as well as the lipid oxidation process.

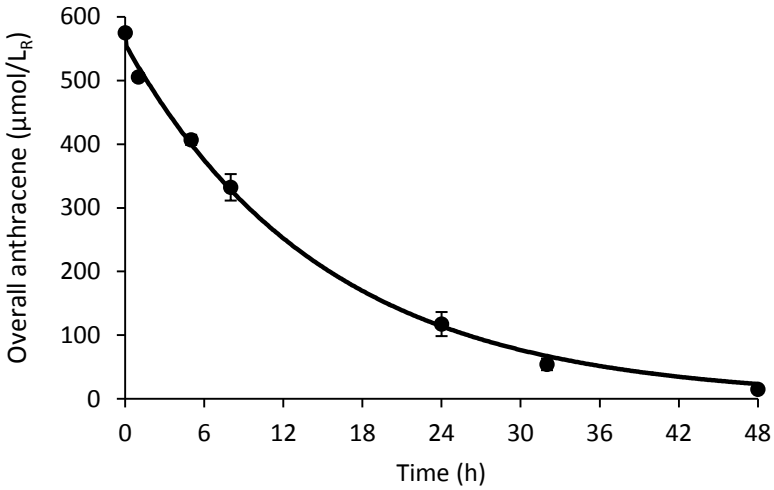

Figure 3.9. Experimental anthracene concentration during oxidation experiment in the reactor (●) and model prediction (—), R^2= 0.998.

The overall mass transfer coefficient K_La resulted in 1573.1 h^{-1}, which is two times higher than the obtained for the analogous TPPB with silicone oil (788.1 h^{-1}) (Chapter 2). The consistency of both values was evaluated under the assumption that the interfacial area between both liquid phases is the parameter that contributes to K_La the most. The interfacial area can be described by equation (3.3):

$$a = \frac{6\phi}{d} \tag{3.3}$$

where Φ is the fraction of the dispersed phase and d is the Sauter mean droplet diameter, which is obtained from the following equation:

$$d = c_1(1 + c_2\phi)W_e^{-c_3}D_i \tag{3.4}$$

where c_1, c_2 and c_3 are constants, D_i is the impeller diameter and W_e is the Weber number, expressed as

$$W_e = \frac{\rho_d N^2 D_i^3}{\sigma} \quad (3.5)$$

where ρ_d is the dispersion density, N is the impeller speed and σ is the interfacial tension.

Since the values of Φ, c_1, c_2, c_3, D_i, and N were the same in the TPPB with silicone oil and with pomace olive oil, the ratio of the interfacial areas in both systems depends on the ratio of $(\sigma/\rho_d)^{-c_3}$. For that reason, the interfacial tension of the systems pomace olive oil—1% Triton X-100 and silicone oil—1% Triton X-100 at 30ºC were measured, resulting in 1.76 mN/m and 5.00 mN/m, respectively. From these data, and taking into account that the value for c_3 is 0.6 (McCabe et al. 1993), the ratio between the interfacial area in the pomace olive oil system and silicone oil system was calculated. This resulted in 1.99, which was approximately the same that the ratio of the K_La predicted for pomace olive oil system and silicone oil system. Therefore, the consistency of the model was demonstrated.

3.4 CONCLUSIONS

The present study investigated the potential use of two vegetable oils, sunflower and pomace olive, as NAPLs in TPPB to carry out the degradation of anthracene by the laccase-HBT system. The superior solubility of anthracene in these oils and the higher enzymatic stability in comparison with silicone oil were demonstrated. Pomace olive oil turned to be the best NAPL for the operation of the TPPB. Oxygen played an important role in the system and the aeration or oxygenation increased the oxidation rate of anthracene. The depletion of HBT during the process required the periodic addition of this mediator to maintain a high oxidation rate of anthracene. The influence of mass transfer and kinetics of enzymatic degradation on the overall process could be elucidated based on anthracene partition between the organic and aqueous phases. Furthermore, experimental data were successfully modeled by applying mass balances in both phases.

Based on these results, TPPB technology can be considered an interesting alternative to regenerate the vegetable oil after its use as extraction solvent in the treatment of PAH-contaminated soils. Further investigations are necessary to test the feasibility of reusing both the aqueous and oil phases throughout several cycles of operation in TPPB as well as to determine the extraction efficiency of the regenerated pomace olive oil in the treatment of anthracene contaminated soil. These issues will be investigated in the next Chapter of the present thesis.

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Chapter 4

COUPLING EXTRACTION AND ENZYME CATALYSIS FOR THE REMOVAL OF ANTHRACENE PRESENT IN POLLUTED SOILS[1]

Summary

In this study, a novel system for removing anthracene from polluted soils using biodegradable vegetable oil was investigated. The process started with the extraction of anthracene from the soil present in a high concentration (1004 mg/kg) by using pomace olive oil as extracting agent. The organic fraction was thereafter treated in a surfactant-assisted two phase partitioning bioreactor (TPPB), operated with a laccase-mediator system. The main outcomes of this study showed high extraction efficiency of anthracene for both fresh and regenerated pomace olive oil (higher than 84%) and almost complete removal of the target pollutant in the TPPB after 48 h. Moreover, a slight reduction in the extraction efficiency of oil was observed for soil subjected to a period of aging of 135 d. Finally, the possibility of reusing both the aqueous and organic phases of the TPPB in successive batches of anthracene degradation in the TPPB was indicative of the low requirements of the degradation system.

[1] **A. Arca-Ramos, G. Eibes, G. Feijoo, J.M. Lema and M.T. Moreira (2015).** Coupling extraction and enzyme catalysis for the removal of anthracene present in polluted soils. Biochemical Engineering Journal 93 (289-293)

OUTLINE

4.1 INTRODUCTION

An interesting possibility for the removal of soil contaminated with polycyclic aromatic hydrocarbons (PAHs) has relied on the use of vegetable oils, which are non-hazardous, natural non-toxic, cost effective and biodegradable solvents (Yap et al. 2010). In addition, its free fatty acids have solubility ratios for PAHs very similar to those of synthetic chemical surfactants (Gan et al. 2009). To date, several types of vegetable oils as sunflower, rapeseed, palm kernel or soybean oils were reported to show a high efficiency in extracting hydrophobic contaminants from soils (Table 4.1).

Table 4.1. Studies on the application of vegetable oil in batch extraction of PAHs from contaminated soils.

Reference	Oil type	PAH	Spiked or field soil	Oil :soil (v:w)	PAH concentration (mg/kg)	% PAH removed
(Gong et al. 2005b)	Sunflower	Mixture	Field	1:1 or 2:1	5453	81-100
(Gong et al. 2005c)	Sunflower	Mixture	Field	1:1	1255	90
			Field	2:1	1255	97
(Chi et al. 2010)	Soybean	Anthracene	Spiked	1:1 or 2:1	50	72-75
					200	74-73
(Lau et al. 2012)	Soybean	Phenanthrene	Spiked	1:1	200	68
					1000	56
		Fluoranthene	Spiked	1:1	200	69
					1000	59
	Palm kernel	Phenanthrene	Spiked	1:1	200	68
					1000	55
		Fluoranthene	Spiked	1:1	200	70
					1000	61
This study	Fresh pomace olive	Anthracene	Spiked	1:1	1000	84
	Regenerated pomace olive	Anthracene	Spiked	1:1	1000	84

The residual oil remaining in the soil after the extraction is biodegradable and mineralization is expected to occur by endogenous microbial activity (Gong et al. 2005a, Lau et al. 2012). In addition, the presence of low concentration of vegetable oils in soil has been demonstrated to facilitate the biological degradation of PAHs present in soils, by enhancing their bioaccessibility to endogenous microorganisms (Scherr et al. 2009).

Nevertheless, the sole vegetable oil extraction only transfers PAHs onto another phase, but not accomplishes their elimination. Furthermore, the regeneration of the oil after the extraction of PAH-contaminated soils is desirable, especially when large amounts of oils are required (Yap et al. 2010). Activated carbon or polyestyrene have been proposed as adsorbents to remove PAHs from organic solvents and oils (Gong et al. 2007, Pannu et al. 2004, Schuur and Mattiasson 2003); however, an additional treatment to recover the contaminated adsorbent should be considered.

The regeneration of the solvent by enzymatic catalysis may constitute an interesting approach to physical separation alternatives, specifically the system based on a two-phase partitioning bioreactor (TPPB) with oxidative enzymes. Although the suitability of fresh pomace olive oil to act as non-aqueous phase in TPPB to remove anthracene has already been proved in Chapter 3, there is still the need to couple the extraction process of the polluted soil with the regeneration of the solvent in the TPPB. In the present chapter, a novel process for removing anthracene from soil based on its initial extraction by pomace olive oil followed by the enzymatic degradation of the dissolved anthracene in a TPPB is proposed (Figure 4.1).

The reuse of both the regenerated pomace olive oil for successive extraction processes and the aqueous and organic phases of the TPPB in subsequent batches of enzymatic oxidation are additional objectives of the study. The effect of aging on the extractability of anthracene by the pomace olive oil was also investigated.

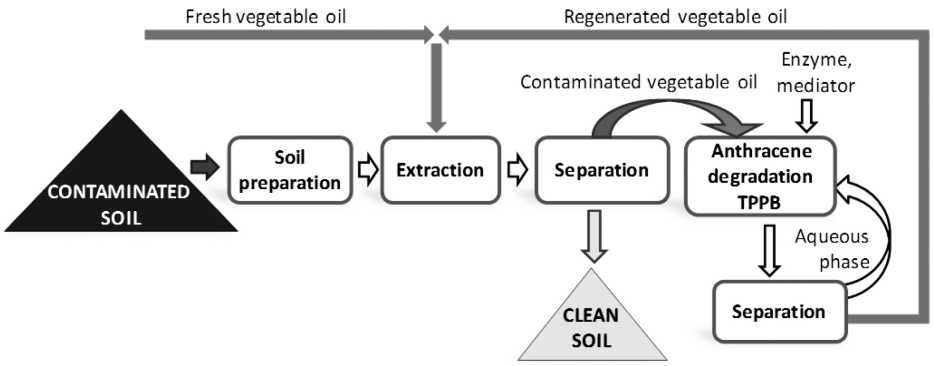

Figure 4.1. Proposed scheme for the remediation of soil contaminated with anthracene using vegetable oil as extraction agent.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and enzyme

Anthracene (99%), Triton X-100 (≥98%) and 1-hydroxybenzotriazole (HBT) (99%) were purchased from Janssen Chimica, Merck and Fluka, respectively. In addition, anthraquinone (97%), 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (≥98%), organic solvents (HPLC grade) and commercial laccase from *Trametes versicolor* were purchased from Sigma-Aldrich. The pomace olive oil utilised in the study was edible commercial oil.

4.2.2 Soil preparation

The soil sample selected was taken from a B horizon of a Cambisol soil. The excavated soil, taken at a depth of 60-70 cm, was homogenized, sieved at 2 mm and air-dried at 25ºC. The main physico-chemical characteristics of the soil are provided in Table 4.2. The soil was spiked with anthracene prepared in an acetone solution for a final concentration of anthracene in soil of 1004 mg/kg (i.e. 5633 µmol/kg). The soil-acetone-anthracene mixture was placed beneath a ventilation hood for 7 d, with periodical stirring until complete evaporation of acetone.

The contaminated soil was stored in the dark at room temperature. A fraction of the soil was used to evaluate the extraction efficiency of pomace

olive oil within the initial 4 days of the preparation. The other fraction was aged for a period of 135 d.

Table 4.2. Physicochemical characteristics of the soil sample

Sand (%)	84.2
Silt (%)	13.6
Clay (%)	2.2
pH (H_2O)	5.12
pH (KCl)	3.99
Total N (g/kg)	1.1
Total C (g/kg)	3.3
Organic carbon (%)	0.3
Ca^{2+} (cmol(+)/kg)	0.09
Mg^{2+} (cmol(+)/kg)	0.04
Na^{+} (cmol(+)/kg)	0.41
K^{+} (cmol(+)/kg)	0.11
Al^{3+} (cmol(+)/kg)	1.03
Cation exchange capacity (cmol(+)/kg)	1.2
Surface area (m^2/g)	10.1

4.2.3 Soil extraction with pomace olive oil

Soil extraction of anthracene by pomace olive oil was performed in Teflon bottles at room temperature (24±2ºC). Fresh or regenerated oil was poured into the bottles containing fresh contaminated soil (36.0 g and 8.5 g for the experiments with fresh and regenerated oil, respectively) with an oil-to-soil ratio of 1:1 (v:w) (Table 4.3). The bottles were sealed and mechanically shaken at 180 rpm on a linear shaker for 24 h, a time period deemed sufficiently long to reach equilibrium extraction for three ring PAHs (Gong et al. 2005b). Thereafter, the content was centrifuged at 4000 rpm for 15 min to separate the oil from the solid phase. An aliquot of 40 µL of the oil was added to a final volume of 4 mL of acetonitrile and mixed in a vortex for 5 min to extract the

anthracene from the oil. A sample of 1 mL of acetonitrile was analyzed by HPLC for anthracene measurement. These experiments were performed in duplicate. Additionally, an analogous experiment using acetonitrile as the solvent instead of vegetable oil was conducted in parallel for comparison purposes.

The effect of aging on the extractability of anthracene in soil was additionally investigated by conducting a similar extraction experiment with fresh pomace olive oil and 36 g of the 135 d-aged contaminated soil, maintaining the oil-to-soil ratio of 1:1 (v:w).

4.2.4 Enzyme activity and anthracene analysis

Laccase activity was spectrophotometrically determined. The concentrations of anthracene and anthraquinone, which was the main product of anthracene oxidation by laccase in the TPPB, were determined by high performance liquid chromatography (HPLC). The equipment and methodologies used were described in Chapter 3.

4.2.5 Regeneration of pomace olive oil in a TPPB

The degradation of anthracene extracted in pomace olive oil was performed at 30ºC and 250 rpm for 48 h in a TPPB consisting of a conventional stirred tank reactor (BIOSTAT®Q reactor, B. Braun-Biotech International). The extract containing the polluted vegetable oil accounted for 10% (v:v) of the total reaction volume. The aqueous phase was comprised of 1200 U/L laccase, 0.1 M sodium acetate (pH 5), 1 mM HBT and 1% Triton X-100 (v:v). Additional operational conditions, determined in Chapter 3, included aeration (periodic pulses 0.2 L/min of air for 1 min every 15 min) and pulses of HBT (1 mM at 8 h and 24 h) to the reaction medium.

The reusability of both aqueous and organic phases in the TPPB was assessed in three consecutive cycles. The first cycle in the TPPB comprised 10% (v:v) of polluted pomace olive oil in a total volume of 300 mL. After each cycle of 48 h, the two phases were separated by centrifugation at 4000 rpm for 15 min. The aqueous phase was supplemented with fresh enzyme (1200 U/L) and HBT (1 mM) prior to its recycling back to the reactor, whereas the oil was

spiked with anthracene (~5600 µM) and added to the TPPB to start a new cycle of anthracene oxidation. The volume of polluted oil at the beginning of the second and third cycle was 24 and 12 mL, respectively, which implied volumes of the aqueous phase of 216 mL and 108 mL. Hence, the proportion of 10% (v:v) oil referred to the total volume was maintained in the three consecutive cycles. Samples from both phases were periodically withdrawn to determine laccase activity as well as anthracene and anthraquinone concentrations.

4.3 RESULTS AND DISCUSSION

4.3.1 Anthracene extractability by pomace olive oil

The extraction efficiency of pomace olive oil to remove anthracene from spiked soil was evaluated in a batch extraction process and compared with that of acetonitrile, a conventional organic solvent with high extraction capacity for PAHs. The concentration of anthracene in the oil after the extraction process was analyzed according to the procedure described in section 4.2.3. Residual anthracene bound to soil particles was not analyzed due to the significant fraction of remaining pomace olive oil in the soil. Moreover, previous studies have reported that the residual concentrations of PAHs in soil samples obtained after extraction processes with palm kernel oil and soybean oil were an order of magnitude lower than the theoretical concentrations calculated by mass balances, as a consequence of analyte loss during the Soxhlet extraction (Lau et al. 2012). Therefore, in the current study the residual concentration of soil-bound anthracene after solvent extraction was estimated with the assumption that concentrations of anthracene in the recovered and non-recovered oil (remaining in soil after centrifugation) were identical (Table 4.3). The extraction efficiency of the oil was determined based on the anthracene bound to soil after the extraction process and the initial amount of pollutant in the soil. For the pomace olive oil, the extraction efficiency resulted in 84.4%, slightly lower than that calculated for acetonitrile (88.4%). Despite the fact that the recovery efficiency depends on the type of pollutant and soil, the concentration of the pollutant and the extract (Zhao et al. 2010), this value was in the range of those reported for other batch experiments using vegetable oils to extract PAHs (Table 4.1).

So far the results are promising, however, several studies have reported that as soil-pollutant contact time increases, pollutant bioavailability and extractability decrease (Luo et al. 2012, Reid et al. 2000). This phenomenon, known as aging, may result from the slow diffusion of the molecules into the small pores in soil aggregates, where they may be retained and become less accessible; and within some components of the organic matter in soils (Hatzinger and Alexander 1995, Luo et al. 2012). For this reason, in order to determine whether the time that anthracene remains in soil affects the ease of its extraction by the pomace olive oil, an additional experiment with 135 days-aged contaminated soil was conducted. The quantity of anthracene extracted from the aged soil with the pomace olive oil described as percentage extracted, suffered a small decrease from 84.4±2.3% with fresh contaminated soil, to 78.6±3.1% for the 135 d-aged contaminated soil. Similar results were described by Pannu et al. (2004), who proposed a peanut oil/water extraction system to remove PAHs from contaminated soils. These authors observed that for a single extraction with 10% (w:w) of peanut oil in water, the efficiency of extraction of total PAHs from weathered soil was 75.6% compared to 85.0% for fresh spiked soil. Nevertheless, Khan et al. (2012) reported a more pronounced reduction, 30%, in the extractability of phenantrene by several organic solvents, after 150 d aging in comparison to the freshly contaminated soil. As previously mentioned, it is well known that the extracting agent, the extraction method, the nature and concentration of the contaminant, and the soil characteristics influence extractability. In the current study, the low content of organic carbon in the soil (Table 4.2) is likely to mitigate the aging effect on anthracene extractability by the pomace olive oil (Luo et al. 2012).

4.3.2 Pomace olive oil regeneration in the TPPB

Once the potential of pomace olive oil as an extractant of anthracene in spiked soil was proven, the regeneration of the contaminated oil was evaluated in the surfactant-assisted TPPB with *T. versicolor* laccase. Figure 4.2 shows the profiles of anthracene and anthraquinone concentrations in the organic and aqueous phases.

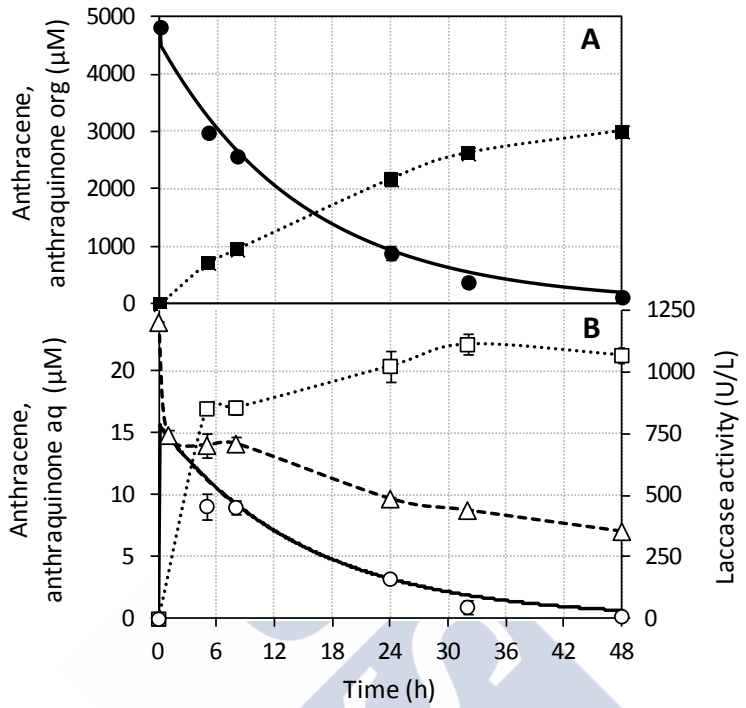

Figure 4.2 (A) Experimental concentrations of anthracene (●) and anthraquinone (··■··) in organic phase and model prediction for anthracene in organic phase (—), R^2=0.997; (B) experimental concentrations of anthracene (○) and anthraquinone (··□··) in aqueous phase, model prediction for anthracene (—), R^2=0.98, and residual laccase activity (- -△- -).

During the first 8 h, 45% of total anthracene was removed at an oxidation rate of 27.0 µmol/(L_R·h). Moreover, the observed percentage of degradation was 97.5% after 48 h. Anthraquinone was detected as the main anthracene oxidation product, although in a lower concentration that the one predicted through stoichiometry. It is noteworthy that no HBT was detected in the organic phase, which revealed that the mediator was only present in the aqueous phase; consequently, its eventual transfer to soil is not expected.

Aiming at comparing the extent of anthracene degradation in the surfactant-TPPB operated with oil spiked with anthracene and with the contaminated oil after the soil extraction process, the model described in Chapters 2 and 3, based on mass balances of anthracene in the organic and in the aqueous phases, was considered. The values of the pseudo first order kinetic constant *k* and the overall mass transfer coefficient K_La (170.7 h^{-1} and

1573.1 h^{-1} respectively) estimated in Chapter 3 for pomace olive oil, were used to predict the concentration of anthracene in the TPPB. Figure 4.2 shows the model predictions for anthracene in both the organic and aqueous phases, and a good fit can be observed (R^2=0.998). Therefore, the extraction process seemed to have no negative effect on the efficiency of the TPPB treating anthracene extracted from soil with oil in comparison with the oil spiked with anthracene.

Regarding laccase stability, the activity profile was similar to the one observed when the spiked oil was used, in Chapter 3. The presence of laccase inhibitors in the soil, such as metal cations (Ca^{2+}, Mg^{2+}) or even halides (Dwivedi et al. 2011), would not constitute a drawback for the enzyme as these ions cannot be extracted by the oil. Nevertheless, before a real application, efforts should be done in order to prevent the decay of enzymatic activity. The immobilization of the enzyme or the use of additives to stabilize laccase could be considered in further steps of the study.

4.3.3 Anthracene extractability by regenerated oil

When it comes to implement the system for a real application, the reuse of the vegetable oil after enzymatic catalysis is of special interest. Hence, the capability of the pomace olive oil regenerated in the enzymatic TPPB to extract anthracene from spiked soil was assessed in a further extraction step.

The main distinctiveness of the regenerated oil was the presence of the reaction product anthraquinone (3001 µmol/L), as well as the presence of a residual concentration of anthracene. The removal efficiency resulted in 84.1%, which was very similar to the value achieved when using fresh pomace olive oil (Table 4.3). Therefore, the dissolved anthraquinone present in the regenerated oil did not affect the anthracene extraction yield.

Although the quinones generated by the enzymatic oxidation present higher polar behavior and solubility in water, which implies that they could be potentially easier to biodegrade by indigenous bacteria present in soils (Cerniglia 1993), no transfer of anthraquinone from the oil to the soil is desirable. For this reason, anthraquinone concentration in the pomace olive oil

after the extraction process was also measured and it was concluded that all the quinone remained in the oil (data not shown).

Table 4.3. Anthracene (ANT) concentrations in soil and solvents and extraction efficiency.

Type of solvent	ANT in contaminated soil (µmol/kg)	Volume of solvent (mL)	ANT in solvent (µmol/L)*	ANT in treated soil (µmol/kg)	Efficiency (%)
Acetonitrile	5633	35.4	5061	653	88.4
Fresh pomace olive oil	5633	35.3	4848±135	879±132	84.4±2.3
Regenerated pomace olive oil**	5633	8.4	5012±69	897±68	84.1±1.2

*Values are given as means of duplicates with standard deviations
**The initial anthracene concentration in the regenerated oil was 218 µmol/L

Another issue of concern is related to the amount of anthracene remaining in the soil after the extraction process. It is well known that biodegradation of PAHs adsorbed in soils is a complex process strongly influenced by a wide variety of environmental and microbial factors (Meulenberg et al. 1997). In this sense, it is expected that the residual oil in the soil after the extraction process could boost PAHs biodegradation (Yap et al. 2010) by enhancing mass transfer of these pollutants from the soil to the degrading microorganisms (Scherr et al. 2009). In addition, it is expected that vegetable oils can be biodegraded by soil microorganisms after their acclimation to the oily soil (Gong et al. 2005a), although this should be evaluated for each particular case.

4.3.4 Feasibility of reusing regenerated pomace olive oil in subsequent regeneration cycles in TPPB

Due to the high quantity of oil used in the soil extraction, the regeneration and reuse of the oil is essential from an economic and environmental point of view. The reuse of the aqueous phase is also of interest to minimize the volume of liquid waste, which must be managed after the process. Therefore, the reusability of both the oil and the aqueous phases in the TPPB was studied with the aim of assessing the possible effect of the

enzymatic reaction products on the process efficiency and the feasibility of the proposed approach. The time profiles of laccase activity and concentrations of anthracene and anthraquinone in three consecutive cycles are shown in Figure 4.3.

The rate of anthracene oxidation at 8 h in the second and third cycles resulted in 38.2 µmol/(L_R·h) and 43.2 µmol/(L_R·h), considerably higher than in the first batch of degradation, which was 30.3 µmol/(L_R·h). This was reflected in the different percentages of degradation after 24 h for each cycle: 81.0%, 87.3% and 100% for the first, second and third cycle, respectively.

In a previous work described in Chapter 3 (section 3.3.2.3), the analysis of anthracene partition for an analogous experiment to that of the first cycle of the TPPB was performed and concluded that under the applied strategy, the rate-limiting step is the reaction kinetics. Consequently, the enhancement of the removal of anthracene observed for the second and third cycles should be associated to increased reaction rates.

Aiming at estimating the pseudo first-order kinetic coefficient (*k*) for the second and third cycles and fit the experimental data, the model was applied maintaining the same value for the global mass transfer coefficient: K_La (1573.1 h^{-1}). This assumption was taken on the basis that most main parameters affecting K_La i.e., agitation rate, oil and surfactant fractions or stirrer diameter were kept constant in all experiments. Moreover, although the unsaturated fatty acids of pomace olive oil were susceptible to oxidation by the laccase-HBT system, the percentage of oxidation was low to consider significant effects on the physical properties of the oil or dispersion (interfacial tension, dispersion density and viscosity). Modeling of the three successive batch experiments is shown in Figure 4.3, where the goodness can also be observed for the second and third cycles (R^2= 0.9990 and R^2= 0.9995, respectively).

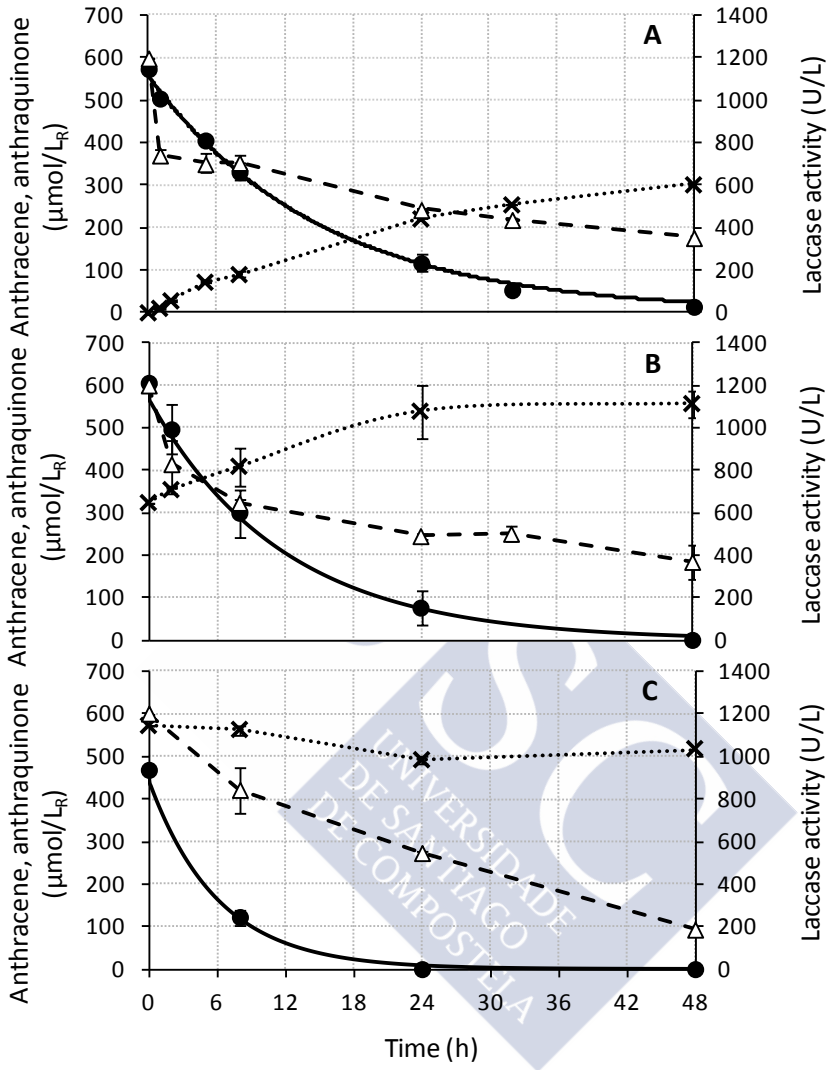

Figure 4.3. Experimental anthracene (●) and anthraquinone (··×··) concentrations referred to the total reaction volume, residual laccase activity in the aqueous phase (--△--) and concentration of anthracene predicted by the model (—) referred to the overall reaction volume in the first (A), second (B) and third cycle (C) of the experiment reusing pomace olive oil and aqueous phase.

The values of *k* for the second and third cycles resulted in 225.6 h^{-1} and 507.5 h^{-1}, respectively. This parameter was expected to depend on several factors such as laccase activity, mediator and oxygen concentration, as well as the possible presence of oxy radicals generated during lipid oxidation.

Similar profiles of laccase activity were observed in the three consecutive batches. Hence, this parameter was discarded to exert any influence in the different values of *k*. The level of dissolved oxygen in laccase catalyzed reactions has an important function on the oxidation rate due to its function as an electron acceptor (Wesenberg et al. 2003). In this study, the frequency and intensity of aeration were maintained constant during the three cycles despite the reduction of the reaction volume through the three consecutive batches due to sampling losses. This caused an increased proportion of oxygen (data not shown) in the second and third cycles that could be responsible for improving the reaction rates.

Another factor, which was presumably beneficial, was the accumulation of anthracene degradation products, mostly anthraquinone, in the reaction medium during the three cycles: 0, 323 and 589 μmol/L_R at the beginning of each cycle, respectively (Figure 4.3). Quinones have been reported to act as electron carriers in redox processes (Cervantes et al. 2001). The positive effect of anthraquinone on the anthracene oxidation by manganese peroxidase (MnP) in monophasic systems had been previously reported by Eibes et al. (2008), who observed that the initial addition of anthraquinone to the system enhanced the degradation of anthracene.

Moreover, the nitroxy radical formed when HBT is oxidized by laccase is a potent electrophile and, consequently, allows the oxidation of unsaturated fatty acids to generate lipid radicals, which are known to cooxidize PAHs (Böhmer et al. 1998). As the reaction proceeds along the three consecutive cycles, the amounts of degradation products and radicals are expected to increase and, hence, anthracene oxidation may be enhanced.

4.4 CONCLUSIONS

The combination of soil extraction by pomace oil and enzymatic catalysis as a novel remediation process for polluted soils was proposed. The vegetable oil, either fresh or regenerated, showed high extraction efficiency. The aging of soil supposed a very small reduction in the extractability of anthracene by the oil in comparison to the fresh contaminated soil. Moreover, the feasibility of reusing both phases in successive operations of the TPPB was demonstrated,

which was advisable from an economic perspective. In addition, reusing the aqueous phase constitutes a strategy to minimize the volume of waste to be managed, as aqueous phase would eventually constitute a residue of the global process. This study may be considered as a basis for the development of a promising technology for removing hydrophobic contaminants from soils under mild conditions. Nevertheless, prior to a full-scale application, further research is required to optimize the different stages of the process such as the extraction procedure to assure minimal oil residues in the treated soil or the enzyme stability in the TPPB. In addition, a comprehensive assessment of the treatment costs and ecological risks should be performed.

4.5 ACKNOWLEDGEMENTS

Authors acknowledge the group GI-1245 Ambiosol from the Department of Edaphology and Agricultural Chemistry, USC, for the donation and characterization of the soil.

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Chapter 5

POTENTIALITY OF A CERAMIC MEMBRANE REACTOR FOR THE LACCASE-CATALYZED REMOVAL OF BISPHENOL A FROM SECONDARY EFFLUENTS[1]

Summary

In the current Chapter, the removal of bisphenol A (BPA) by laccase in a continuous enzymatic membrane reactor (EMR) was investigated. The influence of key parameters, namely, type of laccase, pH and enzyme activity were initially evaluated. Once optimal conditions were determined, the continuous removal of the pollutant in an EMR was assessed in synthetic and biologically treated effluents. The reactor configuration consisted of a stirred tank reactor coupled to a ceramic membrane, which prevented the adsorption of the pollutant and allowed the recovery and recycling of laccase. Nearly complete removal of BPA was attained under both operation regimes with removal yields over 94.5%. In experiments with real wastewater, the removal of BPA remained high while the presence of colloids, certain ions and the formation of precipitates on the membrane potentially affected enzyme stability and made necessary the periodic addition of laccase. Polymerization and degradation were observed as probable mechanisms of BPA transformation by laccase.

[1] **A. Arca-Ramos, G. Eibes, G. Feijoo, J.M. Lema and M.T. Moreira (2015).** Potentiality of a ceramic membrane reactor for the laccase-catalyzed removal of bisphenol A from secondary effluents. Applied Microbiology and Biotechnology. doi:10.1007/s00253-015-6826-4

OUTLINE

5.1 INTRODUCTION

In recent decades, there has been an increasing concern about the potential adverse health and ecological effects of Endocrine Disrupting Compounds (EDCs). These compounds are defined as "a group of chemicals (natural, synthetic, industrial chemicals or by-products) present in the environment and suspected to alter the functions of the endocrine system and, consequently, causing adverse health effects in an intact organism, or its offspring, or (sub) population" (European Commission 2007). Natural and synthetic EDCs are released into the environment mainly through sewage treatment systems since conventional wastewater treatment plants (WWTP) only accomplish their partial degradation (Lloret et al. 2012a). One alternative treatment for their removal relies on the use of oxidative enzymes such as oxidases and peroxidases, which are able to oxidize several EDCs and eliminate the associated hormone-mimicking activity (Cabana et al. 2007). Laccases are promising biocatalysts since, unlike peroxidases, they do not need the addition of H_2O_2 and other cofactors such as veratryl alcohol or Mn^{2+} (Baldrian 2006). In contrast, laccases use molecular oxygen as the final electron acceptor (Kunamneni et al. 2008). The application of enzymes in continuous processes such as wastewater treatment plants must ensure the retention of the biocatalyst in the system (Galliker et al. 2010). Two different approaches, based on free or immobilized laccases, can be considered (Gasser et al. 2014). Although immobilized laccase is typically more resistant against denaturing agents potentially present in wastewaters, it usually exhibits reduced activity towards certain substrates in comparison to free enzymes. In this sense, although some studies reported increased enzymatic activity due to cross linking of laccases (Ammann et al. 2014), such effect was found for standard substrates of laccases, such as 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) or dimethoxyphenol. Regarding bisphenol A (BPA), the model pollutant addressed in the current research, there are several studies concluding that immobilized laccases showed significantly lower rates than free enzyme, probably due to diffusional problems (Hommes et al. 2012, Pang et al. 2015). For this reason, the use of free laccase to avoid mass transfer limitations was proposed. In this scenario, the retention and reuse of the biocatalyst is possible by using an enzymatic membrane reactor (EMR). Such

configuration is based on the use of membranes designed with a pore size smaller than the enzyme, aiming at preventing their washout (López et al. 2004). The semi-permeable membrane facilitates the separation of the enzyme from products and substrates. Moreover, the enzyme remains within the system (reactor) allowing continuous operation, with treated effluent withdrawal and negligible loss of enzyme activity. Beyond the retention of the enzyme, the use of membrane processes such as nanofiltration (NF) for the treatment of WWTP effluents provides a permeate with good physico-chemical and microbiological quality, suitable for reuse in various applications (Acero et al. 2010).

Membrane reactors for enzymatic applications have been typically assembled with polymeric membranes such as polyacrylonitrile, polyethersulfone or polysulfone. In a previous study developed by our research group, an EMR using an ultrafiltration polyethersulphone membrane was successfully applied for the continuous enzymatic transformation of estrogens by laccase from *Myceliophthora thermophila* (Lloret et al. 2013b). However, this system was not suitable for the removal of BPA, due to the adsorption of the micropollutant onto the hydrophobic membrane (Su-Hua et al. 2010). Indeed, several studies have reported the retention of BPA and other compounds on polymeric membranes (Agenson et al. 2003, Zhang et al. 2006). Moreover, it should be noted that the adsorbed compounds could be released in the permeate, especially if the pollutant concentration in the influent presents great variability (Zhang et al. 2006). Other drawbacks related to the hydrophobicity of polymeric membranes are their low membrane flux and poor anti-fouling performance, which have a great effect on their application and usage life (Wu et al. 2008). A more feasible option could rely on the use of hydrophilic ceramic membranes, to minimize the interactions with certain micropollutants as well as foulants present in wastewater, such as natural organic matter. Other important advantages of ceramic membranes in comparison to the polymeric ones are the superior chemical, mechanical and thermal resistance. Although the capital costs of ceramic are higher than those of polymeric membranes, its longer operational lifetime can balance out the costs (Ciora and Liu 2003).

The key goal of the current study was to develop a continuous enzymatic reactor for the removal of a hydrophobic micropollutant. An external filtration module comprising a TiO_2 ceramic membrane was used to retain the enzyme in the system. Although the use of laccase to remove micropollutants from aqueous systems has been reported in several studies as recently reviewed by Gasser et al. (2014), in most studies the enzymatic degradation was performed in buffer solutions, under favorable pH values that are not encountered in WWTPs and high concentrations of the target pollutants. The selection of the xenoestrogen BPA as the model micropollutant was based on its moderately high octanol-water partitioning coefficient (log K_{OW}) that makes it prone to be adsorbed on hydrophobic membrane surfaces (Nghiem et al. 2008). This pollutant is widely used in the production of polycarbonate plastics and epoxy resins (Kim and Nicell 2006a) and has been found at significant concentrations in industrial wastewaters, landfill leachates and even in surface water (Flint et al. 2012).

With the aim of maximizing the removal efficiency of BPA, the progress of the research was planned in two stages: batch and continuous experiments. The selection of the biocatalyst was initially performed according to a number of variables: high and low redox potential laccases, pH range and laccase activity. Subsequently, the outcomes of the batch experiments were the basis for the operation of the EMR with the ceramic membrane. This system was continuously operated at different hydraulic retention times (HRT) with synthetic and real wastewaters.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and enzyme

BPA (≥99%), bisphenol F (≥98%), ABTS (≥98%), organic solvents (HPLC grade), HBT (99%) and the commercial laccase from *Trametes versicolor* (≥10 U/mg) were purchased from Sigma-Aldrich (Spain). Commercial laccase from *M. thermophila* was kindly supplied by Novozymes (Denmark) and produced by submerged fermentation of genetically modified *Aspergillus* sp. with a molecular weight of 56,000 Da.

5.2.2 Determination of enzyme activity and BPA concentration

Samples were withdrawn at different time intervals from the reaction vessel to measure laccase activity and BPA concentration. Laccase activity was determined by a colorimetric assay using ABTS as substrate as described in Chapter 2.

High concentrations of BPA (in the range of mg/L) were quantified by high performance liquid chromatography (HPLC) at a detection wavelength of 278 nm on a Jasco XLC HPLC (Jasco Analitica, Madrid, Spain). This equipment was coupled with a diode array detector 3110 MD, a 4.6x150 mm Gemini reversed-phase column (3 µm C18 110Å) from Phenomenex (supplied by Jasco Analitica, Madrid, Spain) and an HP ChromNav data processor. Gradient elution started with 20% acetonitrile in water, which was kept for 1 min, followed by an increase to 90% acetonitrile within 4 min. This concentration was kept constant for 5 min, and then decreased back to the initial concentration after 14 min. The flow rate was fixed at 0.8 mL/min.

Low concentrations of BPA (in the range of µg/L) were determined by gas chromatography–mass spectrometry (GC-MS). GC-MS analysis required that the samples (50 mL) were acidified at pH 2 with HCl and diluted to 100 mL with distilled water before performing solid phase extraction (SPE). Bisphenol F was added as surrogate internal standard. SPE was carried out using 60 mg OASIS HLB cartridges (Waters closet, Milford, MA, USA) previously conditioned with 3 mL of ethyl acetate, 3 mL of methanol and 3 mL of distilled water adjusted to pH 2 with HCl. The cartridges were then dried with a nitrogen stream and eluted with 3 mL of ethyl acetate. An aliquot of 800 µL of the extract was withdrawn and 200 µL of MTBSTFA (N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide) was added for the derivatization of the species previously to GC–MS analysis. This was conducted in a MS Saturn 2100T system with a CP Sil column (CP Sil 8 CB-MS low bleed 30 m x 0.25 mm x 0.25 µm) (Varian, Walnut Creek, USA). The carrier gas was He with a flow rate of 1 mL /min. The injection was performed in splitless mode (2 min) at 280 ºC. The oven temperature was programmed to rise from 70ºC to 150ºC at 25ºC/min, then at 3ºC/min until 200ºC and finally at 8ºC/min to reach 280ºC, and was maintained at this temperature for 5 min. Electron impact (EI) mass spectra

were generated at 70 eV with a scan range of 50-500 amu. Each sample was analyzed in duplicate.

5.2.3 Batch transformation experiments

The enzymatic oxidation of BPA (nominal concentration of 10 mg/L) was conducted in batch experiments in 100 mL Erlenmeyer flasks at room temperature (24ºC±2ºC) and continuously mixed using magnetic stirring. Laccases from *T. versicolor* and *M. thermophila* with initial activity of 500 U/L were tested to remove BPA in a reaction medium consisting of sodium acetate (100 mM, pH 4) and 5% (v:v) methanol to ensure BPA solubility. Thereafter, the influence of pH and enzyme activity on the removal of BPA (10 mg/L) by *T. versicolor* laccase was studied. Sodium acetate and phosphate buffer solutions (100 mM) were used to adjust pH between 5 and 7. For each pH, three levels of activity were evaluated: 1000, 500 and 100 U/L. Samples were periodically withdrawn for laccase measurements. Subsequently, the reaction was stopped by adding HCl to inactivate the enzyme (pH 2). Samples were frozen until BPA analysis by HPLC. Experiments were performed in triplicate. Control assays were run in parallel in the absence of laccase to verify that the elimination took place only by enzymatic catalysis.

5.2.4 Continuous transformation of BPA in a ceramic EMR

The continuous enzymatic reactor (Figure 5.1) consisted of a conventional stirred tank reactor (2 L, BIOSTAT®Q reactor, B. Braun-Biotech International) coupled to a highly hydrophilic TiO_2 ceramic membrane (Likuid Nanotek, San Sebastián, Spain), which allowed the recovery of laccase and its recycling to the reaction vessel.

In a preliminary assay (data not shown), the capability of two types of membranes with pore sizes of 5 and 1 nm to retain laccase were evaluated. The lowest pore size membrane was necessary to retain the enzyme in the system and was selected for the continuous enzymatic removal of BPA. The surface area of the membrane, with a multitubular morphology, was 0.04 m^2 (Figure 5.2).

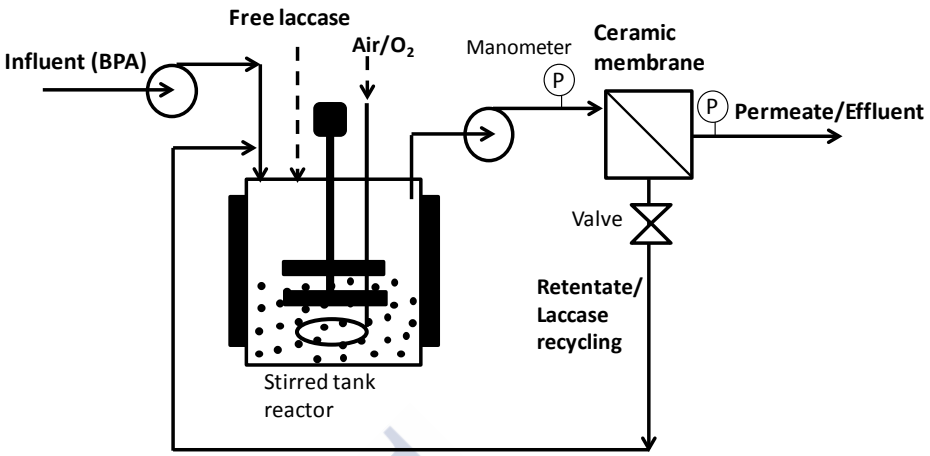

Figure 5.1. Scheme of the enzymatic membrane reactor.

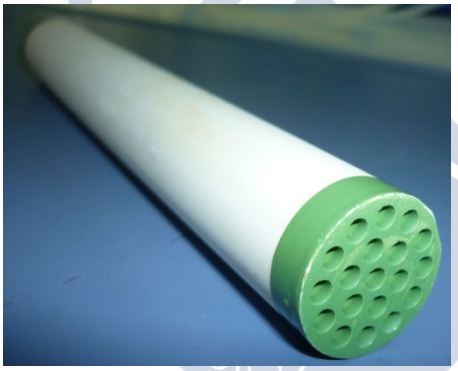

Figure 5.2. Picture of the ceramic membrane.

Polytetrafluoroethylene (PTFE) tubing and Teflon®-coated magnetic stirrers were used to prevent BPA adsorption. The influent containing BPA (75 µg/L in 100 mM sodium phosphate buffer pH 6 or in real secondary effluent from a WWTP) was continuously pumped from a 10-L glass vessel to the reactor, which had a working volume of 1.5 L, through Norprene® tubing using a peristaltic pump. Laccase from *T. versicolor* was added in a single initial pulse of 1000 U/L and supplied when necessary to maintain constant enzymatic activity. A second pump was used to flow the effluent of the vessel through the membrane module and return the enzyme into the reaction vessel with a recycling: feed flow ratio of 4:1. Such ratio was fixed to increase the cross flow velocity, and hence, mitigate fouling or enzyme deposition.

The flow rate of the effluent of the EMR, namely, the membrane permeate, was maintained at the same value as the influent to maintain steady state conditions. A valve located at the end of the membrane module was used to control both the effluent and the recycling flow rates (Figure 5.1). It should be noted that, before the addition of fresh enzyme in the reaction vessel, samples of the membrane permeate were collected to monitor BPA concentration by GC-MS analysis and, hence, verify that the micropollutant was not adsorbed on the membrane or on the tubing. Trans-membrane pressure (TMP) was monitored by two manometers located at the inlet and outlet of the membrane. It should be noted that after each experiment, the system was flushed with 4 L of distilled water at a high flow rate (>100 mL/min) with no permeate production, to remove enzyme potentially attached to the membrane surface. Accordingly, at the start-up of each experiment, it was verified that no laccase activity was detected in the recycling flow.

The real wastewater used in the experiment consisted of the effluent from the secondary clarifier of the municipal wastewater treatment plant of Calo-Milladoiro (Ames, Spain). The collected water was immediately filtered (0.45 µm) to remove particulate matter and suspended solids and it was stored at 4ºC until use. Physico-chemical properties are shown in Table 5.1.

Table 5.1 Characteristics of the secondary effluent used to prepare the reactor feeding. The wastewater was analyzed according to Standard Methods (American Public Health Association (APHA) 1999).

Parameter	Concentration	Anion	Concentration (mg/L)	Cation	Concentration (mg/L)
TOC	3.53 mg/L	Cl^-	27.26	Li^+	<0.05
TC	10.42 mg/L	NO_2^-	<0.05	Na^+	24.92
IC	6.88 mg/L	Br^-	<0.20	NH_4^+	<0.10
TN	4.87 mg/L	NO_3^-	6.26	K^+	5.76
pH	7.2	PO_4^{3-}	<0.05	Mg^{2+}	1.63
		SO_4^{2-}	17.63	Ca^{2+}	5.53

TOC: Total organic carbon; TC: total carbon; IC: inorganic carbon; TN: total nitrogen

Statistical analyses were performed to evaluate whether the percentages of BPA removal were significantly different for the conditions assayed. F-test function of Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) was used to test whether variances were equal (significance level $p<0.05$). Subsequently, the t-test function of Microsoft Excel was applied to determine *p*-values and assess statistical significance (significance level $p<0.05$).

5.2.5 Identification of BPA transformation products

The identification of water soluble products of BPA was attempted in batch experiments performed in sodium phosphate buffer (100 mM, pH 6) and 5% v:v methanol with an initial concentration of pollutant of 10 mg/L and 1000 U/L of *T. versicolor* laccase in a final volume of 100 mL. Two additional samples were obtained in parallel: the control of BPA in buffer without laccase and the blank with enzyme but lacking BPA. Three batch experiments were conducted for 2 h and then stopped by acidifying at pH 2. Then the SPE was carried out prior to GC-MS analysis. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) or MTBSTFA were used as derivatizing agents.

With the aim of identifying high molecular weight metabolites, a 15-mL reaction mixture consisting of 25 mg/L of BPA, 1000 U/L laccase from *T. versicolor*, sodium phosphate buffer (100 mM, pH 6) and 5% v:v methanol was prepared. After 24 h, the enzymatic reaction was stopped at pH 1 by HCl addition. Control and blank samples were run in parallel. These samples were then centrifuged at 5000 rpm for 30 min. The supernatant was discarded and the precipitate was dried under a stream of nitrogen and suspended in chloroform prior to electrospray ionization time of flight mass spectrometry (ESI-TOF MS) analysis in flow injection mode (FIA).

The mass spectrum of the different samples was obtained by means of a mass spectrometer micrOTOF (Bruker, Bremen, Germany). The m/z range was 100-900 Da. The following settings were used: ESI in the negative ion mode, dry gas flow rate of 8 L/min and dry heater at 200ºC. The capillary voltage was set to 4500 V and the collision gas pressure was 2 bar.

5.3 RESULTS AND DISCUSSION

5.3.1 Selection of the optimal transformation conditions

Before the continuous operation of the EMR, a number of process parameters such as type of laccase, pH and enzyme activity were selected from the batch experiments. The redox potential of ligninolytic enzymes has long been believed to play a key role in their prospective ability for oxidizing xenobiotic compounds (Li et al. 1999). High redox potential laccases, such as *T. versicolor* laccase, are associated to higher rates for substrate oxidation than low redox potential ones. Nevertheless, the use of a lower redox potential, but commercially available low-cost enzyme, as *M. thermophila* laccase, is also of interest in the search of a cost-effective technology (Babot et al. 2011). For this reason, the catalytic efficiency of both laccases was evaluated in batch experiments at pH 4, which corresponded to maximum enzyme activity for these enzymes (Lloret et al. 2010, Lloret et al. 2013a). Figure 5.3 depicts the BPA removal profiles and laccase activity for the experiments performed at high BPA concentrations (10 mg/L), which enabled the use of a simple analytical method (HPLC-DAD).

Both enzymes were able to oxidize BPA; nevertheless, the high redox potential laccase was considerably more efficient. For instance, after 8 h, *M. thermophila* laccase converted 64% of initial BPA, whereas *T. versicolor* laccase achieved higher values: 96% and even complete removal before 24 h. These results confirmed that the catalytic efficiency towards BPA was highly dependent on the redox potential of the enzyme. Furthermore, although both laccases showed a high inactivation rate, *M. thermophila* laccase suffered a deeper decay. Hence, laccase from *T. versicolor* was selected to further optimize the conditions of BPA removal.

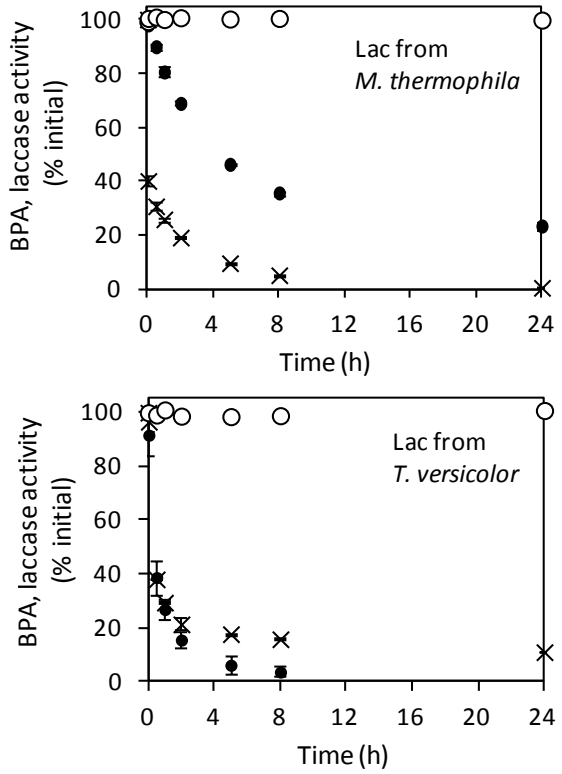

Figure 5.3. Residual BPA concentration (●) and residual enzyme activity profile (×) during the batch treatment by laccase (lac) from *M. thermophila* and by laccase from *T. versicolor*; residual BPA concentration in the control assay without enzyme (○).

It is well known that pH is one of the most important factors affecting the enzymatic activity (Margot et al. 2013b). Although laccases show maximum activity at pH 3-4, a pronounced inactivation occurs when incubated at acid pH, probably as a consequence of the ionizable side-chains of the tertiary structure of the protein (Lloret et al. 2013a). For this reason, the transformation of BPA by *T. versicolor* laccase was evaluated at higher pH values aiming at steady laccase stability, which would likely render into higher reaction yields. Moreover, three levels of enzyme activity were assayed in order to evaluate the dependence of the laccase-catalyzed transformation of BPA on the initial activity of the enzyme.

Table 5.2 shows the transformation rate and the residual concentration of BPA as well as the effectiveness, for the different values of pH and levels of

enzyme activity evaluated. The term effectiveness was defined as the ratio of the amount of BPA transformed and the loss of enzyme activity after 1 h.

Table 5.2 Conversion rate, percentage of BPA removal and effectiveness after 1 h treatment of BPA (nominal concentration of 10 mg/L) by laccase from *T. versicolor.*

pH	Activity (U/L)	Conversion rate (mg/(L·h))	Residual BPA (%)	Effectiveness (mg/U)
pH 5	100	Non evaluated		
	500	6.7±0.2	23.4±2.1	0.018±0.001
	1000	7.9±0.1	4.1±1.2	0.013±0.003
pH 6	100	3.0±0.3	65.0±3.9	0.045±0.008
	500	6.8±0.1	22.6±1.2	0.024±0.001
	1000	7.8±0.03	3.7±0.3	0.036±0.003
pH 7	100	1.5±0.2	83.5±1.9	0.066±0.010
	500	5.5±0.2	42.7±2.2	0.082±0.004
	1000	7.0±0.1	16.8±1.2	0.046±0.002

The conversion rates of BPA were quite similar at pH 5 and 6 and the percentage of transformed BPA was higher when using 1000 U/L. At neutral pH, laccase was more stable, which led to higher effectiveness; however, the removal rate of BPA was lower than at acid pH. This is in accordance with previous work that reported the optimum pH for BPA degradation in the range between 5 and 6 (Kim and Nicell 2006a, Margot et al. 2013b). At pH 5, laccase suffered significant inactivation, which resulted in a considerably lower effectiveness than at pH 6 or 7. For this reason, it was not possible to evaluate the laccase addition of 100 U/L at pH 5. For pH 6 and 7, and 100 U/L, the conversion rate of BPA referred to the first hour resulted in 3.0 and 1.5 mg/L·h, respectively, which evidenced that the decrease of laccase activity led to a lower conversion rate.

Laccase catalyzed transformation of BPA was also dependent on the initial activity of the enzyme and the higher transformation rates were obtained for the highest enzymatic activity. Nevertheless, the amount of BPA removed per quantity of enzyme applied diminished when the initial concentration of laccase increased due to the non-proportional dependency between the

laccase dose and the conversion rate in the assayed range. A similar behavior has been previously observed for the enzymatic transformation of triclosan by laccase (Kim and Nicell 2006b).

Summarizing, high laccase activity (1000 U/L) and pH between 5 and 6 were required to attain high removal percentages (higher than 95%) and provided the highest removal rates. Since the effectiveness at pH 6 and 1000 U/L was almost 3-times higher than at pH 5, the selected conditions to perform the continuous enzymatic transformation of BPA were pH 6 and 1000 U/L.

5.3.2 Identification of BPA transformation products

Identification of the BPA water soluble products was conducted by GC-MS analysis using BSTFA or MTBSTFA as derivatizing agents. When using MTBSTFA, three peaks in the sample not present in the control or blank samples were observed (Figure 5.4), whereas with BSTFA only two peaks could be detected (data not shown).

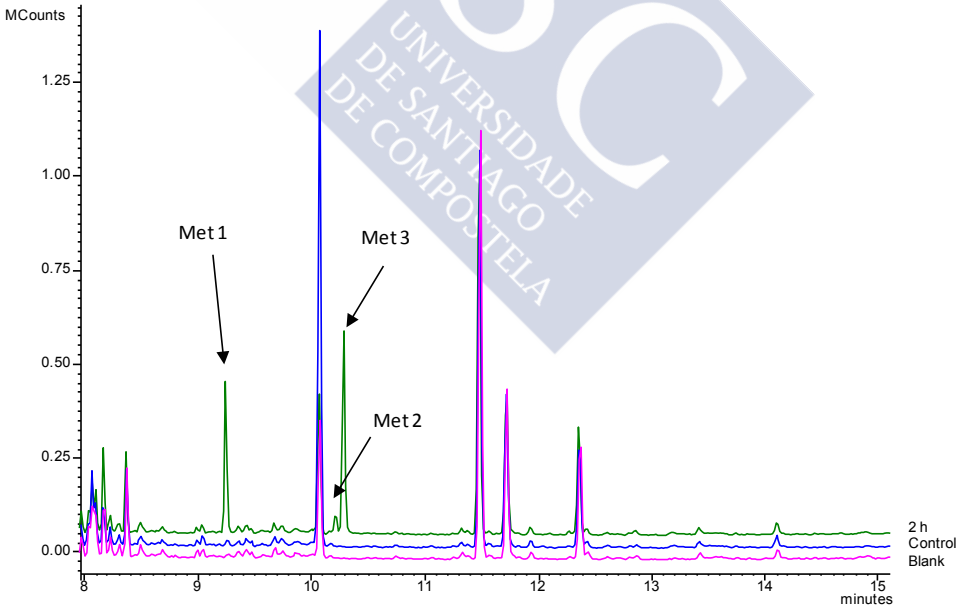

Figure 5.4. Chromatograms of blank (without BPA), control (without laccase) and sample after 2-h laccase treatment. To facilitate the visualization of the new peaks, only the first part of the chromatogram is shown. The retention time of BPA was 21.16 min.

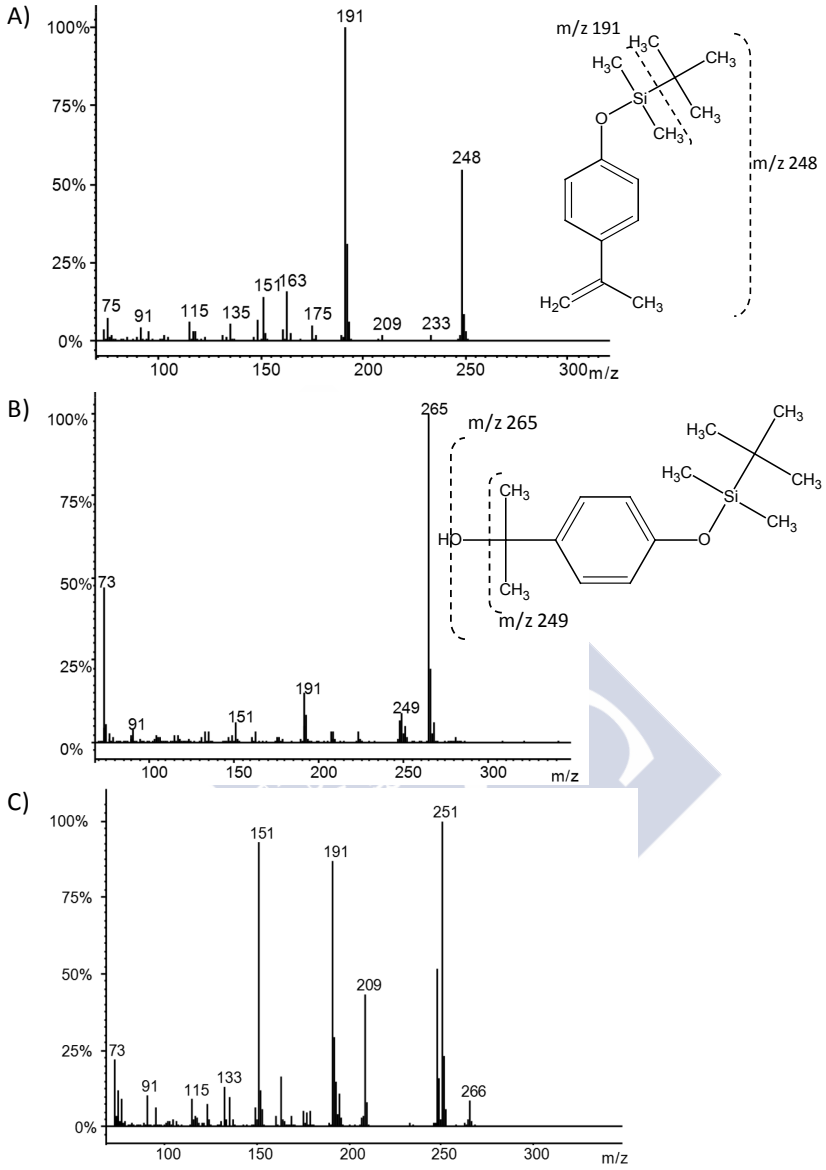

Figure 5.5. Mass-spectrum of Met 1 (A) identified as 4-isopropenylphenol via GC-MS after derivatization with MTBSTFA; Met 2 (B) which might corresponds to 4-(2-hydroxypropan-2-yl)phenol; and Met 3 (C) formed after incubation (2h at pH 6) with laccase.

The first peak, observed in GC-MS chromatograms with both MTBSTFA (Figure 5.5A) and BSTFA (Figure 5.6) was identified as 4-isopropenylphenol. The ion fragments of the O-trimethylsilylated compound, which are depicted in

(Figure 5.6) are almost identical to the fragmentation pattern obtained for 4-isopropenylphenol by (Galliker et al. 2010).

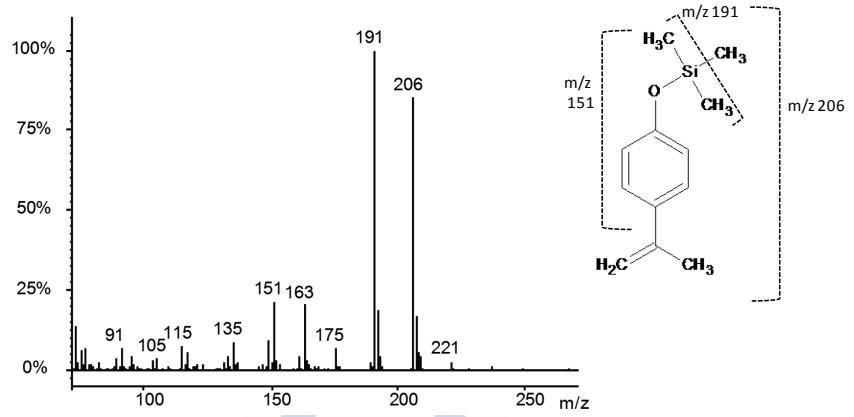

Figure 5.6 Mass-spectrum of one of the metabolites formed after incubation of BPA with laccase from *T. versicolor* (1000 U/L) at pH 6, identified as 4-isopropenylphenol via GC-MS after derivatization with BSTFA.

The second peak detected in Figure 5.4 might correspond to 4-(2-hydroxypropan-2-yl)phenol although the identification could not be confirmed (Figure 5.5B). Formation of 4-isopropenylphenol and 4-(2-hydroxypropan-2-yl)phenol from the degradation of BPA by other laccases or peroxidase has been reported in previous studies (Fukuda et al. 2004, Huang and Weber 2005). Regarding the third new peak (Figure 5.5C), its identification was not possible

Figure 5.7 shows the ESI(-)MS spectrum of chloroform soluble compounds present in 24-h samples. In this case, eight new ions, which did not appear in the parallel samples lacking BPA or enzyme (blank and control), were observed (indicated with arrows). The m/z values of 453 and 679 may correspond to BPA dimer and trimer with molecular weights of 454 and 680, respectively. For both peaks, accurate masses were in agreement with the calculated ones with errors of -3.3 and 2.9 ppm, respectively. A third peak with m/z of 93 might correspond to phenol. However, due to the low intensity of the peak, the error could not be calculated. The formation of phenol from enzymatic transformation of BPA by horseradish peroxidase or laccase has been reported in previous studies (Galliker et al. 2010, Huang and Weber 2005).

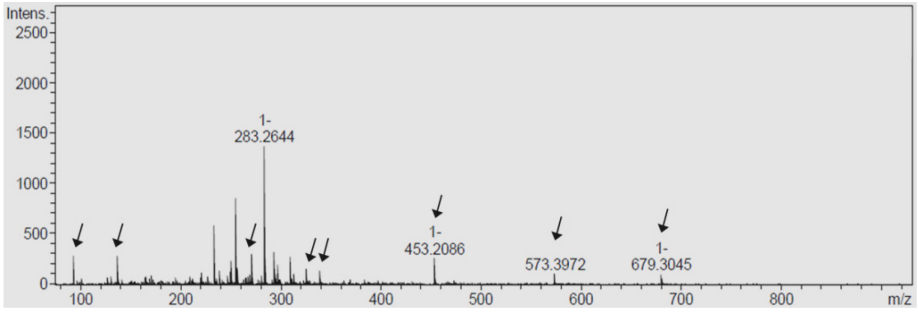

Figure 5.7. ESI(-)MS full scan spectrum of high molecular weight products of laccase-catalyzed transformation of BPA at a pH 6 and 1000 U/L of *T. versicolor* laccase.

The proposed pathway of BPA polymerization reported in other works consisted of a polymerization mechanism of BPA through C-C or C-O bonds to form oligomers (Cabana et al. 2007). While 4-isopropenylphenol could be released from the degradation of the formed oligomers (Fukuda et al. 2004, Uchida et al. 2001), other authors suggested that this compound was formed in a separate biodegradation route although it could also participate in the parallel pathway of polymerization (Michizoe et al. 2005). It should be noted that previous research demonstrated that BPA products after laccase treatment lack estrogenic activity (Fukuda et al. 2004, Torres-Duarte et al. 2012) and BPA polymerization renders into precipitates, which is beneficial to reduce its environmental impact (Torres-Duarte et al. 2012). In addition, with the technology presented in the current research, the discharge of BPA polymerization products in the effluent of the EMR is not expected, since the nanofiltration membrane should remove this type of compounds.

5.3.3 Continuous transformation of BPA in a ceramic EMR

Once the most appropriate conditions to remove BPA were obtained from batch experiments, such conditions were applied in the continuous EMR. The proposed reactor consisted of a stirred tank reactor associated to the membrane filtration unit. In such configuration, the effluent of the reactor is flown in the tangential direction along the membrane and then, part of the flow containing the enzyme is recycled into the reaction vessel, according to the feed/flow ratio. This type of EMR, with the oxidation reaction and the separation device placed in series, presents high versatility since both compartments can be controlled independently. In a first experiment, the

capability of two nanofiltration membranes of pore sizes of 5 and 1 nm to retain the enzyme in the system was tested. The 1 nm pore size membrane was necessary to retain laccase, which has been reported to have an average molecular size of ~6.1 nm × 5.0 nm × 4.9 nm (Gascón et al. 2014). Therefore, the lowest pore size membrane was selected to conduct the subsequent experiments.

5.3.3.1 Effect of hydraulic retention time

Experiments were performed at a BPA concentration closer to environmental levels: 75 µg/L and different HRTs were evaluated: 2.5, 5, 7.5 and 10 h, corresponding to BPA addition rates of 30, 15, 10, 7.5 µg/(L·h), respectively. Before the addition of the biocatalyst in the reaction vessel, the feed was pumped through the system for several hours and samples from feed and permeate were collected. The analysis revealed that no BPA adsorption on the membrane occurred. For each experiment, the EMR was operated for at least five times the value of the HRT. The TMP increased with the permeate flow rate. For instance, for the HRT of 2.5 h, corresponding to a permeate flux of 15 L/(m^2·h), the TMP was around 1.6 bar; whereas for the HRT of 10 h, corresponding to a flux of 3.75 L/(m^2·h), the TMP did not exceed 0.1 bar. For each experiment, these values were kept constant throughout the operation. Table 5.3 shows the percentages of BPA transformation in steady state for each of the conditions studied (experiments 1 to 4).

Table 5.3. Bisphenol A removal (%) in the EMR operated in continuous mode.

Experiment	HRT (h)	Feed addition rate (µg/(L·h))	Matrix	*Removal (%)
1	2.5	30	Phosphate buffer pH 6	93.1±1.2
2	5	15	Phosphate buffer pH 6	96.2±1.0
3	7.5	10	Phosphate buffer pH 6	98.3±0.6
4	10	7.5	Phosphate buffer pH 6	99.5±0.5
5	5	15	**Phosphate buffer pH 6	96.2±0.5
6	10	7.5	Secondary effluent	94.6±2.0

* Mean ± standard deviation of n data in steady state (n≥7). Mean values are significantly different at $p<0.05$, except for HRT of 5 h without and with oxygenation

**Continuous oxygenation

As it can be observed, the removal percentage of BPA was above 93% for all the HRT evaluated. Nevertheless, the increase in the HRT, which implies a longer contact time between BPA and the enzyme, improved the elimination of the micropollutant (Figure 5.8) and the differences in the percentage of BPA removed for the different HRTs were significant (as determined by t-Student test; p <0.05). In fact, complete conversion of BPA was almost achieved at a HRT of 10 h while maintaining the enzymatic activity fairly constant.

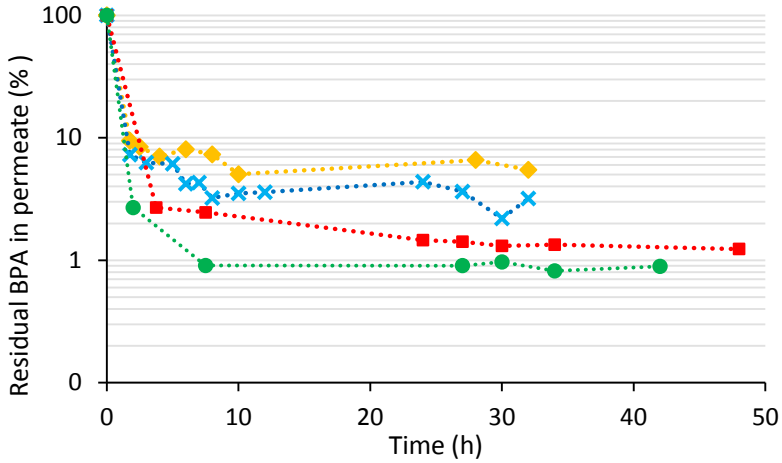

Figure 5.8. Residual BPA profiles in the effluent (membrane permeate) of the EMR operated at different HRT: 2.5 h (◆), 5 h (×), 7.5 h (■), 10 h (●).

In a recent study, Nguyen et al. (2014) evaluated the degradation of BPA in an EMR with a submerged hollow fiber membrane module of polyacrylonitrile by commercial *M. thermophila* laccase. Starting with feed addition rates of 23.7 and 15.8 µg/(L·h) corresponding to HRTs of 8 and 12 h, respectively, BPA removal percentages between 85% and 90% were observed in synthetic water. In the current study, the use of a high redox potential laccase and pH 6 likely contributed to higher removal yields (more than 93.1%), despite the lower concentration of BPA in the influent (2.5 fold lower).

It is well known that oxygen participates in the catalytic cycle of laccase, acting as the final electron acceptor and the increase in the dissolved oxygen concentration may improve the transformation of pollutants by laccase. In Experiments 1-4 (Table 5.3), the aeration was not considered because agitation was sufficient to maintain the level of dissolved oxygen close to saturation

values (6.5-7.5 mg/L). Nevertheless, Lloret et al. (2012a) evaluated the effect of aeration and oxygenation on the laccase-catalyzed transformation of estrogens in an EMR and observed that, whereas aeration had no effect on the transformation of estrogens, the supply of O_2 gas in pulses considerably enhanced the degradation percentage. For this reason, in an attempt to achieve complete removal of BPA in a shorter HRT (5 h), the continuous oxygenation of the reaction medium was assayed (experiment 5, Table 5.3). In such experiment, despite dissolved oxygen concentration was above 18 mg/L during the operation (data not shown), the transformation percentage of BPA did not increase.

5.3.3.2 Effect of the wastewater matrix

In view of a real application, the effect of a real wastewater matrix on the catalytic action of laccase was assessed. The secondary effluent from a wastewater treatment plant filtered through 0.45 μm was used. The aim of the filtration was to remove particulate solids and other microorganisms, which could contribute to the elimination of BPA by adsorption or undefined biotransformation, and hence, the evaluation of the sole enzymatic transformation by laccase could be possible (Lloret et al. 2013b). Figures 5.9A and 5.9B show the residual BPA concentration and the laccase activity profiles along the continuous operation of the ceramic EMR operated with buffer solution (pH 6) and secondary effluent (pH~7.2) matrices, respectively (Experiments 4 and 6 in Table 5.3).

In the experiment with buffer solution, laccase maintained an activity over 850 U/L after 50 h, when the reactor was stopped (Figure 5.9A), and almost complete transformation of BPA was observed during the operation of the reactor. In contrast, in the operation with real wastewater (Figure 5.9B), laccase activity remains over 800 U/L during the first 22 h, but after that period, a gradual drop in the activity was observed. It should be mentioned that laccase activity in the permeate was periodically checked and a low value (in the range of 4-8 U/L) was detected when the buffer matrix was evaluated. From a mass balance (taking into account the permeate flow rate, the initial laccase activity in the vessel and the working volume) the loss of laccase activity during 50 h of operation was estimated in 51 U (3.4% of the initial

activity). In the operation with real wastewater, no laccase in the membrane permeate was detected. In order to maintain the enzymatic activity in values around 1000 U/L, a pulse of laccase was added after 32 h. As a consequence of membrane fouling, the TMP increased along the operation from 0.1 to approximately 1 bar.

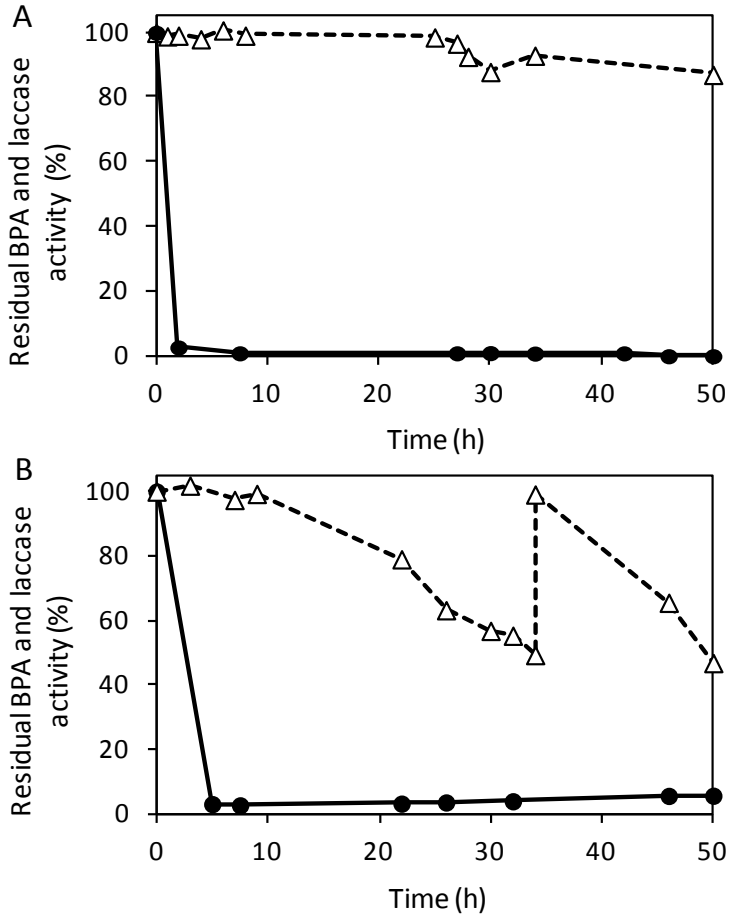

Figure 5.9. Residual BPA concentration (●) and residual activity profile (△) of laccase during the continuous treatment in the ceramic EMR operated at a HRT of 10 h. A) 100 mM phosphate buffer (pH 6) and B) real biologically treated wastewater, both spiked with 75 µg/L BPA.

These results are in agreement with previous research which evaluated the long term stability of free laccase from *Coriolopsis polyzona* in wastewater and reported a residual activity of 2.5% after 29 days (Zimmermann et al.

2011). In the present study, the negative impact of the wastewater matrix along the operation may be associated to the retention of colloidal organic matter by the nanofiltration membrane and its subsequent accumulation in the reactor vessel, which could promote the inactivation of the enzyme and reduction of the removal efficiency. For instance, humic acids, which are commonly present in effluents from biological treatment plants, have been reported as membrane foulants that may inactivate laccase (Keum and Li 2004). Membrane clogging and fouling due to the accumulation and adsorption of polymers, proteins or even biofilms on the membrane surface can partially solved by using a frequent backwash.

Secondly, the composition of the secondary effluent could be also playing an important role on the inactivation process. Indeed, it is well known that laccase is susceptible to the inhibitory action of small anions such as halides and metal cations or proteolytic attack (Cabana et al. 2007). The significant concentration of Cl^-, Ca^{2+} or Mg^{2+} detected in the wastewater may enhance laccase denaturation (Table 5.1).

Margot et al. (2013a) evaluated the inhibitory effect of NaCl on *T. versicolor* laccase and reported enzyme inhibition of 20% at a salt concentration of 5 mM, corresponding to 177 mg/L Cl^-. However, the chloride concentration in the current wastewater matrix was lower: 27.3 mg/L (Table 5.1). The presence of Ca^{2+} (5.5 mg/L) could have a higher impact on the enzyme stability. In this sense, Lloret et al. (2012b) reported a residual activity for free laccase from *M. thermophila* of 45% after 30 min of incubation in phosphate buffer (pH 7) containing 10 µM $CaCl_2$ (0.4 mg/L Ca^{2+}).

On the other hand, the presence of exocellular enzymes and specifically, proteolytic enzymes in secondary effluents from wastewater treatments plants has been previously reported (Westgate and Park 2010). Such proteins may progressively accumulate in the reaction vessel, although this hypothesis should be confirmed in further research.

Despite the laccase activity in the EMR operated with real secondary effluent was below the threshold value, the percentage of BPA transformed was close to 95%. Therefore, the amount of BPA discharged in the membrane

permeate was below the lowest non observed effect concentration (NOEC) reported for BPA, which ranged from 16-1280 µg/L (Staples et al. 2011).

5.4 CONCLUSIONS

The main parameters affecting the laccase-catalyzed transformation of BPA, i.e, redox potential of laccase, pH and laccase activity, were evaluated in batch experiments. Nearly complete removal of BPA was attained in the operation of a continuous EMR using a ceramic membrane, which allowed the recycling of laccase avoiding BPA adsorption. The identification of several BPA transformation products revealed that polymerization and biodegradation were the removal mechanisms. Enzymatic reactors coupled to ceramic membranes could be considered as an alternative technology to efficiently remove BPA and other organic micropollutants from highly contaminated wastewaters. Nevertheless, further research should focus on increasing laccase stability aiming at reducing the amount of enzyme periodically added to the reactor. Additionally, the effects of laccase treatments on wastewater toxicity under realistic reaction conditions should be assessed. Finally, prior to implementing this technology at pilot scale, the evaluation of the robustness of the EMR in a long-term operation is required. Nevertheless, this work may be considered as a basis for the development of a promising technology to allow the extensive removal of persistent and emerging EDCs in water effluents.

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Chapter 6

ASSESSING THE USE OF NANOIMMOBILIZED LACCASES TO REMOVE MICROPOLLUTANTS FROM WASTEWATER[1]

Summary

Enzymes immobilization is a useful way to allow enzyme reuse and increase their stability. A high redox potential laccase from *Trametes versicolor* (*TvL*) and a low redox potential, but commercially available low-cost laccase from *Myceliophthora thermophila* (*MtL*), were successfully immobilized and co-immobilized onto fumed silica nanoparticles (fsNP). Enzyme loads of 1.78±0.07, 0.69±0.03 and 1.10±0.01 U/mg fsNP were attained for the optimal doses of *TvL*, *MtL* and co-immobilized laccases, respectively. In general, the laccase-fsNP conjugates showed higher resistance against acidic pH and higher storage stability, especially when incubated in the secondary effluent from a municipal wastewater treatment plant (WWTP). The ability of the laccase-fsNP to remove a mixture of 14C-bisphenol A (BPA) and 14C-sodium diclofenac (DCF) from spiked secondary effluent was assessed in batch experiments. The catalytic efficiency was highly dependent on both the biocatalyst source and state. The high redox potential *TvL* as free enzyme attained a percentage of BPA transformation 4-fold higher than free *MtL*. Compared to free laccases, immobilized enzymes led to much slower rates of BPA transformation. For instance, after 24 h, the percentages of BPA transformation by 1000 U/L of a mixture of free and co-immobilized enzymes were 67.8±5.2 and 27.0±3.9%, respectively. Nevertheless, the use of 8000 U/L of co-immobilized laccase led to nearly complete removal of BPA, despite the unfavorable conditions for laccase catalysis (pH~ 8.4). DCF transformation was not observed for any of the enzymatic systems showing that this compound is highly recalcitrant towards laccase oxidation under realistic conditions.

[1] **A. Arca-Ramos, E.M. Ammann, C.A. Gasser, P. Nastold, G. Eibes, G. Feijoo, J.M. Lema, M.T. Moreira, P.F.-X. Corvini.** Assessing the use of nanoimmobilized laccases to remove micropollutants from wastewater *(submitted).* Part of this work was performed in the Institute for Ecopreneurship of the University of Applied Sciences and Arts Northwestern Switzerland (FHNNW), in Basel, Switzerland.

OUTLINE

6.1 INTRODUCTION

The occurrence of emerging pollutants such as pharmaceuticals and other hormonally active chemicals in the aquatic environment has become an increasing concern due to their potential adverse effects on human health and aquatic ecosystems (Auriol et al. 2007, Deblonde et al. 2011). Wastewater treatment plant (WWTP) effluents are a major source of micropollutants released to the environment (Luo et al. 2014). Numerous WWTP effluents were reported to contain several of these pollutants at potentially harmful concentrations, i.e., above predicted no-effect concentrations (PNEC) (Gasser et al. 2014a). Therefore, improved removal of these chemicals is of increasing interest and will likely require a tertiary wastewater treatment (Gasser et al. 2014b). In this context, many technologies have been explored, e.g., oxidation, filtration, adsorption and biodegradation (Corvini and Shahgaldian 2010). Enzyme catalyzed transformation processes have attracted interest in recent years (Cabana et al. 2007, Demarche et al. 2012). More specifically, laccase has received special attention due to their oxidative versatility and low catalytic requirements: they use oxygen and release water as the sole by-product (Camarero et al. 2012).

Despite the proven potential of laccase to remove a great variety of pollutants, the large-scale use of free enzyme in continuous systems such as WWTPs is limited by several factors. The retention and reuse of the biocatalyst in the system is a crucial factor to ensure the technical and economic feasibility of the enzymatic treatment. A possible strategy to retain the free laccase, investigated in Chapter 5, consists on the use of a membrane coupled to the reactor, which permits the retention of the enzyme in the reaction vessel and the release of permeable substrates and products with the effluent. Nevertheless, aiming to ensure the total retention of the biocatalyst, a small pore size ultrafiltration or even nanofiltration membrane is required, and it is well known that energy costs rise with decreasing membrane pore size (Schäfer et al. 2001). Besides the cost, fouling problems and the accumulation in the reactor of products, which can negatively affect both laccase catalytic action and stability, must be considered. In order to mitigate these drawbacks, immobilization and insolubilization techniques for laccases have been lately

investigated to improve enzymatic stability in wastewater and facilitate the reuse of the biocatalyst (Ba et al. 2013, Gasser et al. 2014a).

The main immobilization methods could be classified as follows: i) immobilization on supports by physical, ionic or covalent binding, mainly through the interaction with the amino groups of the protein; ii) entrapment of the enzyme by its physical retention in a porous solid matrix such as polyacrylamide, collagen or alginate; iii) encapsulation in a polymeric network synthesized in the presence of the enzyme, resulting in its physical retention; and iv) self-immobilization by the use of bifunctional cross-linkers (Brady and Jordaan 2009, Fernández-Fernández et al. 2013). Figure 6.1 illustrates some of these methods schematically.

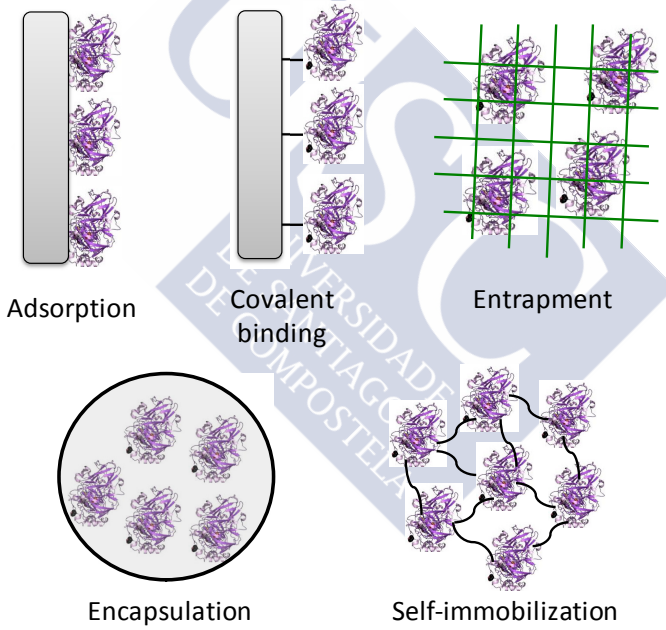

Figure 6.1. Scheme of the immobilization of enzymes by different methods (adapted from Fernández-Fernández et al. (2013)).

In general, the choice of a suitable method will depend on the enzyme and its purpose. An important requirement for immobilizing enzymes is that the matrix must provide an inert and biocompatible environment (Cipolatti et al. 2014).

Over the last years, nanoparticles have received increasing attention because of their ideal characteristics for enzyme immobilization. Firstly, they provide a high specific surface area for enzyme attachment, which allows high enzyme loading capacity. In addition, mass transfer limitations are generally lower than in conventional macroscale matrixes (Cipolatti et al. 2014, Hou et al. 2014). Although nanosupports are usually associated to high cost, this parameter mainly depends on the material used. In this sense, fumed silica nanoparticles (fsNP) are an inexpensive nanostructured material (Cruz et al. 2011). These fsNP have been successfully used as support for the immobilization of single or combined laccases via sorption-assisted immobilization (Ammann et al. 2014, Hommes et al. 2012). However, in these previous studies, the characterization of the laccase-fsNP conjugates (i.e., pH and thermal stability, reusability) was not conducted despite being of major interest for practical application of the biocatalyst.

In the current Chapter, two laccases with different redox potential, namely, laccase from *Trametes versicolor* (*TvL*) and *Myceliophthora thermophila* (*MtL*), were individually immobilized and co-immobilized onto fsNP. *MtL* is a low redox potential (0.47 V) but commercially available low-cost laccase. Its combination with *TvL*, a high redox potential laccase (0.78 V), was of interest regarding the development of a cost-effective technology. The free and immobilized laccases were characterized in terms of optimal pH and temperature regarding enzymatic activity. Additionally, other characteristics were also determined such as storage, thermal and pH stability, as well as reusability. Finally, the capability of both free and immobilized laccases to remove two model micropollutants: the plasticizer ^{14}C-bisphenol A (BPA) and the pharmaceutical ^{14}C-sodium diclofenac (DCF) from biologically treated wastewater at high pH, was assessed.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and enzyme

FsNP (surface area: 390±40 m^2/g; aggregates of particles with a size of 7 nm) from Sigma–Aldrich (Switzerland) were used as immobilization support material. 3-Aminopropyltriethoxysilane (APTES), glutaraldehyde (50%),

2,2'azinobis-(3-ethylbenzothiazoline-6-sulfonate)2,2' (ABTS) and commercial laccase from *Tv* (≥ 10 U/mg) were purchased from Sigma-Aldrich (Switzerland). Commercial laccase from *Mt* was kindly supplied by Novozymes (Denmark) and produced by submerged fermentation of genetically modified *Aspergillus* sp. with molecular weight of 56,000 Da.

Radioactive BPA [phenol ring U-^{14}C] was purchased from Hartmann Analytic (Germany) with a specific radioactivity of 3.92x10^{9} Bq/mmol and a concentration of 218.58 μg/mL in ethanol. Radioactive DCF [phenyl acetic acid ring- U-^{14}C] was supplied by Perkin Elmer (USA) with a specific radioactivity of 2.32x10^{9} Bq/mmol as a crystalline solid and subsequently dissolved in ethanol with a concentration of 260 μg/mL.

6.2.2 Enzyme assay

Activity of free and immobilized laccases was determined by a colorimetric assay using ABTS as substrate as described by Zimmermann et al. (2011). Briefly, an aliquot of 50 μL laccase-containing sample previously diluted in citrate-phosphate buffer at pH 3 (i.e. soluble laccase or suspended conjugates) was added to 150 μL of 0.267 mM ABTS solution (citrate-phosphate buffer at pH 3) in a 96-well-plate. The absorbance at 420 nm (ε_{420} = 30,800 $M^{-1}cm^{-1}$) over 420 s at 6 s intervals was measured using a plate-reader (Synergy™ 2 Multi-Mode Microplate Reader, BioTek Instruments, Inc., Switzerland) and the Gen5 1.08 Data Analysis Software (BioTek Instruments, Inc., Switzerland). One unit (U) of activity was defined as the amount of enzyme catalysing the production of 1 μmol of $ABTS^{\bullet+}$ per min.

6.2.3 Immobilization procedure

Surface modification of fsNP were performed as previously reported (Hommes et al. 2012). According to such protocol, 800 mg of fsNP were suspended in 40 mL of phosphate buffer pH 7. Subsequently, 150 μL of APTES were added (0.8 mmol APTES/ g fsNP), and the solution was incubated under agitation (100 rpm) for at least 12 h at room temperature. The unreacted APTES was washed away by four centrifugation/re-suspension steps at 4,500×g for 3 min. The APTES concentration in the supernatants was monitored as follows: 50 μL of a 5.3 mM glutaraldehyde solution was added to 150 μL

supernatant. The yellow coloration due to the imine bond resulting from the chemical reaction of APTES with glutaraldehyde was measured spectrophotometrically at 390 nm. After four washing steps, no APTES was detected. The aminofunctionalized nanoparticles were kept in the fridge until its use.

The subsequent laccase immobilization was carried out following the sorption-assisted immobilization (SAI) method (Ammann et al. 2014) (Figure 6.2).

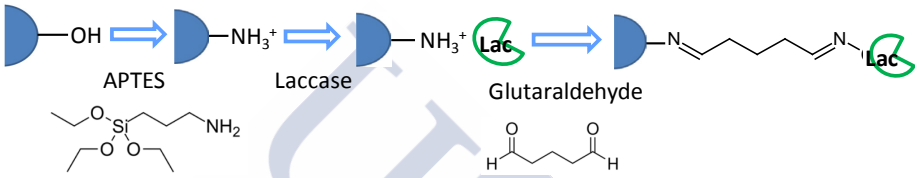

Figure 6.2. Scheme of the functionalization with APTES and immobilization of laccase with glutaraldehyde.

According to SAI method, a solution of laccase in phosphate buffer pH 7 was directly added to the amino-functionalized fsNP and the mixture was incubated under agitation (100 rpm) for 2 h at 4ºC. Next, glutaraldehyde (1 mmol glutaraldehyde/g fsNP) was added dropwise to the mixture of fsNP and laccase, and the solution was incubated under agitation (100 rpm) and 4ºC for at least 18 h. The unreacted glutaraldehyde and the excess and not stable bound enzymes were washed away by five centrifugation/re-suspension steps at 4,500×g for 10 min. The supernatants containing free laccase and the fsNP–laccase conjugates suspensions (pellets re-suspended in the initial volume of phosphate buffer pH 7) were collected after every washing step for further analysis.

The amount of protein applied was determined using bicinchoninic acid (BCA) protein assay kits (Pierce®, Thermo Scientific, Germany), according to the instruction manual. The enzymatic activity of both fsNP-laccase conjugates suspensions and supernatants was measured to determine the immobilization yield, washing loss and enzyme load. The immobilization yield was defined as the ratio between the measured activity of the immobilized laccase (bound to

the support after five washing steps) and the initial laccase activity in solution before immobilization (equation 6.1):

$$IY(\%) = \frac{A_f}{A_i} \cdot 100 \qquad (6.1)$$

Enzyme load was defined as the apparent laccase activity in U per mg fsNP of the produced nanobiocatalyst.

6.2.4 Characterization of free and immobilized laccase

6.2.4.1 Optimum pH and temperature

Laccase activity (free and immobilized) at different pHs was investigated by adding 50 µL of sample containing laccase, previously diluted in the buffer at the corresponding pH, to 150 µL of 0.267 mM ABTS in 100 mM citrate-phosphate buffer (for the range pH 2.4-5) or sodium phosphate buffer (for the range pH 6-8). The relative activity was calculated as the ratio between the activity at each pH and the maximum attained. The effect of temperature (22-60ºC) on laccase activity was tested by determining the activity at the corresponding temperature under standard conditions. The relative activity was calculated as the ratio between the activity at each temperature and the maximal activity obtained for optimal temperature.

6.2.4.2 pH and thermal stability

The effect of pH on the stability of the immobilized and free laccases was studied by incubating 1 mL-aliquots of 1000 U/L of the biocatalyst in 100 mM citrate–phosphate buffer (pH 3, 7 and 8.5) at room temperature (25±2ºC). Thermal stability of the enzymes was evaluated by incubating 1000 U/L of the biocatalyst in 100 mM citrate–phosphate buffer (pH 7) at selected temperatures: 10, 25 and 60ºC, respectively. Samples were taken at specific time intervals and transferred to standard conditions to measure the residual enzymatic activity. At the end of the experiment, remaining samples were centrifuged to separate the supernatant from the pellets of nanoparticles. The activity associated to the supernatant was also measured to check for free laccase.

Statistical analyses were performed to evaluate whether activity of the free and immobilized laccases were significantly different for the conditions assayed. The paired t-test function of Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) was used to determine *p*-values and assess statistical significance (significance level $p < 0.05$).

6.2.4.3 Reusability of immobilized laccases

The reusability of the immobilized laccases was assayed by means of consecutive cycles of ABTS (2 g/L) oxidation in 0.1 M phosphate buffer at pH 7. At the end of each 4-min oxidation cycle, the immobilized enzyme was removed by centrifugation and the absorbance of the supernatant was measured (420 nm). The immobilized laccase was collected and washed twice with phosphate buffer pH 7. Thereafter, the procedure was repeated with a fresh aliquot of substrate. The activity of the immobilized enzyme was considered to be 100% in the initial cycle. These experiments were performed in duplicate.

6.2.4.4 Storage stability

The storage stability was assessed by incubating free and immobilized enzymes in a phosphate buffer at pH 7 and 4ºC for five weeks with periodical withdrawal for the measurement of residual activity.

6.2.4.5 Long-term stability in wastewater

Stability of free and immobilized laccases in real wastewater was evaluated by incubating 1000 U/L of biocatalyst in 30 mL of secondary effluent, obtained after an activated sludge treatment (sequencing batch reactor process), from the WWTP located in Birsfelden (Switzerland). Incubation was performed at pH~8.4 after adding the biocatalyst, magnetic stirring (210 rpm) and room temperature (25±2ºC). The composition of the secondary WWTP effluent contained: 0.5±0.2 of ammonium nitrogen, 6.2±2.2 total nitrogen, 0.7±0.2 total phosphorus, 7.8±1.8 dissolved organic carbon, and 6.9±4.7 mg/L of total suspended solids. Chemical oxygen demand was 30.1±2.9 mg O_2/L whereas pH was 8.7±0.7. Samples were taken at five different times over two weeks and the residual enzymatic activity was measured.

6.2.5 Enzymatic transformation of BPA and DCF

The enzymatic transformation of ^{14}C-BPA (78 µg/L) and ^{14}C-DCF (93 µg/L) present in biologically treated wastewater was conducted at room temperature (i.e. 25±2ºC) with magnetic stirring (210 rpm) for 24 h in 100 mL Erlenmeyer flasks. The final volume of the reaction mixture was 28 mL. The secondary effluent from the WWTP was filtered by 2.7 µm pore size borosilicate glass filters, and then spiked with 100 µL of a solution (original solution diluted 1:10 in methanol) of ^{14}C-BPA and 100 µL of a solution (original solution diluted 1:10 in methanol) of ^{14}C-DCF. Free and immobilized *MtL* and *TvL*, or the combination of both, were used as biocatalysts. The initial laccase activity was adjusted to 1000, 4000 or 8000 U/L. Control experiments without enzyme were run in parallel.

To measure the radioactivity, 50 µL samples of the reaction medium were taken at different times, suspended in 5 mL of liquid scintillation cocktail (Irgasafe Plus; PerkinElmer, Switzerland) and analyzed in a liquid scintillation counter (LSC) (Tri-Carb 2800TR; PerkinElmer, Switzerland).

In order to determine the residual concentration of ^{14}C-BPA and ^{14}C-DCF, samples of 2 mL were periodically taken in duplicate (0, 0.5, 1, 2, 4, and 24 h) and analyzed according to the following procedure. Solid phase extraction (SPE) cartridges OASIS HLB 6 cc (for BPA determination) and OASIS MCX 3cc (for DCF determination after acidification with sulfuric acid), previously conditioned with heptane-acetone-methanol and water (at pH 7.5 for HLB cartridges and pH 2.8 for MCX cartridges) were used for the initial processing of 2 mL-samples. The cartridges were dried by a nitrogen stream. Subsequently, the substances retained in the OASIS HLB cartridges were eluted four times with 2 mL methanol and the solvent was evaporated by heating at 64ºC and under a moderate nitrogen stream. On the other hand, the substances retained in the OASIS MCX cartridges were eluted two times with 2 mL of acetone and then the solvent volume reduced to approximately 500 µL by heating at 56ºC under a moderate nitrogen stream. Subsequently, 1 mL of methanol was added and the evaporation process went on until the complete depletion of the solvent. Residues were carefully re-dissolved in 100 µL acetonitrile and transferred into HPLC vials for further analysis. Liquid

chromatography analysis was conducted using an HPLC 1200 Series (Agilent Technologies, Switzerland) coupled to a radioisotope detector "Ramona Star" (Raytest, Germany). A Nucleodur C18 Pyramid column (150x4 mm, 3 µm particle size, Macherey and Nagel) was thermostated at 40ºC and the flow rate was set to 0.8 mL/min. Aliquots of 50 µL recovered from the SPE were injected in the HPLC and eluted with a water:acetonitrile gradient starting with a water fraction of 55%, which was decreased to 2% in 17 min. Elution solvents were acidified with formic acid to a final concentration of 0.1%.

6.2.6 BPA mineralization assay

BPA mineralization experiments were conducted in glass tubes containing tris buffer pH 7, 1 µL of the original stock of BPA (222.10^6 DPM/mL), which corresponded to 87.5 µg BPA/L, and 1000 U/L of free *TvL* in a total volume of 2.5 mL. Two-milliliter centrifugation tubes filled with 1 mL of 1 M NaOH were fixed by copper wire in the headspace of the flasks to trap formed $^{14}CO_2$. The tubes were sealed with an air-tight rubber plug and incubated at room temperature (25±2ºC) on a rotary shaker at 130 rpm for 16 h. A parallel experiment lacking laccase was used as control. Samples from the reaction medium and NaOH were diluted in the liquid scintillation cocktail and analyzed in the LSC. The radioactivity recovered in the NaOH traps was used to estimate the amount of BPA mineralized.

6.3 RESULTS AND DISCUSSION

6.3.1 Immobilization of laccases

Different amounts of laccases from the basidiomycete *Tv* and from the ascomycete *Mt* were immobilized onto fsNP. The optimal concentrations of APTES (0.8 mmol/g fsNP) and glutaraldehyde (1 mmol/g fsNP) have previously been established (Hommes et al. 2012). Although the use of a high redox potential laccase as *TvL* often results in a high rate of substrate oxidation (Hahn et al. 2014, Madhavi and Lele 2009), the use of a low redox potential laccase from *Mt* was also considered, as this enzyme can be heterologously expressed in *Aspergillus oryzae,* which makes it a commercially-available low-cost laccase (Babot et al. 2011). Co-immobilization of both laccases was also studied in order to obtain a biocatalyst with intermediate cost, combining

features from both enzymes. Table 6.1 shows the immobilization yield and the enzyme load for the different amounts of laccase considered. For both enzymes, the enzyme load increased with enzyme dosage, while the immobilization yield declined. The choice of the optimal protein amount for both laccases was done based on a balance between a high enzyme load and high immobilization yield.

Table 6.1. Comparison of different laccase-fsNP conjugates produced with different starting amount of laccase (*n*=3).

Lac	Applied laccase (mg protein/mg fsNP)	Applied laccase (U/mg fsNP)	Washing loss (% of activity)	Washing loss (% of protein)	Immobilization yield (%)	Enzyme load (U/mg fsNP)
Tv	0.025	2. 8	23.9±1.0	24.1±0.4	88.1±1.6	1.79±0.04
Tv	0.018	2.0	5.6±1.3	6.7±1.5	106.0±6.1	1.78±0.07
Tv	0.009	1.0	2.12±0.3	3.5±1.4	105.0±0.5	0.87±0.01
Mt	0.027	1.0	21.9±12.0	23.1±1.2	78.0±12.0	0.73±0.06
Mt	0.021	0.8	10.0±1.4	12.9±1.6	84.5±5.4	0.69±0.03
Mt	0.011	0.4	4.6±0.1	5.2±0.4	100.6±1.9	0.38±0.01
Mt&Tv	0.011 *MtL* 0.009 *TvL*	0.4 *MtL* 1.0 *TvL*	6.5±0.3	7.5±0.5	89.7±0.2	1.10±0.01

Washing losses were determined in two different ways, i.e., laccase activity discarded during washing relative to the laccase activity before washing (100 %) and as protein loss in washing solution divided by the protein initially applied (100 %).

For *TvL*, the enzyme load of 1.79 U/mg fsNP appeared to be close to the saturation value for the carrier. Based on the immobilization yield, the dose of applied laccase of 2.0 U/mg fsNP (corresponding to 0.018 mg protein applied/mg fsNP) was selected to further produce a larger amount of biocatalyst for the subsequent experiments. It should be mentioned that for *TvL*, the enzymatic activity after the reaction with glutaraldehyde (before the washing procedure) was higher than the initial applied laccase activity, and this was the reason of attaining immobilization yields above 100%, despite protein loss during the washing steps (Table 6.1).

The increased enzymatic activity of certain immobilized laccases toward standard substrates such as ABTS or dimethoxyphenol has been observed in previous research (Ammann et al. 2014). Cabana et al. (2011) reported the hyperactivation of laccase from *T. versicolor* after the formation of covalent bonds with chitosan; whereas Matijošytė et al. (2010) described a similar behavior for laccase from *Trametes villosa* (activity recovery up to 148%) after the formation of cross linking enzyme aggregates (CLEAs®). Such an increase of the laccase activity may be a consequence of a change in conformation of the soluble laccase during the cross linking reaction, which makes it more active towards certain substrates (Cabana et al. 2011). Another explanation is a higher concentration of substrate at the surface of the support material, i.e. creation of a concentration gradient.

In the case of *MtL*, the specific activity, defined as the activity of laccase in units per mg of protein, was lower than that of the *TvL.* Therefore, despite applying a higher protein load per mg of carrier (Table 6.1), the enzyme load in terms of units was considerably lower and reached the saturation at values close to 0.73 U/mg fsNP. The applied laccase load of 0.80 U/mg fsNP (corresponding to 0.021 mg protein applied/mg fsNP), which led to an immobilization yield of 84.5±5.4%, was selected to produce a large amount of immobilized *MtL*.

Finally, the co-immobilization of both laccases was possible. This was demonstrated by comparing the enzyme load of the biocatalyst containing both enzymes (1.10±0.01 U/mg fsNP) with the activities of the biocatalysts resulting from using the same amounts of individual laccases: 0.38±0.01 and 0.87±0.01 U/mg fsNP for *MtL* and *TvL* (Table 6.1), respectively. The enzyme loads obtained in the current study are in the range reported for other laccases using fsNP as support (Ammann et al. 2014, Hommes et al. 2012), while much lower load of *TvL* (0.14 U/mg, measured using ABTS in pH 3 phosphate-citrate buffer solution as well) was attained using TiO_2 nanoparticles as support for the covalent immobilization of the enzyme (Hou et al. 2014). The considerable lower specific surface area of the TiO_2 nanoparticles (60.1±0.3 m^2/g) in comparison to the fsNP (390±40 m^2/g) is probably the reason of the much higher enzyme loads observed in the current study. The importance of the specific surface area of the support on the enzyme load has been reported in

several works. For instance, Cabana et al. (2009) has attributed the low enzyme load of laccase from *Coriolopsis polyzona* immobilized onto Celite R-633 (0.0008 U/mg, determined using ABTS in pH 3 as substrate) to the low specific surface area of such support (1.3 m^2/g).

6.3.2 Characterization of immobilized laccase

6.3.2.1 Optimum pH and temperature

The dependence of pH on activity was tested for free and immobilized laccase in a pH range from 2.4 to 8. As can be observed in Figure 6.3A, the immobilization process did not change the optimal pH value, which was pH 3 in all six tested combinations.

This observation is in accordance with previous studies (Lloret et al. 2013, Lloret et al. 2012), reporting optimal pH of 3 for free *MtL* and *TvL* using ABTS as substrate and no change in optimal pH for *MtL* after immobilization onto Eupergit supports. In the current study, for each laccase the differences in the relative activity, for the free and immobilized form were not significant (the *p*-values calculated by statistical analysis using Student t-test were above the significance level of 0.05). These observations are in contrast with a study of Patel et al. (2014), which found a considerable increase in the relative activity for the *TvL* immobilized onto SiO_2 nanocarriers in comparison to the free enzyme. At neutral pH, *MtL* exhibited higher relative activities compared to *TvL* and the biocatalyst containing both enzymes: 14.3±0.3% and 10.6±0.1% for the free and immobilized *MtL*; whereas for *TvL* the relative activity was below 1.0% for both immobilized and free laccase. The co-immobilized laccases showed an intermediate value of 3.5±0.2%.

Figure 6.3B reveals that the optimal catalytic temperature for all the biocatalysts was in the range 40-60ºC. The differences between 40 and 60ºC were not statistically significant for any of the biocatalysts (*p*-value above 0.05). In addition, for each enzyme, there were no significant differences between the free and the immobilized form.

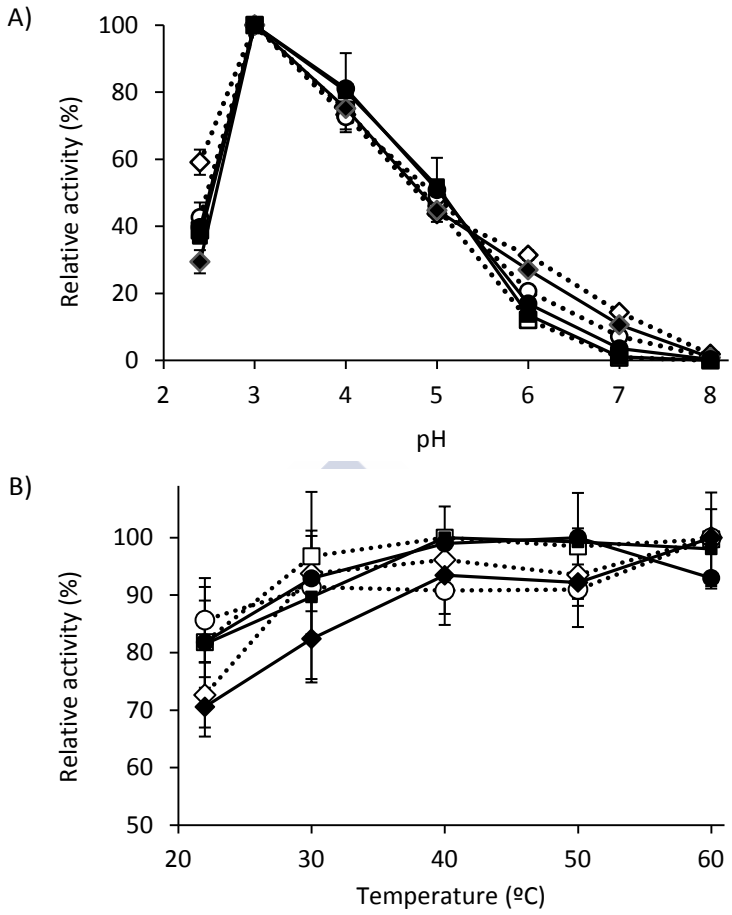

Figure 6.3. Effect of pH (A) and temperature (B) on the activity of free laccases from *Mt* (◇), *Tv* (□) and mixture (○) and on immobilized laccases from *Mt* (◆), *Tv* (■) and co-immobilized (●). Error bars represent the standard deviation of replicate experiments.

6.3.2.2 Stability against pH and temperature

One of the most important factors affecting the enzymatic activity is pH (Margot et al. 2013b). The effect of the pH on the enzyme stability was investigated by incubating laccase formulations at pH 3, 7, and 8.5 (close to the pH of the WWTP secondary effluents used in this study). The stability of the enzyme against pH was different depending on the type of laccase (Figure 6.4).

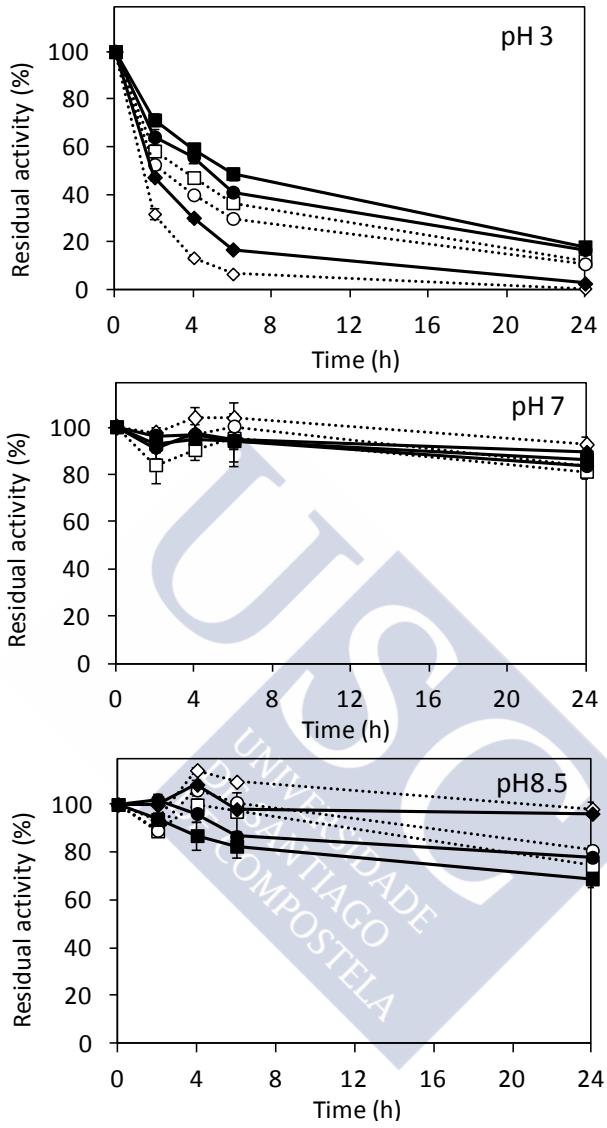

Figure 6.4. Residual activity of free laccases from Mt (◇), Tv (□) and mixture (○), immobilized laccases from Mt (◆), Tv (■) and co-immobilized (●) laccases incubated at room temperature (25º±2 °C). Error bars represent the standard deviation of replicate experiments.

At pH 3 pronounced deactivation occurred for all the biocatalysts. This circumstance was probably a consequence of the ionizable side-chains of the tertiary structure of the protein (Lloret et al. 2013). At acidic pH, laccase from *Tv* exhibited higher resistance than laccase from *Mt*. For instance, after 6 h,

the residual activity of immobilized *TvL* was 48.8±2.5%, whereas for immobilized *MtL* resulted to be 16.8±1.0%. In both cases, the activity of the immobilized enzymes was significantly higher than the free form: 36.4±0.7% and 6.5±0.1% for free *TvL* and *MtL*, respectively) (*p*-values below the significance level of 0.05). Once again, the co-immobilized laccases showed intermediate behavior between *TvL*-fsNP and *MtL*-fsNP.

At pH 7 all the biocatalysts showed similar profiles, maintaining a residual activity above 81% after 24h. At basic pH, *MtL* exhibited higher stability than *TvL*. In this case, although the free enzymes seemed to be more stable, the t-test revealed no significant differences between free and immobilized enzymes (*p*-values above the significance level of 0.05 at 24 h) (Figure 6.4). Although enzyme stability is one of the properties that have been generally considered to be improved via immobilization, this is not always true (Singh et al. 2013). For instance, Rekuć et al. (2009) observed that laccase from *Cerrena unicolor* covalently immobilized onto mesostructured siliceous cellular foams exhibited a lower pH stability that the free laccase.

In order to evaluate the thermal stability of the biocatalysts: free and immobilized laccases were incubated at 10, 25 and 60ºC. At the lowest temperature, all the enzymes maintained the initial activity for 24 h (Figure 6.5); whereas at 25ºC a slight enzymatic decay was observed, although the residual activity was above 81% after 24 h for the six series of experiments (Figure 6.4, pH 7).

At 60ºC, laccase from *Mt* showed higher stability than *TvL* (Figure 6.5). This was an expected behavior since *MtL* was derived from a thermophilic fungi (Xu et al. 1996). In addition, it was observed that for each laccase, the immobilized form exhibited higher thermal stability during the first 6 h of incubation (*p*-values below the significant level of 0.05). This might be a consequence of the reduction of conformational flexibility in immobilized enzyme due to multipoint attachment (Mateo et al. 2007). Nevertheless, after 24 h, all six biocatalysts were almost completely deactivated.

The improvement on the stability during the first hours was in the range of that reported in previous research. For instance, Kunamneni et al. (2008) used epoxy carriers to immobilize laccase from *Mt* and observed that the

stability at 60ºC was increased by 15% in the immobilized enzyme relative to the free one. Lloret et al. (2012) reported similar improvement for *MtL* immobilized in Eupergit® supports. As expected, the co-immobilized laccases showed an intermediate behavior between laccase from *Mt* and *Tv*, but closer to the high redox potential one (Figure 6.5).

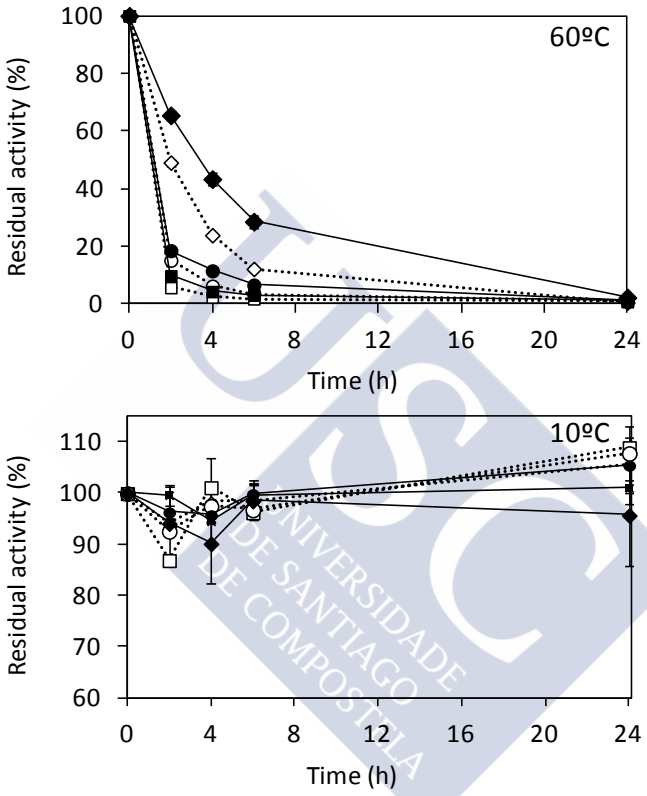

Figure 6.5. Residual activity of free laccases from *Mt* (◇), *Tv* (□) and mixture (○) and immobilized laccases from *Mt* (◆), *Tv* (■) and co-immobilized (●) incubated in phosphate buffer pH 7. Error bars represent the standard deviation of replicate experiments.

6.3.2.3 Reusability of the laccase-fsNP conjugates

An important parameter to be considered in view of a large-scale application of the laccase-fsNP conjugates is the reusability. This was examined by applying consecutive cycles of ABTS oxidation. As can be seen in Figure 6.6, immobilized laccase from *Tv* and the co-immobilized enzymes showed residual

activities of 58.2±5.2% and 62.8±9.9% after 10 cycles. In addition, after seven cycles, the activity of the co-immobilized laccase remained constant.

Laccase from *Mt* maintained a lower residual activity of 41.5±16.2% after the same number of cycles. Reduction in the catalytic efficiency can be a consequence of partial enzyme inactivation and release into the supernatants along the consecutives cycles of substrate oxidation. Other studies in the literature reported residual activities of 65% (Rekuć et al. 2009) and 70% (Tavares et al. 2013) after 10 cycles of ABTS oxidation for different laccases immobilized onto silica carriers.

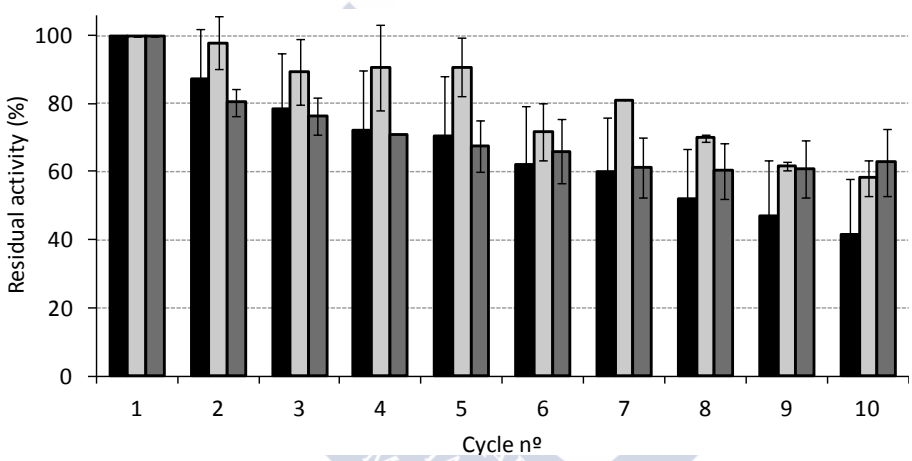

Figure 6.6. Residual activity (%) of immobilized laccases from *Mt* (■), *Tv* (■) and co-immobilized (■) on fsNP in subsequent cycles of ABTS oxidation. Error bars represent the standard deviation of replicate experiments.

6.3.2.4 Storage stability

The retention of enzyme activity for free and immobilized laccases during five-week storage was investigated. Storage stability between free and immobilized laccases did not differ considerably for single enzymes. Activity of free and immobilized laccase from *Mt* decreased to 77.1±0.6% and 83.7±0.6%, respectively, indicating a slight improvement in stability through immobilization (Figure 6.7).

In comparison, *TvL* was more stable. Residual activities of free and immobilized *TvL* were similar and above 93%. In case of co-immobilized

laccases, the storage stability was considerably enhanced by immobilization. Namely, the residual activities were 81.4±0.3% and 101.6±0.7% for the free and co-immobilized enzymes, respectively.

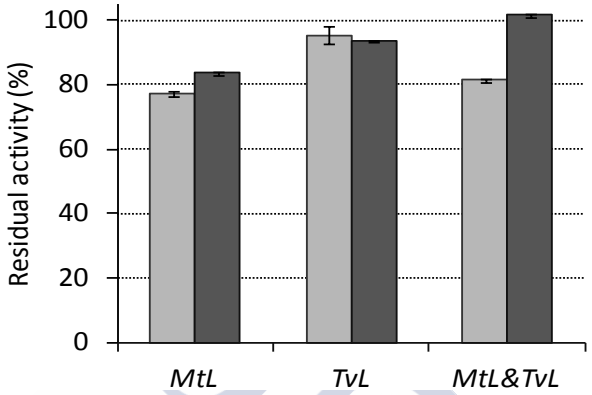

Figure 6.7. Residual activity (%) of free (■) and immobilized laccases (■) on fsNP after 5 weeks storage in sodium phosphate buffer pH 7 and 4ºC. Error bars represent the standard deviation of replicate experiments.

6.3.2.5 Long-term stability in wastewater

The stability of the biocatalyst is an essential feature, especially regarding its application in wastewater treatment. For this reason, the stability of the laccase-fsNP conjugates incubated in the secondary effluent from a municipal wastewater treatment plant was evaluated over two weeks and compared with that of the free laccases. Figure 6.8 shows the activity profiles for the six enzymatic systems. It can be observed that the residual activity for the immobilized laccases was considerably higher than for the free ones.

For instance, after 14 days, 40.2±2.5%, and 16.8±1.0% of the initial activity remained for the co-immobilized laccases and the mixture of free laccases, respectively. The difference in stability between immobilized and free laccases was even more pronounced for the single laccases. This is in accordance with previous results (Hommes et al. 2012) where the residual activity of laccase from genus *Thielavia* immobilized onto fsNP in biologically treated wastewater was 81.1% after 7 days, which was drastically higher than for the free laccase (1.5% after 2 days). A further study reported a residual

activity above 75% for *C. polyzona* laccase immobilized on fsNP after 15 days of incubation in aerobically treated effluent from a municipal WWTP (Zimmermann et al. 2011).

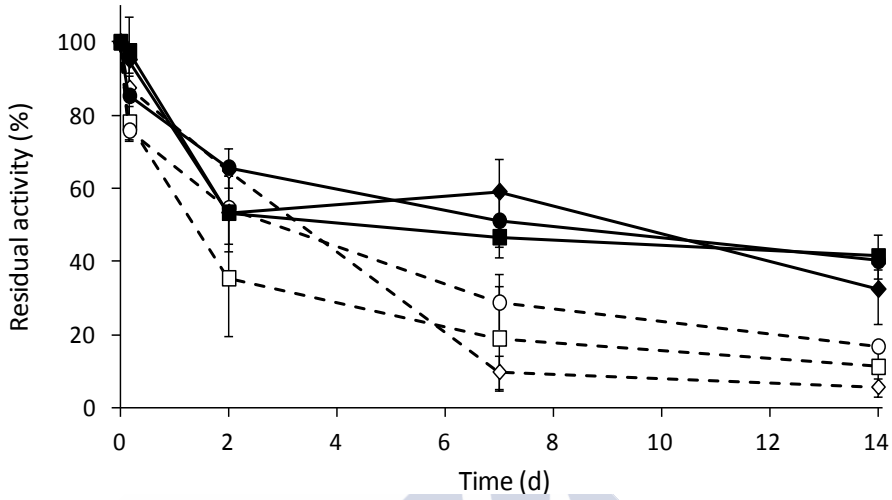

Figure 6.8. Residual activity (%) of free *MtL* (◇), *TvL* (□) and mixture (○) and immobilized *MtL* (◆), *TvL* (■) and co-immobilized laccases (●) along the stability assay in biologically treated wastewater (pH~8.4 after adding the biocatalyst). Error bars represent the standard deviation of replicate experiments.

6.3.3 Biotransformation of ^{14}C-BPA and ^{14}C-DCF by free and immobilized laccases

The use of free or immobilized laccase to remove micropollutants from aqueous systems has been reported in several works (Gasser et al. 2014a). However, in most of these studies the enzymatic degradation was conducted in buffer solutions, under favorable conditions of pH not encountered in WWTPs and high concentrations of target pollutants. In the present study, the ability of the immobilized enzymes to remove model micropollutants was evaluated in a real wastewater matrix, under unfavorable but realistic conditions of pH. **Table 6.2** shows the percentage of removal and transformation rate of BPA after 2 h of treatment by laccase.

The catalytic efficiency was highly dependent on both the biocatalyst source and state. The high redox potential *TvL* in free form attained the highest percentage of transformation, more than 4-fold higher than that of the

free *MtL*. This is in accordance with a previous study (Li et al. 1999) comparing several laccases regarding phenol red transformation in the presence of mediators. The same group observed much higher conversion rates for laccase from *T. villosa*, with a similar redox potential to *TvL* (Morozova et al. 2007), in comparison to *MtL*. Other researchers also reported that laccase from *Tv* is more suitable than bacterial laccases from the genus *Streptomyces* to remove several micropollutants (Margot et al. 2013a).

Table 6.2. Percentage of BPA transformation by 1000 U/L of biocatalyst and transformation rate (2 h of enzymatic treatment), (n=2).

Type of biocatalyst	BPA transformation (%)	BPA transformation rate (µg/L·h)
Mt free	13.5±10.1	5.3±3.9
Mt-fsNP	0.6±0.4	0.2±0.2
Tv free	61.8±6.5	24.1±2.5
Tv-fsNP	10.2±5.7	4.0±2.2
Mix free	43.0±5.1	16.8±2.0
Co-immobilized	6.7±2.7	2.6±1.1

Regarding the state of the biocatalyst, the immobilized laccase showed considerably lower activity towards BPA than the free enzyme. This is in accordance with a recent study by Pang et al. (2015), which reported that the transformation rates of BPA and catechol by laccase immobilized onto carbon nanoparticles was almost 10-fold lower than those for the free enzyme. The authors hypothesized that the aggregation of the nanoparticles reduced the substrate accessibility, which led to low reaction rates.

Regarding DCF, none of the enzymatic systems attained appreciable degradation of the micropollutant after 24 h (data not shown). Several studies have demonstrated the ability of laccase to transform the aniline moiety of DCF (Lloret et al. 2010, Lloret et al. 2013, Marco-Urrea et al. 2010, Margot et al. 2013b). However, the extent of the reaction was found to be greatly dependent on pH, being the optimal pH range below 5.5, whereas at basic pH

the transformation rate decreased drastically and at pH 8.9 no transformation occurred within 26 h (Margot et al. 2013b). It should be mentioned that in the current research, the presence of BPA did not seem to enhance the removal rate of DCF. These results are in contrast with a previous study, where the presence of certain micropollutants, among them BPA, increased the transformation of DCF by laccase, due to a mediator effect of the other oxidized compounds (Nair et al. 2013). The low concentration of DCF, the use of a real wastewater matrix with high pH (around 8.4 after adding the enzyme solutions) and the low content of organic matter, were likely hampering the transformation of DCF by laccase in the present study.

In an attempt to improve the removal rate of the micropollutants by the immobilized enzyme, the dose of co-immobilized laccases was increased. Figure 6.9 shows the profiles of residual BPA concentration for the different doses of biocatalyst. Increasing biocatalyst amounts leads to increasing BPA removal. In fact, when 8000 U/L of co-immobilized laccases were employed, almost complete removal of BPA took place within 24 h, despite the unfavorable conditions of the reaction media for laccase catalysis. Nevertheless, DCF oxidation was not observed, showing that this compound is highly recalcitrant towards laccase oxidation under realistic conditions.

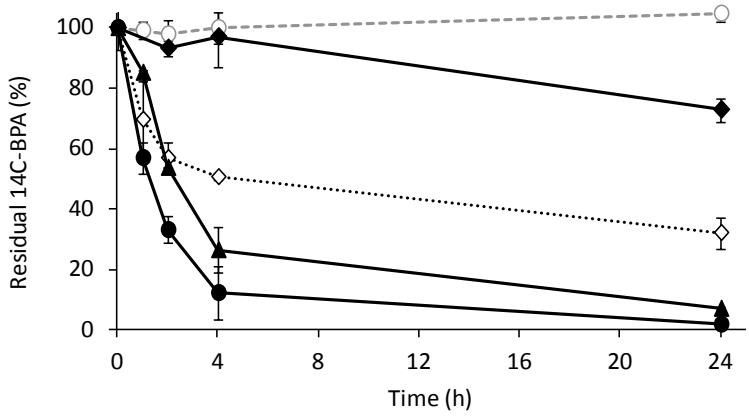

Figure 6.9. Residual ^{14}C-BPA concentration during the batch experiments of removal by 1000 U/L of the mixture of free laccases (◇) and 1000 U/L (◆), 4000 U/L (▲) and 8000 U/L (●) of co-immobilized laccases. Control without enzyme (○). Error bars represent the standard deviation of replicate experiments.

Regarding the products of laccase-catalyzed transformation of BPA, a new signal with a retention time shorter than BPA was observed in the HPLC radiochromatograms (data not shown). This signal was visible in all the samples regardless the type of biocatalyst, provided that the percentage of BPA removed was higher than 30%. This peak likely corresponded to 4-isopropenylphenol as this was previously detected as a degradation product of BPA by immobilized laccase (Galliker et al. 2010) and also by free laccase (Chapter 5). Oligomers usually occurring as BPA transformation products by free or immobilized laccase (Galliker et al. 2010) were removed during sample cleanup and hence, they were not detected. Previous reports demonstrated that BPA products after laccase treatment lack estrogenic activity (Fukuda et al. 2004). In addition, the formation of precipitates, as consequence of BPA polymerization, is favorable to reduce its environmental impact (Torres-Duarte et al. 2012).

Finally, an additional aspect worth noting was that the radioactivity of the reaction media evolved in different ways depending on the use of free or immobilized laccases Figure 6.10.

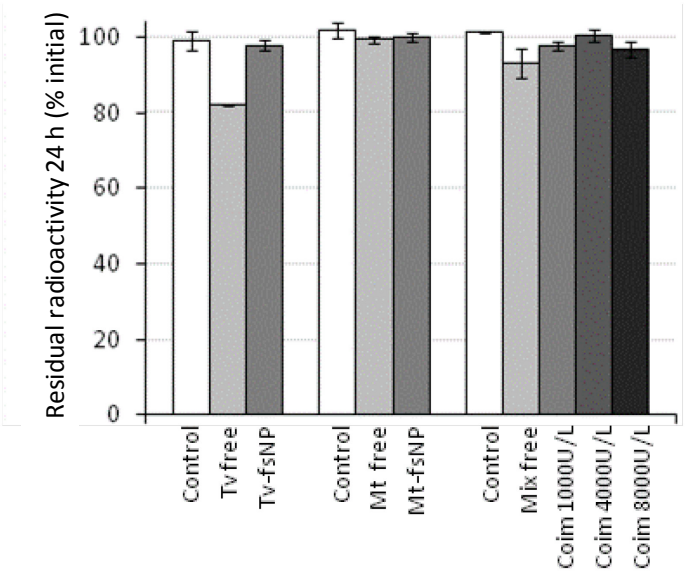

Figure 6.10. Residual radioactivity (% initial) in the reaction media after 24h.

For the mixture of free laccases and especially for free *TvL*, a slight reduction in the radioactivity of the reaction media was observed. In contrast, despite the high percentage of BPA transformation when using 8000 U/L of co-immobilized laccase, the radioactivity after 24 h did not change significantly. In an attempt to find an explanation for this fact, a mineralization assay of BPA by free *TvL* was conducted. Whereas in the control (experiment lacking enzyme), the radioactivity of the medium remained constant, in the experiments containing laccase a radioactivity decrease of 42.6±5.8% was observed (Table 6.3). Such a reduction was considerably higher than for the degradation experiments in the secondary effluent due to two main factors. The first one was that in the experiments with real effluent not only ^{14}C-BPA but also ^{14}C-DCF contributed to the overall radioactivity, being the percentage of their contribution of 66.2 and 33.8%, respectively. Hence, since ^{14}C-DCF was not appreciably degraded, it is expected that at least, the radioactivity associated to such compound remained in the reaction media after 24 h. In addition, the mineralization assay was conducted in buffer tris pH 7, instead of real biologically treated wastewater, which translated into a more favorable pH and the absence of dissolved compounds in water, diminishing the catalytic efficiency of laccase.

Table 6.3. Total radioactivity associated to the reaction media (RM) and sodium hydroxide (CO_2 traps) for the treatment of ^{14}C-BPA by free *TvL* and in control lacking enzyme (*n*=2).

Time (h)	Control RM (Bq)	Control (NaOH) (Bq)	Free *TvL* treatment RM (Bq)	Free *TvL* treatment (NaOH) (Bq)
0	4069 ± 56	-	3735±79	-
16	4158 ± 23	1.9±0.6	2141±261	14.0±1.1

The radioactivity recovered in the NaOH traps, which would correspond with the amount of BPA mineralized, was significantly higher for the experiment containing laccase in comparison to the control. Nevertheless, the value corresponded to approximately only 0.88±0.03% of the total radioactivity diminution observed in the reaction medium (or 0.38±0.04% of the total initial radioactivity) (Table 6.3). Therefore, although a small fraction

of BPA could be mineralized by free *TvL*, the reason behind the high reduction in the radioactivity of the reaction medium is most likely linked to the nature of the products formed during laccase treatment. The high molecular weight polymerization products may be less soluble than the parent compound and hence may be adsorbed onto the reactor walls or precipitate when using free laccase. Therefore, such products would not be present in the liquid fraction used to determine the radioactivity in the LSC. When using immobilized laccase, these products may adsorb more readily on the carrier material (fumed silica) instead of the reactor walls. Consequently, as the reaction medium was homogeneous, the nanobioacatalyst and the adsorbed radioactive products would be present in the sampled liquid fraction. To the best of our knowledge, this is the first time the possibility that BPA may be mineralized by laccase is reported. Nevertheless, the amount of BPA transformed into $^{14}C\text{-}CO_2$ was very low and further research to elucidate the fate of the reaction products would be interesting.

6.4 CONCLUSIONS

Laccases from *Tv* and *Mt* were successfully immobilized and co-immobilized onto fsNP and the resulting biocatalyst were applied to remove ^{14}C-BPA and ^{14}C-DCF from spiked biologically treated wastewater. The main advantages of the laccase-fsNP conjugates were the higher stability in WWTP effluents and at extreme pH values. Furthermore, they can be retained and reused if applied in a membrane reactor. However, the transformation rate of BPA was slower for the immobilized enzymes and higher enzymatic activities of laccase-fsNP than of free enzymes were necessary to achieve the same degradation rate. The catalytic efficiency toward BPA was also dependent on the microbial source of the enzyme, and was considerably higher for *TvL*. The transformation of DCF could not be achieved with the tested biocatalysts under real WWTP effluent conditions and improvements should be considered to expand the substrate range (adjust pH, addition of natural mediators, etc.). Finally, in view of a real application, estimation of costs is also necessary to evaluate whether it is preferable to use free enzymes, adding fresh enzymes more or less continuously, or immobilized enzymes that can be used for a longer time; and to select the more suitable enzyme: *MtL*, *TvL*, or a mixture of laccases.

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Chapter 7

PRODUCTION, CHARACTERIZATION AND USE OF MAGNETICALLY SEPARABLE CROSS LINKED LACCASE AGGREGATES [1]

Summary

Insolubilization of laccases as cross-linked enzyme aggregates (CLEAs) is a simple technique to increase stability and reusability of biocatalysts. In the present work, the use of magnetic mesoporous silica microbeads (MMSMB) as supports was proposed to produce mechanically resistant and magnetically-separable CLEAs (MCLEAs). The effects of several parameters such as cross linking time, addition of bovine serum albumin (BSA) as protein feeder, pH, glutaraldehyde concentration and laccase:MMSMB ratio on the immobilization yield and enzyme load were investigated. The best evaluated conditions based on enzyme load: 2 h of cross linking time, lack of BSA, pH 7, glutaraldehyde of 5 mM and laccase: MMSMB ratio of 1:2 (w:w), allowed the rapid preparation of MCLEAs with high enzyme load: 1.53 U laccase/mg MCLEAs. The stability of MCLEAs was improved with regard to low pH, presence of chemical denaturants and real wastewater matrix, compared to free laccase. In addition, the novel biocatalyst exhibited good operational stability, maintaining up to 70% of its initial activity after 10 successive batch reactions operated with magnetic separation. Finally, MCLEAs demonstrated its catalytic potential to transform acetaminophen and various non-phenolic pharmaceutical active compounds (PhACs) as mefenamic acid, fenofibrate and indomethacin from biologically treated wastewater, with similar or even higher efficiency than free laccase.

[1] Part of this work was performed in the Department of Civil Engineering of the University of Sherbrooke (Quebec, Canada).

OUTLINE

7.1 INTRODUCTION

Although laccases constitute promising biocatalysts in industrial and bioremediation processes due to their wide substrate range and simple requirements of catalysis (presence of substrate and O_2) (Majeau et al. 2010), two major drawbacks limit their application in continuous bioprocess. On one hand, the susceptibility of free enzymes to inactivation over time due to unfavorable conditions (i.e. unfavorable pH, chemical/biological denaturing agents or temperature) (Cabana et al. 2011, Gasser et al. 2014). Another major obstacle is related to the retention and reusability of the biocatalyst (Ba et al. 2013). In order to mitigate these drawbacks and enhance the economy of biocatalytic processes, immobilization and insolubilization techniques for enzymes have been lately investigated (Brady and Jordaan 2009). In this sense, the insolubilization of enzymes as cross-linked enzyme aggregates (CLEAs) is a simple technique to increase stability and reusability of biocatalyst (Arsenault et al. 2011, Cao 2005, Sheldon 2011). The preparation of CLEAs usually involves the precipitation from buffered solution followed by a cross-linking step using a bifunctional reagent, such as glutaraldehyde (Figure 7.1)

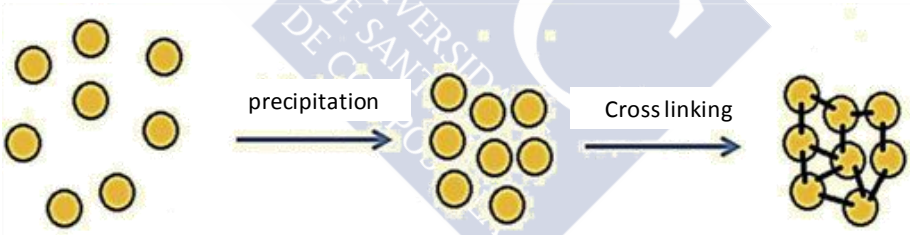

Figure 7.1. Enzyme immobilization using CLEAs technique (Talekar et al. 2013).

Since precipitation is a commonly used method for enzyme purification, the CLEAs production combines purification and immobilization into a single operation (Sheldon 2011). In addition, it is amenable to rapid optimization, leading to low costs and short time-to-market processes.

However, CLEAs may suffer from several drawbacks as softness or poor mechanical stability, which make them difficult to handle both in conventional stirred tanks or in packed bed reactors, and often causes leaching of enzyme in the reaction medium during the biocatalysis (Wilson et al. 2002). In addition,

the processes commonly applied to recover CLEAs from reaction medium, namely, centrifugation or filtration, lead to increase in CLEAs size and clusters formation (clumping), resulting in internal mass transfer limitations (López et al. 2014). Hence, for many applications, CLEAs may require physical support to improve mechanical properties (Brady and Jordaan 2009, Garcia-Galan et al. 2011). CLEAs entrapment within rigid Lentikats (polyvinyl alcohol hydrogel) (Wilson et al. 2002) or their preparation on a siliceous support (Lee et al. 2005) are examples of such approach. More recently, the use of magnetic supports was proposed to produce mechanical resistant and magnetically-separable CLEAs, which can be easily recovered using a magnet instead of centrifugation or filtration methods (López et al. 2014). For instance, magnetic CLEAs of α-amylase were successfully applied to hydrolyze starch (Talekar et al. 2012). More recently, Tudorache et al. (2013) conducted the precipitation of lipase and subsequent cross-linking of the enzyme aggregates onto magnetic particles, and applied the biocatalyst to the synthesis of glycerol carbonate. In a recent work done by Kumar et al. (2014), this approach was applied for the first time to laccase. Stable and magnetically separable biocatalysts were obtained by immobilizing laccase CLEAs onto iron-based magnetic nanoparticles previously functionalized with 3-aminopropyltriethoxysilane (APTES).

In the current study, magnetic mesoporous silica microbeads (MMSMB) already containing amino groups were used as support to produce robust magnetically-separable laccase CLEAs (MCLEAs) according to the scheme depicted in Figure 7.2. A large accessible surface area (>100 m^2/g) combined with a minimal pore size of approximately 3 times the average enzyme diameter (>30 nm) is desirable for better enzyme loading, retention and reduced diffusion constraints (Cao 2006).

The MCLEAs were characterized in terms of pH, temperature, kinetics, stability and reusability. Finally, a bioremediation application was proposed to evaluate the oxidative potential of MCLEAs in environmentally relevant matrices. In this sense, although laccase-based treatments have been successfully applied towards the elimination of many xenobiotics in solutions, most of works deal with single-compound solutions comprising laccase substrates (i.e. mainly phenolic).

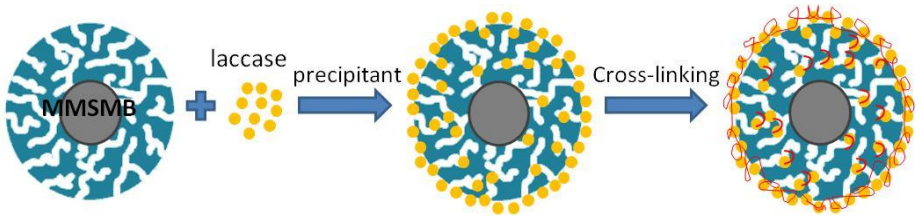

Figure 7.2. Scheme of MCLEAs preparation.

On the other hand, the transformation of non-laccase substrates requires the presence of mediators (Lloret et al. 2013, Nguyen et al. 2013). Recent studies reported that the substrate spectrum of laccase could be extended in mixtures when phenolic compounds are present (Margot 2015, Touahar et al. 2014). The latter may involve cross coupling reactions with non-phenolic compounds. Thereby, this effect would compensate the absence of specific laccase mediators (e.g., 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 1-hydroxybenzotriazole (HBT)). Therefore, in a second part of the study the catalytic potential of the MCLEAs to remove a mixture of pharmaceutical active compounds (PhACs) from real wastewater was investigated.

7.2 MATERIALS AND METHODS

7.2.1 Chemicals and enzyme

Commercial laccase from *Trametes versicolor* (≥10 U/mg), bovine serum albumin (BSA), ABTS, glutaraldehyde (25%) and PhACs were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl acetate, dichloromethane, formic acid, and methanol were of analytical grade and were purchased from Fisher Scientific (Ottawa, ON, Canada).

7.2.2 Magnetic mesoporous silica microbeads

Magnetic mesoporous silica microbeads (MMSMB) were kindly provided by Materium Innovations (Granby, Quebec, Canada). These consisted in magnetite nanoparticles (40-50 nm) encapsulated in hollow mesoporous silica microcapsules with an average size around 20 µm. The material used is bearing

both amino and vinyl groups on their surface (information provided by Materium Innovations).

Magnetization curves were recorded on a SQUID magnetometer (MPMS-5S XL Quantum Design magnetometer). Fourier transform infrared (FTIR) analysis was performed on a Nicolet FT-IR spectrophotometer.

7.2.3 Enzyme activity

Laccase activity was measured by monitoring the oxidation of ABTS to its cation radical ($ABTS^{\bullet+}$) at 420 nm (ε_{max} =3.6 × 10^4 M^{-1} cm^{-1}). Fifty microliters of laccase or MCLEAs containing sample (diluted in 0.1 M acetate buffer, pH 4.0) were added to 150 µL of 0.6 mM ABTS (in 0.1 M acetate buffer, pH 4.0) in a 96-well plate. The ABTS oxidation was monitored by measuring the absorbance at 420 nm over 60 s (6 s intervals). One unit (U) of activity was defined as the amount of enzyme forming 1 µmol of $ABTS^{\bullet+}$ per min. Activity measurements were carried in a 96-well plate using a double-beam UV–Vis spectrophotometer (SpectraMax Plus 384, Molecular Devices Corp., Sunnyvale, CA).

7.2.4 Magnetic CLEAs preparation

The initial conditions for preliminary immobilization assays were established based on the work of Kumar et al. (2014) for the synthesis of magnetically separable CLEAs. According to these, pH value of 4, co-aggregation with BSA (ratio laccase:BSA of 1:1 w:w), propanol as precipitant agent and glutaraldehyde (5 mM) as cross linking agent were initially assayed. The initial ratio laccase:MMSMB was 1:2 (w:w). 100 µL of stocks of laccase and BSA (10 mg/L of protein in 0.1 M sodium acetate buffer pH 4), freshly prepared at the beginning of each experiment, were added to 200 µL of a solution of MMSMB (10 mg/mL in 0.1 M sodium acetate buffer, pH 4) on 1.5 mL centrifuge tubes and shaken for 90 min at room temperature (24±2ºC). Then, 1 mL of chilled propanol was added and stirred for 90 min. When precipitation occurred, glutaraldehyde was added into the suspension to reach a final concentration of 5 mM and then shaken again for 2, 6 or 22 h at room temperature (24±2ºC). After cross-linking, the MCLEAs were magnetically

separated from the mixture and washed thrice with sodium acetate buffer (pH 4) to remove excess of glutaraldehyde and any unbound enzyme.

Subsequently, the effect of several parameters onto the MCLEAs formation were investigated: i) a preliminary sorption step consisting in mixing laccase, BSA and MMSMB solutions for 2 h before precipitation with propanol; ii) presence or absence of BSA, iii) pH (0.1 M acetate buffer pH 4, or 0.1 M phosphate buffer pH 7), iv) concentration of glutaraldehyde (1, 2.5, 5 and 10 mM), or v) laccase:MMSMB ratio (1:1; 1:2; 1:3 and 1:4 w:w).

The immobilization yield (IY) of the MCLEAs was estimated based on the apparent laccase activity of the MCLEAs formed (A_f) relative to the initial applied laccase activity (A_i) according to the following equation:

$$IY(\%) = \frac{A_f}{A_i} \cdot 100 \qquad (7.1)$$

At the same time, washing losses (WL) were determined as the relative difference between the apparent (measured) laccase activity in MCLEAs after exhaustive washing and the apparent laccase activity before washing (100 %). Finally, the term enzyme load was defined as the apparent laccase activity per mg of immobilized biocatalyst. The dry weight of the MCLEAs was obtained by lyophilizing the MCLEAs.

7.2.5 Characterization of MCLEAs

7.2.5.1 Optimum pH and temperature

The pH of maximum laccase activity (free enzyme and MCLEAs) was investigated using 0.45 mM ABTS in a 0.1 M citrate–phosphate buffer (pH 2.6–8). The relative activity was calculated as the ratio between the activity at each pH and the maximum activity attained.

The effect of temperature (30-60ºC) on the activity of free laccase and MCLEAs was tested by determining the activity at the corresponding temperature under standard conditions i.e., 0.45 mM ABTS in 0.1 M acetate buffer pH 4. The relative activity was determined as the ratio between the activity at each temperature and the maximum activity attained.

7.2.5.2 Determination of kinetic parameters

Apparent kinetic parameters of free laccase and MCLEAs were determined by measuring the laccase activity under standard conditions using ABTS as substrate in the range 1.5-600 µM. Experimental data were fitted to determine the Michaelis-Menten parameters by minimizing the sum of squared residuals.

7.2.5.3 Storage stability and leaching test

The storage stability was investigated by incubating free laccase and MCLEAs in 0.1 M phosphate buffer at pH 7 and 4ºC for one month and measuring the residual activity periodically.

In order to determine any leaching of protein from MCLEAs, these were washed (to remove traces of free enzymes in the prepared MCLEAs suspension) and incubated in 0.1 M phosphate buffer pH 7 for 6 h at 250 rpm and room temperature (24±2ºC). After the incubation period, the MCLEAs were separated from the buffer with an external magnetic field. Subsequently, the laccase activity in the buffer was measured to determine the amount of laccase leached from MCLEAs biocatalyst.

7.2.5.4 Long term stability in wastewater

Stability of free laccase and MCLEAs in wastewater was evaluated by incubating 1000 U/L of the biocatalyst in 5 mL of secondary effluent collected from the municipal Wastewater Treatment Plant (WWTP) in Magog (Qc, Canada) (Table 7.1). The collected sample was filtered through 0.45 µm, and adjusted to pH 7 as these were the conditions selected for the subsequent degradation experiments. Samples were taken over a one month period and the residual enzymatic activity was measured.

Table 7.1. Properties of the effluent from the wastewater treatment plant (WWTP).

Parameter	Value
Biological oxygen demand (BOD)	1.8 mg/L
Chemical oxygen demand (COD)	16.0 mg/L
Total suspended solids	4.2 mg/L
Volatile suspended solids	2.3 mg/L
Total Kjeldahl nitrogen	1.1 mg/L
Ammonia content	0.43 mg/L
Nitrate content	7.1 mg/L
Phosphate content	0.06 mg/L
Total phosphorus	0.13 mg/L
pH	7.4

7.2.5.5 MCLEAs reusability

Reusability of the MCLEAs was assayed by means of consecutive cycles of ABTS (0.5 g/L) oxidation in 0.1 M phosphate buffer at pH 7. At the end of each 8-min oxidation cycle, the immobilized enzyme was removed by applying a magnetic field and the absorbance of the supernatant was measured (420 nm). The immobilized laccase was collected and washed once with the phosphate buffer, and the procedure repeated with a fresh aliquot of ABTS. The activity of the immobilized enzyme was considered to be 100% in the initial cycle. These experiments were performed in duplicate. A control assay with deactivated MCLEAs was conducted in parallel to verify that no ABTS oxidation occurred in absence of active laccase.

7.2.5.6 Stability of MCLEAs against denaturation

The effect of the pH on the enzyme stability was studied by incubating laccase in 0.1 M citrate-phosphate buffer (pH 3, 5, 7 and 8.5) at room temperature (24±2ºC) during 24 h. Samples were transferred to standard reaction mixtures in order to determine the laccase activity according to the

activity assay. The residual activity was calculated referred to the value of the initial activity at each pH.

The thermal stability study was carried out by incubating samples of the biocatalysts in 0.1 M citrate-phosphate buffer (pH 7) at selected temperatures: 20, 45 and 60ºC and enzyme activity was periodically measured. The results were expressed in relative form, assigning 100% activity to the initial measurement.

The stability of free laccase and MCLEAS laccase against chemical denaturation was tested by incubating 500 U/L in the presence of deactivating reagents $CaCl_2$, $CoCl_2$, $ZnCl_2$ (25 mM) and hydrophilic organic solvents: acetone, acetonitrile, and methanol (50%); at pH 7 and room temperature (24±2ºC). The incubations lasted for 1 h and subsequently activities were measured. The results for stability were given in percentage, allocating 100% activity to the value in phosphate buffer at pH 7.

7.2.6 Pharmaceuticals degradation in secondary effluent

A sample of biologically treated wastewater was collected on May 2015 from the effluent of the municipal WWTP in Magog (QC, Canada) and filtered through 0.45 µm. The aim of the filtration was to remove particulate solids and other microorganisms, which could contribute to the elimination of the target pollutants by adsorption or undefined biotransformation, and hence, the evaluation of the sole enzymatic transformation by laccase could be possible (Lloret et al. 2013b). The pH (originally 7.4) was adjusted to pH 7 with acetic acid 1 M and then spiked with each of the following PhACs to attain initial concentrations in the range of 10-50 µg/L: acetaminophen, ketoprofen, cyclophosphamide, mefenamic acid, caffeine, indomethacin, naproxen, fenofibrate, trimethoprim and ibuprofen. Treatment of the PhACs by both free laccases or MCLEAs was performed in batch mode at 30°C in 50 mL Erlenmeyer flasks with orbital shaking at 150 rpm for 6 h. The final volume of samples was 10 mL. Biocatalysts (free laccase or MCLEA) were added to obtain a final laccase activity of 1000 U/L. For controls, the biocatalyst solutions were substituted by deactivated MCLEAS or phosphate buffer solution. For each compound, the enzymatic removal by MCLEAs and free laccase was calculated

according to the concentration detected in controls with inactivated MCLEAs and without enzyme, respectively. The total removal of each compound by MCLEAs (including the possible sorption onto the biocatalyst) was calculated using as reference the control without enzyme. Tests were performed in duplicate.

After 6 h of reaction, MCLEAs were magnetically separated and 1 g of NaCl was added to the sample and thorough mixing. Then, 5 mL of ethyl acetate were added and the mixture was vortexed for 2 min. After the phase separation in a glass funnel, the organic phase was transferred to a glass vial. In a second extraction step, 5 mL dichlorometane were added to the aqueous phase and vortexed for 2 min. After decantation and phase separation, the dichlorometane was transferred to the same glass vial containing ethyl acetate. The mixture of solvents was evaporated to dryness at 40ºC under a gentle stream of nitrogen prior to re-suspension in 1 mL of a solution of water-methanol 50-50% (v:v) and 0.1% (v:v) formic acid. The mixture was then sonicated for 5 min and filtered through 0.2 µm PTFE (polytetrafluoroethylene) membrane filters before transferring into UPLC vial for quantitative analysis. Samples were stored at 4°C until analysis by ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS).

The analysis of PhACs was performed according to the methodology developed and described by Touahar et al. (2014) and Ba et al. (2014). An Acquity UPLC XEVO TQ mass spectrometer (Waters Corporation, Milford, MA) with an Acquity UPLC HSS-T3 column (100 mm x 2.1 mm, 1.8 µm) (Waters Corporation, Milford, MA) was used for the PhACs analysis. The solvent flow rate was set to 0.4 mL/min and the column temperature was maintained at 35ºC. The sample volume injected was 5 µL. Mobile phase was 0.20% formic acid/water (A) and 0.20% formic acid/methanol-acetonitrile (75-25 v/v) (B) (LC/MS grade). The adopted elution gradient started with 5% of eluent B, increasing to 90% in 8 min and then back to initial conditions in 4 min. The mass spectrometry analysis was performed using a positive electrospray ionization (ESI+) source in Multi-Reaction-Monitoring mode. The optimized parameters were obtained by direct infusion of analytical standard solutions at 10 µg/mL as follows: desolvation gas (nitrogen), 800 L/h; cone gas (nitrogen), 50 L/h; collision gas (nitrogen), 0.22 mL/min; capillary voltage 2.5 kV; source

temperature, 150ºC and desolvation temperature, 550ºC. Two daughter traces (transitions) were used. The most abundant transition was used for quantification, whereas the second most abundant was used for confirmation. The concentration of pharmaceutical was determined by comparing the peak areas obtained with those of standard solutions of known concentrations

7.3 RESULTS AND DISCUSSION

7.3.1 MCLEAs preparation

Preparation of traditional CLEAs consists of two main steps, the aggregation by precipitation, followed by the cross linking of the enzymes (Figure 7.1). It is expected that in the presence of a support containing amino groups, not only intermolecular cross-linking of the sorbed enzyme occurs, but also cross-linking to the amino groups of particles.

Starting with the conditions established by Kumar et al. (2014), a series of parameters were tested to maximize the enzyme load while maintaining a high immobilization yield. Several cross linking times were initially evaluated aiming at establishing the time required to obtain the highest enzyme load, as it has been demonstrated that whereas a too short cross linking time could result insufficient and lead to poor immobilization yield and operational stability, prolonged cross linking may be detrimental to enzyme activity (Talekar et al. 2013). As hardly differences were observed at 2, 6 and 24 h, (Table 7.2, run 1-3), the lowest cross linking time was selected to design a rapid process for the MCLEAs preparation. Omitting the sorption step led to an slight increase in the immobilization yield (9.3%) (Table 7.2, run 4), probably due the lower time of laccase exposure to acidic pH. Although several studies reported that the use of protein feeders rich in lysine amine residues, such as BSA or egg white in the preparation of CLEAs resulted in higher activity recovery and stability (Jiang et al. 2014, Shah et al. 2006), it was observed that in absence of BSA, the immobilization yield was 2-fold higher than with the albumin (run 4 and 5). This is in accordance with previous studies of Cabana et al. (2007) and Guauque Torres et al. (2014), who stated the same negative effect of BSA on laccase and lipase CLEAs, respectively. The inadequate exposure of the catalytic centers to ABTS owing to the shielding effect of BSA and mass

transfer limitations from the substrate solution to the laccase active site (Cabana et al. 2007), may be behind this fact. Besides, in the presence of albumin, laccase and BSA compete for binding to the support, resulting in a lower available surface for laccase attachment.

Table 7.2. Effects of experimental parameters on MCLEAs formation.

Run	Sorption step (h)	pH	BSA: Lac	Glut (mM)	Lac: MMSMB	Cross linking time (h)	WL (%)	IY (%)	Enzyme load (U/mg MCLEAs)
1	2	4	1:1	5	1:2	2	27.7±9.1	3.5±1.5	0.11±0.03
2	2	4	1:1	5	1:2	6	29.2±0.9	2.2±0.01	0.06±0.001
3	2	4	1:1	5	1:2	22	10.9±0.4	3.0±0.03	0.12±0.001
4	-	4	1:1	5	1:2	2	45.5±3.6	9.3±0.4	0.25±0.001
5	-	4	-	5	1:2	2	31.7±10.7	19.2±1.7	0.59±0.05
6	**-**	**7**	**-**	**5**	**1:2**	**2**	**31.2±5.0**	**39.0±0.9**	**1.53±0.04**
7	-	7	-	1	1:2	2	51.2±7.4	27.6±4.4	0.89±0.16
8	-	7	-	2.5	1:2	2	39.4±3.0	36.6±2.0	1.28±0.11
9	-	7	-	10	1:2	2	28.0±4.9	35.1±3.0	1.19±0.28
10	-	7	-	5	1:1	2	67.5±3.2	16.9±2.0	1.12±0.03
11	-	7	-	5	1:2	2	22.8±6.0	42.1±2.0	0.94±0.003
12	-	7	-	5	1:3	2	17.0±0.9	41.5±0.9	0.75±0.001

Regarding pH, a considerable increase in enzyme load and immobilization yield was attained when the MCLEAs were produced at pH 7 (Table 7.2, run 6). The higher laccase stability and the more favorable reactivity of glurataraldehyde with amino groups at pH 7 are likely to enhance the immobilization yield (Migneault et al. 2004). In this sense, it was reported that reversibility was observed at pH above 3, except in the range of pH 7 to 9 where only a little reversibility is observed, and a high immobilization yield in CLEAs is expected (Talekar et al. 2013). Apart from reversibility issues, it was found that in aqueous solution at acidic pH, glutaraldehyde is mostly present in a monomeric form, whereas alkaline pH promotes the existence of a polymeric form. This one is preferable to obtain CLEAs with more space among the aggregates, preventing the occurrence of diffusional restrictions (Talekar et al. 2013).

The concentration of glutaraldehyde was fixed in 5 mM as, whereas lower glutaraldehyde concentrations increased washing losses, attaining lower enzyme loads (0.89 and 1.28 U/mg biocatalyst for 1 and 2.5 mM of glutaraldehyde, respectively); increasing the glutaraldehyde concentration to 10 mM had a negative impact on laccase activity. Finally, different laccase:microparticles (Lac:MMSMB) ratios were evaluated (Table 7.2, run 10-12) using the best conditions previously established. Decreasing the amount of microparticles to a ratio of 1:1 led to higher washing losses, which resulted into lower immobilization yield (16.9%). On the other hand, the increase in the amount of microparticles resulted into much lower enzyme load.

According to these results, the conditions assayed in run 6 were applied to produce a large amount of MCLEAs for the subsequent experiments.

7.3.2 Characterization

7.3.2.1 Microparticles and MCLEAs properties

The FTIR spectra of the MMSMB with amine and vinyl groups and final MCLEAs are illustrated in Figure 7.3.

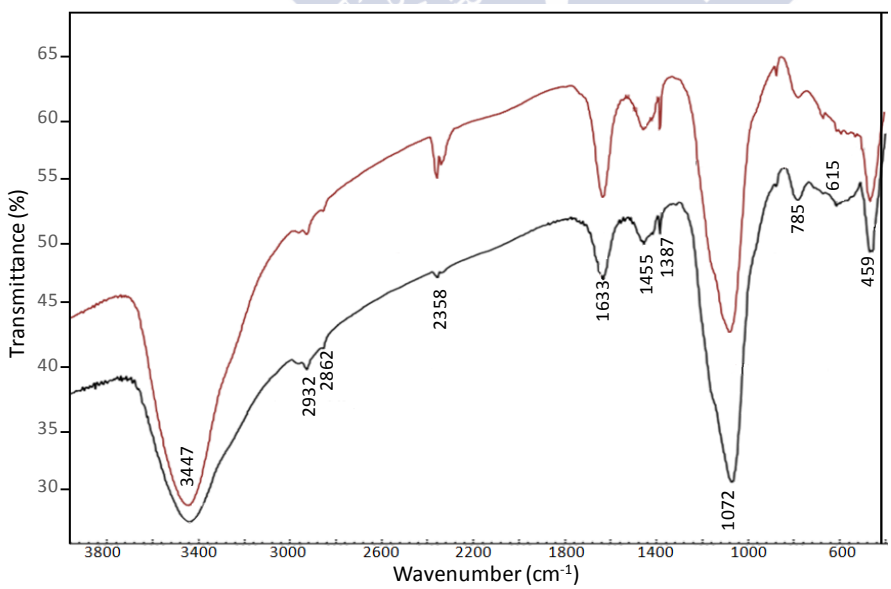

Figure 7.3. FTIR spectra of MMSMB (black line) and MCLEAs (red line).

The vibration bands around 450 and 600 cm^{-1} determine the characteristics of (Fe–O) vibrations. The successful presence of amine groups has been verified by stretching vibrations of amine in the region of around 3300 cm^{-1}. The silica coated magnetite sample shows a band around 1050 cm^{-1} corresponding to the stretching vibrations of (Si-O–Si) and it is associated with the motion of oxygen in Si-O-Si antisymmetric stretch due to the asymmetric stretching bonds of Si-O-Si in SiO_2. The band around 450 cm^{-1} is for Si-O-Si or O-Si-O bending modes. The absorption band at 2100 cm^{-1} is associated with C-H band of vinyl groups and the absorption band around 1600 and 1400 cm^{-1} was ascribed to the stretching vibration of C=C and bending vibration of CH_2.

The saturation magnetization (M_s) was 8.0 emu/g and 8.6 emu/g for the MCLEAs and MMSMB, respectively, which resulted enough to show a rapid response to an external magnetic field (Figure 7.4B).

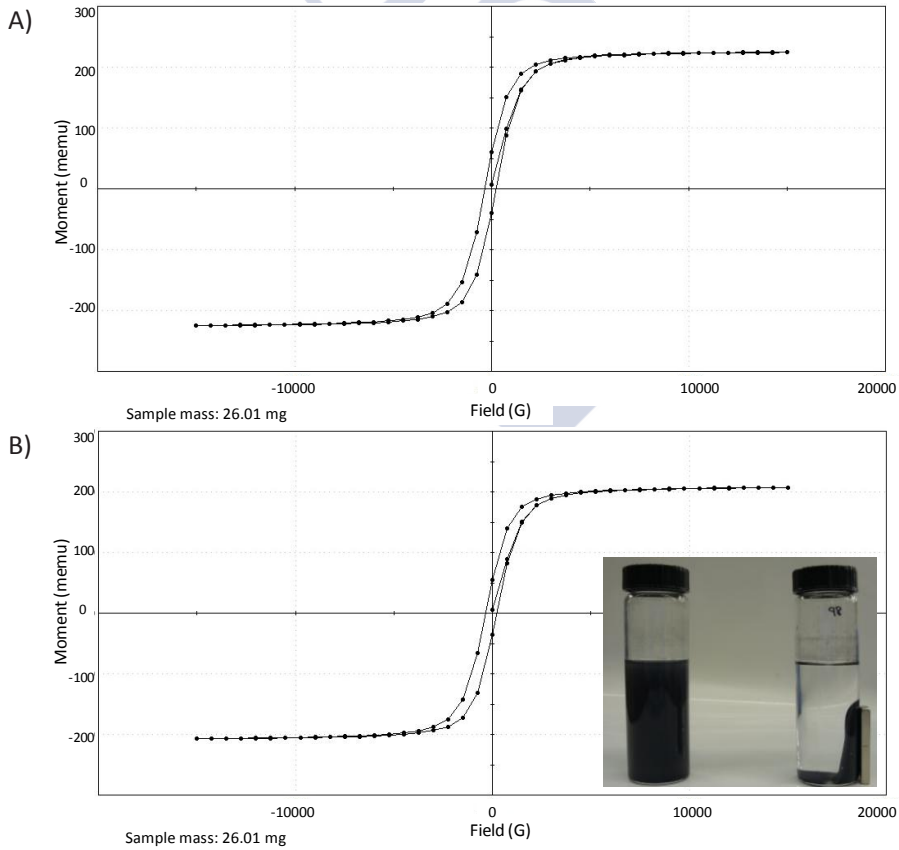

Figure 7.4. Magnetization versus magnetic field for MMSMB (A) and MCLEAs (B).

The slight decline in the magnetization moment of MMSMB after laccase attachment was the consequence of the decrease in the amount of the magnetic moment per unit weight of MCLEAs. The magnetization curves, depicted in Figure 7.4, showed slight hysteresis characterized by coercive fields (H_{ci}) around 285 Oer and remnant magnetization (M_r) of 1.7 and 1.9 emu/g for the MCLEAs and MMSMB, respectively. This trend reveals a super paramagnetic behavior.

7.3.2.2 Optimum pH and temperature

A comparative study between free laccase and MCLEAs was conducted aiming to study the effect of pH and temperature on the activity. Figure 7.5 depicts the activity profiles for both biocatalysts at pH between 3.0 and 8.0 at 25ºC. Free laccase showed the maximum activity at pH 3 whereas the optimal pH for MCLEAs was found around 2.6. In the range 3-6, free laccase showed a slightly higher relative activity (no more than 8% of difference); whereas at pH above 6, the relative activity of both biocatalyst forms was identical.

Regarding optimal temperature, both free and MCLEAs laccases displayed maximal catalytic activities in the range 40-50ºC.

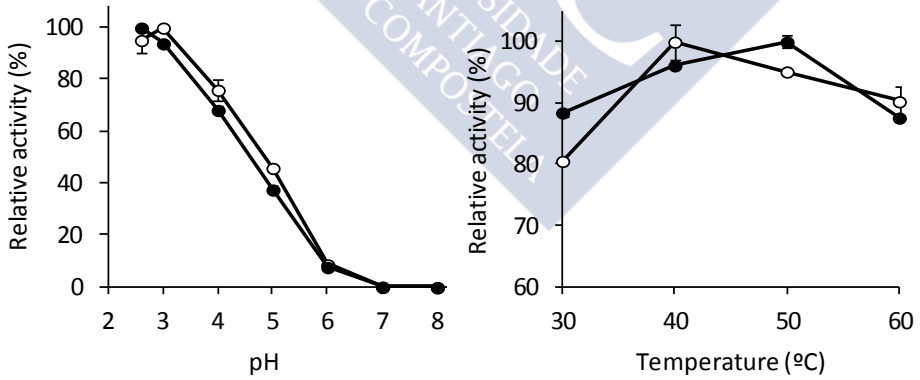

Figure 7.5. Effect of pH and temperature on free laccase (○) and MCLEAs (●).

7.3.2.3 pH and thermal stability

One of the most important factors affecting the enzymatic stability is pH. For this reason, its effect on laccase stability was investigated by incubating

the MCLEAs and free enzyme in citrate-phosphate buffer at pH 3, 5, 7 and 8.5 for 24 h (Figure 7.6).

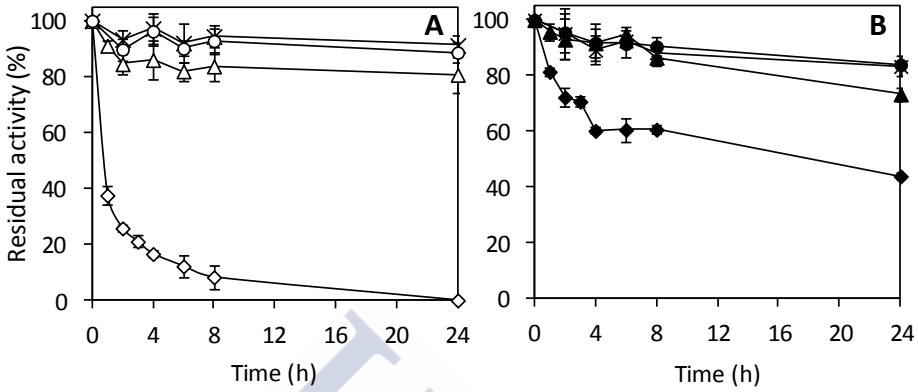

Figure 7.6. Effect of pH on free laccase (A) and MCLEAs (B). Symbols: pH 3 (diamond), pH 5 (triangle), pH 7 (multiplication symbol) and pH 8 (circle).

A drastic increase in the stability of laccase at pH 3 was observed after MCLEAs formation; at the end of the incubation period, MCLEAs exhibited a residual activity above 43%, whereas free laccase was completely inactivated, which confirmed the usefulness of insolubilization as a way to protect the enzyme. This is in accordance with most of published studies, which report increase in acid stability after laccase immobilization (Songulashvili et al. 2012, Zhang et al. 2015). At higher pH values, no significant differences were found between free laccase and MCLEAs.

Regarding thermal stability, no improvement was observed for MCLEAs in comparison with free laccase and both were completely deactivated after 3 h of incubation at 60ºC and neutral pH (Figure 7.7). The lower stability for the immobilized laccase in comparison with the free one at 45ºC is difficult to explain, although a similar behavior was previously reported by Masuda et al. (2013) for the enzyme formaldehyde dehydrogenase immobilized onto mesoporous silica (MPS). These authors hypothesized that the enzyme immobilized on the MPSs suffered a faster denaturalization owing to thermal energy transmitted from silica wall.

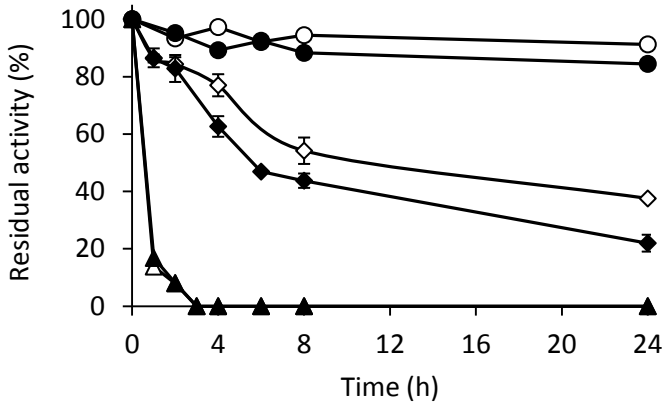

Figure 7.7. Thermal stability of free laccase (white symbols) and MCLEAs (filled symbols) at room temperature (24±2ºC) (circle), 45 ºC (diamond) and 60 ºC (triangle).

7.3.2.4 Resistance against inhibitors

Figure 7.8 shows residual activities of free laccase and MCLEAs to high concentrations (25 mM) of halide salts, $ZnCl_2$, $CoCl_2$, $CaCl_2$ and organic solvents (50% v:v) at neutral pH.

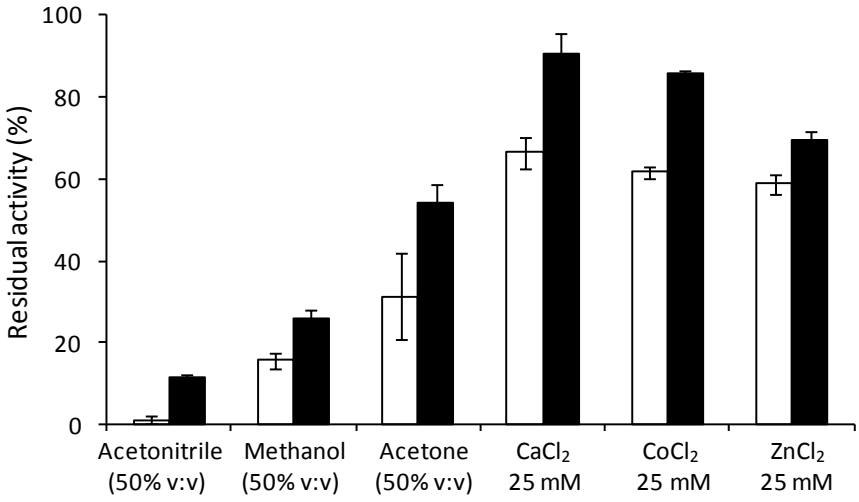

Figure 7.8. Residual activity (A/A_0) of laccase CLEAs after 1-h incubation with denaturants (mean of triplicates±standard deviation)

It is evident that MCLEAs displayed higher resistance against organic solvents than free laccase. For instance, after 1 h of incubation in the presence

of acetonitrile (50% v:v), free laccase was almost deactivated whereas MCLEAs exhibited 12% of residual activity. In acetone, the residual activity of MCLEAs was 1.7 fold higher than for free laccase. The higher stability of enzyme in the presence of organic cosolvents after CLEAs formation has been observed in several works, and it was attributed to the rigidity of the biocatalyst formed (Ba et al. 2014, Tyagi et al. 1999). In the same way, the MCLEAs showed higher residual activity than the free enzyme in presence of the chaotropic salts $ZnCl_2$, $CoCl_2$ and $CaCl_2$. These results confirmed that MCLEAs formation increases laccase resistance against chemical inhibitors.

7.3.2.5 Storage stability and leaching test

Figure 7.9 depicts the activity profile of free laccase and MCLEAs stored at 4ºC and neutral pH. Storage stability did not differ considerably for free laccase and MCLEAs. For the first 18 d, activities were almost coincident whereas after 30 d, free laccase exhibited a slightly superior stability: 82.2±4.5% and 74.3±4.0% for free laccase and MCLEAs, respectively.

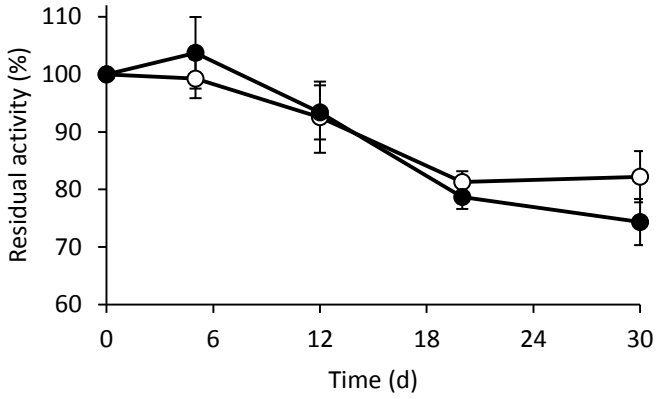

Figure 7.9. Residual activity (A/A_0) of free laccase (○) and MCLEAs (●) during one month of storage in phosphate buffer pH 7 and 4ºC.

In relation to the leaching test, negligible leaching of laccase from MCLEAs was detected after incubation for 6 h at 250 rpm.

7.3.2.6 Reusability of laccase

Apart from stability, the reusability of the biocatalyst is an important feature to be considered in view of its large scale application. The reusability of MCLEAs was investigated by applying consecutive cycles of ABTS oxidation. As can be seen from Figure 7.10, the activity of MCLEAs was above 100% during the first 5 cycles, indicating that no significant deactivation or leaking occurred during oxidation or washing procedure. The increase in the activity up to third cycle could be explained considering the different forms that laccase can present: native intermediate, resting oxidized, and partly reduced (Shleev et al. 2006). Accordingly, laccase molecule may be passing from the resting state to the catalytic state (Tavares et al. 2015). Such behavior had already been observed in previous reports. For instance, Cristóvão et al. (2012) observed the activation of laccase immobilized on green coconut fibber along the first four cycles of ABTS oxidation.

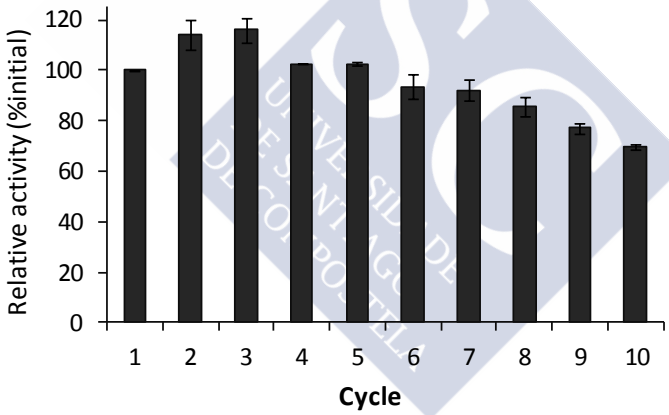

Figure 7.10. Residual activity (%) of MCLEAs after cycles of ABTS oxidation in phosphate buffer pH 7 in batch reactions.

After the 6th cycle, a gradual loss of activity started, probably because of enzyme deactivation during the consecutive batches, and after 10 cycles of ABTS oxidation and separation, laccase MCLEAs retained 70% of the initial activity. This is in the range reported by other studies evaluating the immobilization of laccase onto silica carriers. For instance, Patel et al. (2014) immobilized laccases onto SiO_2 nanocarriers and observed a residual activity of 93.7% and 82.5% after 5 and 10 cycles of ABTS oxidation, respectively,

whereas Rekuć et al. (2009) and Tavares et al. (2013) reported activities of 65% and 70%, respectively, after 10 cycles of ABTS oxidation for different laccases immobilized onto silica carriers.

7.3.2.7 Kinetic parameters

Table 7.3 presents the Michaelis–Menten kinetic parameters for free and MCLEAs laccases using ABTS as substrate.

Table 7.3. Apparent kinetic parameters for free laccase and MCLEAs.

	Free laccase	**MCLEAs**
K_M (μM)	38.5 ± 3.1	64.3 ± 6.7
K_{cat} (s^{-1})	153.7 ± 1.3	134.6± 6.7
K_{cat} /K_M (s^{-1} μM^{-1})	4.00 ±0.29	2.10 ± 0.11

In comparison to free laccase, a lower affinity of MCLEAs for ABTS was found as indicated by a slight increase in the K_M value (0.038 ± 0.003 mM and 0.064 ± 0.006 mM, respectively). These results are in agreement with several studies reporting laccase immobilization (Bayramoglu et al. 2010, Patel et al. 2014), and could be attributed to several factors as diffusional limitations, conformational changes on the protein molecule after immobilization or steric hindrance (Lloret et al. 2012). Nevertheless, the affinity for the substrate was still higher than that of other enzymes immobilized onto porous supports, which have displayed higher K_M values (Fernández-Fernández et al. 2013, Rekuć et al. 2009, Songulashvili et al. 2012). In contrast, Cabana et al. (2007) did not find appreciable changes in K_M value after the insolubilization of laccase from *Coriolopsis polyzona* in CLEAs; and Kumar et al. (2014) reported higher affinity of functionalized magnetic nanoparticles bonded to laccase CLEAs for ABTS, in comparison to the free laccase from *T. versicolor*. The turnover number (K_{cat}) for the free laccase and MCLEAs showed that the maximal rate for free laccase is slightly higher than for MCLEAs. As result, the catalytic efficiencies K_{cat}/K_M for MCLEAs laccase was 1.9-fold lower than for free laccase. Even so, we can state that the laccase insolubilization and further immobilization provided efficient catalytic ability.

7.3.2.8 Stability in wastewater

The stability of the biocatalyst in a real environmental matrix must be evaluated in order to estimate the operational lifetime in a real bioremediation process. For this reason, the stability of the MCLEAs and free laccase in real wastewater was tested by exposing the biocatalysts to a sample of biologically treated wastewater (characteristics in Table 7.1). From Figure 7.11, it is evident that MCLEAs are more resistant against real wastewater matrices than the free laccase. The same conclusion was drawn in Chapter 6 for laccases immobilized onto fumed silica nanoparticles.

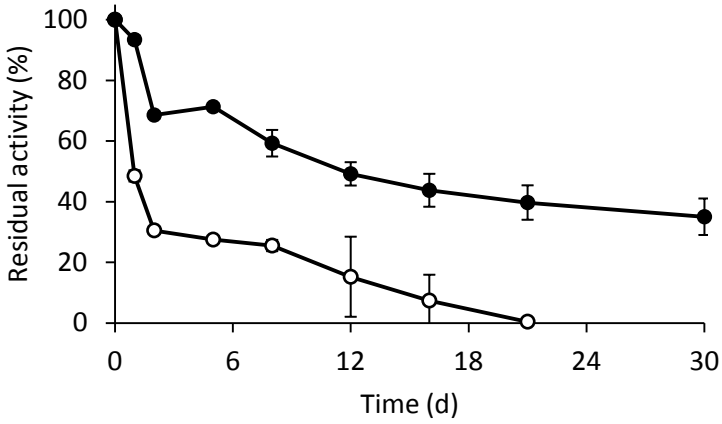

Figure 7.11 Residual activity of free laccase (○) and MCLEAs (●) during 30 d of incubation in a wastewater effluent collected from the WWTP of Magog (Qc, Canada).

7.3.3 Performance of MCLEAs in removal of pharmaceuticals from biologically treated wastewaters

In order to test the catalytic potential of the biocatalyst in real matrices, free laccase and MCLEAs were applied for the removal of a mixture of PhACs from spiked biologically treated wastewater. Although laccase mainly targets phenolic, aromatic and aliphatic amines, it was shown that the spectrum of laccase substrates can be broaden in mixtures when reactive radicals (e.g., phenoxyl radicals) are generated (Jeon et al. 2012). For this reason, the solution of PhACs was designed to include phenolic and aniline compounds, which are directly oxidizable by laccase, as well as non-laccase

substrates. Table 7.4 shows the PhACs concentrations in controls and reacting mixtures after the treatment. The enzymatic activity at the end of the experiment of 6 h-duration was 771±29 U/L and 898±63 U/L for free laccase and MCLEAs, respectively.

Table 7.4. Detected PhACs concentrations in the controls and reacting mixtures containing free laccase or MCLEAs after 6 h treatment

Compound	Control without biocatalyst (µg/L)	Control with deactivated MCLEAs (µg/L)	Free laccase (µg/L)	MCLEAs (µg/L)
Mefenamic acid	26.1 ± 1.3	24.1 ±1.27	22.3±1.6	15.0±0.5
Fenofibrate	32.6 ± 0.4	27.5±0.1	26.8±2.3	17.2±2.9
Indomethacin	19.7 ± 1.1	17.5 ±0.1	14.5±1.1	14.5±0.4
Acetaminophen	12.8 ±0.0	11.4 ±0.3	2.5±0.7	8.4±0.0
Caffeine	27.1 ±2.6	26.4 ±1.3	27.4±3.2	27.4±0.6
Ibuprofen	31.5 ±2.0	32.0±0.0	30.9±0.0	33.8±0.0
Naproxen	38.1 ±0.1	37.5±0.6	39.1±1.3	37.3±1.0
Ketoprofen	33.5 ± 2.2	33.2 ±2.3	33.5±5.0	31.5±1.5
Cyclophosphamide	27.5 ±1.1	27.1 ±0.3	28.4±2.3	28.5±1.7
Trimethoprim	15.5 ±1.2	15.7 ±2.0	15.3±3.4	15.8±0.0

The removal of the selected PhACs by free and immobilized laccases after 6-h treatment is depicted in Figure 7.12. For MCLEAs, the removal percentage may result from two mechanisms, sorption onto the MCLEAs and enzymatic transformation, although the contribution of sorption was low or negligible for the majority of the PhACs. Significant removal of 4 out 10 of the investigated PhACs was observed. Free laccase was capable of removing 80% of acetaminophen after 6 h of contact time; whereas for the MCLEAs the removal percentage was considerably lower: 34%. Acetaminophen is a phenolic compound susceptible to be degraded by laccase, as it has been demonstrated in previous research (Georgieva et al. 2010, Lu et al. 2009, Touahar et al. 2014). Lu et al. (2009) found that the laccase-catalyzed transformation of acetaminophen followed second-order kinetics on the concentrations of both

the substrate and the enzyme, and it reported a removal percentage of 73% (7.56 mg/L acetaminophen) by 1000 U/L of laccase after 1 h-treatment at pH 7.

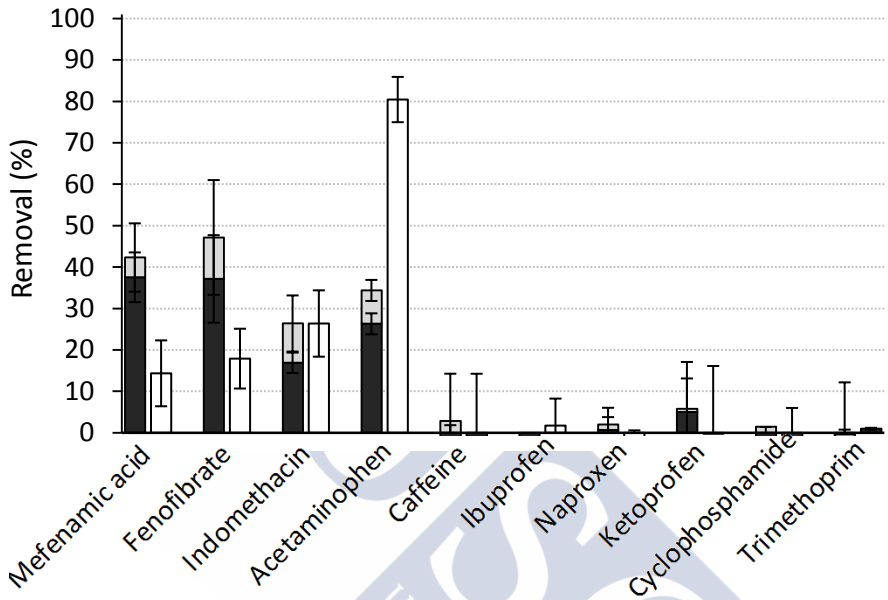

Figure 7.12. Removal of PhACs by adsorption (grey bar) and enzymatic transformation (black bar) by MCLEAs; and free laccase (white bar) in filtered secondary effluent at pH 7 from Magog WWTP for a contact time of 6 h and 30ºC.

The non-phenolic PhACs mefenamic acid, fenofibrate and indomethacin were partially removed by laccase. This fact may be explained based on the chemical structures. In general, electron donating groups (EDG) such as amine ($–NH_2$), hydroxyl (–OH), alkoxy (–OR), alkyl (–R) and acyl (–COR) are prone to oxidative attack whereas electron withdrawing groups (EWG) such as amide ($–CONR_2$), carboxylic (–COOH), halogen (–X), and nitro ($–NO_2$) group generates an electron deficiency and diminish the reactivity of the molecules (Yang et al. 2013). Mefenamic acid is an aniline compound and is known to be a substrate of laccase. Margot et al. (2013) studied the removal of mefenamic acid at high concentrations from buffered solutions and demonstrated the influence of initial activity and pH on the removal rate. For instance, for pH 7.2 and 888 U/L of laccase, the percentage of degradation was 39%; whereas for pH 5.7 and 562 U/L, 98% of mefenamic acid was transformed after 5.5 h. On the other hand, fenofibrate and indomethacin contain functional groups with electron donor properties in the laccase reaction as acyl in fenofibrate or alkoxy and

alkyl in indomethacin (Tran et al. 2010, Yang et al. 2013). Radicals resulting from the laccase treatment of the phenolic PhAC acetaminophen could interact with electron donor groups present on non-phenolic PhACs, enhancing their transformation (Touahar et al. 2014). Removal of 20% of fenofibrate (1 mg/L) after 14-h reaction at pH 5 and 750 U/L of free laccase was reported by the only previous work evaluating the laccase-catalyzed transformation of fenofibrate in a mixture of pharmaceuticals (Touahar et al. 2014). Tran et al. (2010) reported the complete removal of indomethacin from a mixture of PhACs under much more favorable conditions: 6000 U/L of free laccase in acetate buffer at pH 4.5. To our knowledge, no studies were found evaluating the laccase catalyzed-transformation of fenofibrate or indomethacin as single component.

Interestingly, the removal of mefenamic acid and fenofibrate was much higher for the MCLEAs (above 40% for both compounds) than for the free laccase. A possible reason could be the creation of a concentration gradient of substrate onto the surface of the MCLEAs. In this sense, the high hydrophobicity of mefenamic acid (K_{OW} of 5.12) and fenofibrate (K_{OW} of 5.19) may be promoting their absorption onto MCLEAs due to the presence of free vinyl groups, making these PhACs more available for enzymatic attack. Nevertheless, this is not evident from Figure 7.12, since sorption contribution for mefenamic acid and fenofibrate was similar to that for acetaminophen (which was removed faster by free laccase). Other possible explanation is relating to conformational changes and/or chemical modifications of enzymes after immobilization. In this way, it is common to observe a certain decline in enzyme activity towards its natural substrate, as it was observed for ABTS (Section 7.3.2.7). Nevertheless, in many cases the target substrate is quite far from the physiological one and it is not unlikely to obtain biocatalysts with superior specific activity towards a particular substrate (Rodrigues et al. 2013, Schoevaart et al. 2004). Such random hyperactivation produced by a particular immobilization method towards a particular substrate may be based on the casual generation of a more active enzyme form, and it would be more likely to occur with enzymes having a flexible active center. For instance, it was reported that porous CLEAs of papain displayed higher catalytic efficiency than the free enzyme towards BSA, but for the substrates N-benzoyl-L-arginine ethyl

ester (BAEE) and ovalbumin, free papain exhibited higher catalytic efficiency than the insolubilized enzyme (Wang et al. 2011). Therefore, the increased activity of MCLEAs towards mefenamic acid and fenofibrate may be justified based on this random hyperactivation. In the case of indomethacin, the percentage of transformation by free laccase and MCLEAs was almost identical, around 19% for both.

The remaining PhACs showed low or negligible enzymatic degradation (≤5%) after the 6-h treatment under the evaluated conditions. Whereas slight removal of naproxen by laccase was reported (Marco-Urrea et al. 2010a, Spina et al. 2013, Tran et al. 2010); ibuprofen and ketoprofen showed different behavior depending on its treatment as single pollutants, where no removal was appreciated (Marco-Urrea et al. 2010b, Marco-Urrea et al. 2009) or in a mixture, where partial removal was observed (Spina et al. 2015, Tran et al. 2010). In the current study, the low concentration of PhACs (in the range of μg/L) may be diminishing the chance to have cross reaction between radicals and pollutants, and the synergistic effect of other compounds mediating transformation of non-phenolic molecules could be limited (Margot 2015). The wastewater matrix could also affect someway the laccase-catalyzed transformation of PhACs. For instance, the low content of organic matter may hamper the transformation of PhACs.

Finally, caffeine and trimethoprim seems recalcitrant during the laccase treatment of a mixture of pharmaceuticals (Touahar et al. 2014). The *in vitro* degradation of cyclophosphamide by laccase had not been studied before but, in a recent work this antineoplasic drug (10 mg/L) showed neither degradation nor sorption by *T. versicolor* pellets after 8 d-treatment (Ferrando-Climent et al. 2015).

7.4 CONCLUSIONS

In this study, a rapid method to produce robust magnetically-separable CLEAs of laccase was developed. The MCLEAs showed higher stability against inhibitors, acidic pH and wastewater than the soluble laccases. In addition, they can be reused after the easy recovery by a magnetic field. The MCLEAs were applied for the elimination of a cocktail of PhACs from real secondary

effluent. The novel biocatalyst showed the ability to transform the phenolic compound acetaminophen and certain non-phenolic PhACs such as mefenamic acid, fenofibrate and indomethacin, with similar or even higher efficiency than free laccase. The use of MCLEAs could be an interesting alternative to remove targeted micropollutants from specific effluents after optimizing the removal conditions. On the contrary, if the aim is to decrease the concentration of a wide range of pollutants, as those present in secondary effluent from WWTPs, the use of single laccase does not seem an attractive option due to the limited range of substrates, and its combination with other enzymes could be proposed to solve this limitation.

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GENERAL CONCLUSIONS

This Thesis contributes to the development of continuous or semicontinuous technologies exploring the oxidative potential of laccases for the elimination of pollutants from real matrices.

The work developed in the present Thesis addressed the enzymatic removal of two types of pollutants. On the one hand, polycyclic aromatic hydrocarbon (PAHs), and more specifically, anthracene, which are toxic and persistent pollutants in soils due to their high hydrophobicity. The second type consisted in emerging organic contaminants (EOCs), such as endocrine disruptors or pharmaceutical active compounds (PhACs), which mainly enter the environment through wastewaters.

The following conclusions are drawn on these two main topics of this thesis:

Laccase-catalysis of the poor soluble compound anthracene:

1. Three different configurations of laccase-based reactors: micellar, biphasic and the combination of both, allowed the treatment of concentrations of anthracene, the hydrophobic model compound, considerably higher than that restricted by the solubility in aqueous phase.
2. The two-phase partitioning bioreactor (TPPB) with the addition of a surfactant (Triton X-100) at concentrations above the CMC was proved to be the best configuration to remove anthracene by *T. versicolor* laccase and the mediator 1-hydroxybenzotriazole (HBT).
3. The addition of Triton X-100 (1% v:v) improved mass transfer of anthracene from the organic phase, which consisted of 10%

silicone oil saturated in anthracene (1900 µM), to aqueous phase. Moreover, the surfactant enhanced the solubility of pollutant in the aqueous phase (from 0.08 to 9.4 mg/L), which favored the degradation kinetic. Additionally, Triton X-100 increased laccase stability, protecting it against deactivation, which was caused mainly by the mediator radicals. The combination of these effects led to the removal of higher loads of anthracene at fast oxidation rate (16 µmol/L_R·h), which was 1.5 times higher than that of micellar system with only Triton X-100 and 10 times higher than the achieved in the analogous two phase system lacking surfactant.

4. Anthraquinone was obtained as the main product of anthracene oxidation by laccase-HBT.
5. A model, based on the assumption that PAH must be first dissolved in the aqueous phase to be available for the catalytic oxidation of the enzyme, accurately predicted anthracene degradation in the TPPB. This consisted of mass balances in the organic phase, which considered the output rate of anthracene due to partition into the aqueous phase; and in the aqueous phase, taking into account both, input rate of anthracene due to its solubilization, as well as a pseudo first order kinetic for enzymatic oxidation.
6. The aqueous phase, containing the laccase-mediator system, could be reused in two additional oxidative cycles in the surfactant-assisted TPPB, with no extra addition of laccase or HBT.
7. The feasibility of reusing the silicone oil to dissolve anthracene and carry out subsequent degradation cycles was demonstrated.
8. The vegetable oils sunflower and pomace olive oils could act as reservoirs of anthracene in surfactant-assisted TPPB due to the high solubility of anthracene in the sunflower oil (15,000 µM) and pomace olive oil (27,350 µM).
9. Pomace olive oil was the best organic phase in TPPB to conduct the degradation of anthracene by the laccase-HBT system. The superior solubility of anthracene in the pomace olive oil than in sunflower and the higher enzymatic stability in comparison with silicone oil were demonstrated. In addition, oxy radicals generated during the peroxidation of unsaturated fatty acids of pomace olive oil by

laccase-HBT, likely contributed to the rapid degradation of anthracene in the system.

10. Oxygen played an important role in the system and the aeration or oxygenation increased the oxidation rate of anthracene. The depletion of HBT during the process required the periodic addition of this mediator to maintain a high oxidation rate of anthracene. The optimized conditions led to a removal rate of 30.3 μmol/L_R·h.
11. The experimental data were successfully modeled by applying mass balances in both phases. The higher value of the kinetic constant (170.7 h^{-1}) respect to the previous work with silicone oil (52.2 h^{-1}) could be just attributed to the higher concentration of mediator in the reaction medium as well as the lipid oxidation process. The overall mass transfer coefficient K_La (1573.1 h^{-1}) was two times higher than the obtained for the analogous TPPB with silicone oil (788.1 h^{-1}) due to the lower interfacial tension of the system pomace olive oil—1% Triton X-100 in comparison to the system silicone oil—1% Triton X-100.
12. A novel process based on the combination of soil extraction by pomace oil and enzymatic catalysis in a surfactant assisted TPPB was developed to remediate polluted soils. The vegetable oil, either fresh or regenerated, showed remarkably high efficiency (superior than 84.0%) in extracting anthracene from contaminated soil (1004 mg/kg). The aging of soil caused a slight reduction in the extractability of anthracene by the oil in comparison to the freshly contaminated soil.
13. The surfactant assisted TPPB operated with the laccase-HBT system allowed the regeneration of the contaminated pomace olive oil (elimination of anthracene) in 48 h.
14. The feasibility of reusing both the aqueous phase and the pomace olive oil in successive operations of the TPPB was demonstrated, which was advisable from an economic perspective. In addition, reusing the aqueous phase constituted a strategy to minimize the volume of waste needing to be managed, as aqueous phase would eventually constitute a residue of the global process.

Laccase-catalysis of EOCs from secondary effluents:

1. The redox potential of laccase, pH and laccase activity significantly, affected bisphenol A (BPA) transformation by laccase as it was demonstrated in batch experiments with model solutions. The best evaluated conditions were pH 6 and 1000 U/L of laccase from *T. versicolor*. Polymerization and biodegradation were the removal mechanisms of BPA by laccase.
2. The continuous removal of BPA (75 µg/L) by free laccase was possible by using an enzymatic membrane reactor (EMR), based on a continuous stirred tank reactor coupled to a ceramic nanofiltration membrane to retain the biocatalyst.
3. In buffered solution (pH 6), complete removal of BPA was attained for a hydraulic retention time of 10 h. In experiments with spiked real secondary effluent, the removal of BPA remained high while the matrix composition affected enzyme stability and made necessary the periodic addition of laccase.
4. Laccases from *Trametes versicolor* and *Myceliophthora thermophila* were successfully immobilized and co-immobilized onto fumed silica nanoparticles (fsNP). The resulting biocatalyst was reusable and exhibited higher stability than free laccases in WWTP effluent and at acid pH.
5. The transformation rate of BPA from spiked secondary effluent was slower for the immobilized enzymes and higher enzymatic activities of laccase-fsNP than of free enzymes were necessary to achieve the same degradation rate. The transformation of DCF could not be achieved with the tested biocatalysts under real WWTP effluent conditions and improvements should be considered to expand the substrate range (adjust pH, addition of natural mediators, etc.).
6. A rapid method to produce robust magnetically-separable cross linked enzyme aggregates (MCLEAs) of laccase from *T. versicolor*, was developed using magnetic mesoporous silica microbeads as support.

7. The MCLEAs showed higher stability against inhibitors, acidic pH and wastewater than the free laccase. In addition, they could be reused after the easy recovery by a magnetic field.
8. When applied for the elimination of a cocktail of PhACs from real secondary effluent, MCLEAs were able to transform the phenolic compound acetaminophen and certain non-phenolic PhACs as mefenamic acid, fenofibrate and indomethacin, with similar or even higher efficiency than the dissolved laccase; whereas several PhACs did not suffer any transformation. Therefore the use of MCLEAs could be an interesting alternative to remove target micropollutants from certain effluents containing remarkable concentrations of these contaminants rather than the use of the enzymatic system for large flows of WWTP effluents.

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CONCLUSIONES GENERALES

Esta tesis contribuye al desarrollo de tecnologías en continuo o semicontinuo, explotando el potencial oxidativo de las lacasas para la eliminación de contaminantes de matrices reales.

El trabajo desarrollado en la presente Tesis aborda la eliminación enzimática de dos tipos de contaminantes. Por un lado, los hidrocarburos aromáticos policíclicos (HAPs), y más específicamente, antraceno, compuestos de carácter tóxico y altamente persistentes en suelos debido a su alta hidrofobicidad. El segundo tipo consistió en contaminantes orgánicos emergentes, tales como compuestos disruptores endocrinos o compuestos con actividad farmacéutica, liberados al ambiente principalmente a través de aguas residuales.

De los dos temas principales de la tesis, se extraen las siguientes conclusiones específicas:

<u>Eliminación enzimática del compuesto de baja solubilidad antraceno</u>:

1. Tres configuraciones de reactores: micelar, bifásico y la combinación de ambos, permitieron el tratamiento de concentraciones de antraceno, el compuesto modelo por su alta hidrofobicidad, considerablemente superiores a las limitadas por la solubilidad en fase acuosa.
2. El reactor bifásico con la adición de un surfactante (Triton X-100) en concentraciones superiores a la concentración micelar crítica (CMC) demostró ser la mejor configuración para eliminar

antraceno por lacasa de *T. versicolor* y el mediador 1-hidroxibenzotriazol (HBT).

3. La adición de Triton X-100 mejoró la transferencia de masa de antraceno desde la fase orgánica, consistente en un 10% (v:v) de aceite de silicona saturado en antraceno (1900 µM), a la fase acuosa. Además, el surfactante incrementó la solubilidad del antraceno en la fase acuosa (de 0,08 a 9,4 mg/L), lo que favoreció la cinética de degradación. Adicionalmente, el Triton X-100 incrementó la estabilidad de la lacasa, protegiéndola contra la desactivación, causada mayoritariamente por los radicales del mediador. La combinación de estos efectos condujo a la eliminación de altas cargas de antraceno a una elevada velocidad de oxidación (16 µmol/L_R·h), 1,5 veces superior a la del sistema micelar con sólo Triton X-100, y 10 veces superior a la alcanzada en el reactor bifásico sin surfactante.
4. El principal producto de la oxidación de antraceno por el sistema lacasa-HBT fue la antraquinona.
5. Un modelo, basado en la premisa de que el HAP debe primero disolverse en la fase acuosa para estar disponible a la oxidación enzimática, predijo de modo satisfactorio la degradación de antraceno en el reactor bifásico con surfactante. Dicho modelo consistió en balances de masa en la fase orgánica, considerando la velocidad de reparto de antraceno en la fase acuosa; y en la fase acuosa, teniendo en cuenta la velocidad de transferencia del antraceno desde la fase orgánica, y una cinética de oxidación enzimática de pseudo primer orden con respecto al antraceno.
6. La fase acuosa, que contenía el sistema lacasa-mediador, pudo reutilizarse en dos ciclos oxidativos adicionales en el reactor bifásico con surfactante, sin adición extra de lacasa o HBT.
7. Asimismo, se demostró la viabilidad de reusar el aceite de silicona para disolver antraceno y llevar a cabo varios ciclos consecutivos de degradación.
8. Los aceites vegetales de girasol y de orujo de oliva pudieron usarse para disolver antraceno en el reactor bifásico con surfactante,

debido a la elevada solubilidad del antraceno en el aceite de girasol (15000 µM) y de orujo (27350 µM).

9. El aceite de orujo de oliva fue la mejor fase orgánica en el reactor bifásico para llevar a cabo la degradación de antraceno por el sistema lacasa-HBT. En el aceite de orujo se demostró una mayor solubilidad de antraceno que en el aceite de girasol y una mayor estabilidad enzimática en comparación con el aceite de silicona. Además, los oxi-radicales generados durante la peroxidación de los ácidos grasos insaturados del aceite de orujo por el sistema lacasa-HBT contribuyeron a la rápida degradación de antraceno en el sistema.
10. El oxígeno jugó un papel importante en el sistema y la aireación u oxigenación aumentaron la velocidad de oxidación del antraceno. El consumo de HBT durante el proceso requirió la adición periódica de este mediador para mantener una velocidad de oxidación elevada. Las condiciones optimizadas condujeron a una velocidad de eliminación de 30,3 µmol/L_R·h.
11. Los datos experimentales fueron modelados con éxito mediante la aplicación de balances de masa en ambas fases. El mayor valor de la constante cinética (170,7 h^{-1}) con respecto al trabajo previo con el aceite de silicona (52,2 h^{-1}) podría atribuirse a la mayor concentración de mediador en el medio de reacción, así como al proceso de oxidación lipídica. El coeficiente global de transferencia de masa K_La (1573,1 h^{-1}) fue dos veces superior que el obtenido para el mismo reactor bifásico con aceite de silicona (788,1 h^{-1}) debido a la menor tensión interfacial del sistema aceite de orujo - 1% Triton X- 100 en comparación con la del sistema aceite de silicona-1% de Triton X-100.
12. Se desarrolló un proceso novedoso para la remediación de suelos contaminados. Este se basó en la combinación de la extracción de suelo con aceite de orujo y la catálisis enzimática en un reactor bifásico con surfactante. El aceite vegetal, bien fresco o regenerado, mostró una elevada eficacia de extracción (superior al 84,0%) de antraceno del suelo contaminado (1004 mg/kg). El envejecimiento de suelo provocó una ligera reducción en la

extractabilidad del antraceno por el aceite en comparación con el suelo recién contaminado.

13. El reactor bifásico con surfactante operado con el sistema lacasa-mediador permitió la regeneración del aceite de orujo contaminado (eliminación del antraceno) en 48 h.
14. Se demostró la factibilidad de reutilizar tanto la fase acuosa como la orgánica en sucesivos ciclos de operación del reactor bifásico, lo cual es aconsejable desde un punto de vista económico. Además, la reutilización de la fase acuosa constituyó una estrategia para minimizar el volumen de residuo a gestionar, dado que dicha fase acaba constituyendo un residuo del proceso global.

Eliminación enzimática de compuestos orgánicos emergentes de efluentes secundarios por lacasa:

1. El potencial oxidativo de la lacasa, el pH y la actividad enzimática afectaron de modo significativo a la transformación de bisfenol A (BPA) por lacasa, como se demostró en experimentos en discontinuo con disoluciones modelo. Las mejores condiciones evaluadas fueron pH 6 y 1000 U/L de lacasa de *T. versicolor.* La polimerización y la biodegradación fueron los mecanismos de eliminación de BPA por lacasa.
2. La eliminación en continuo de BPA (75 µg/L) por lacasa libre fue posible mediante el empleo de un reactor enzimático de membrana (REM), basado en un reactor en continuo de tanque agitado acoplado a una membrana cerámica de nanofiltración para retener el biocatalizador en el sistema.
3. En una disolución tamponada (pH 6), se logró una eliminación completa de BPA para un tiempo de residencia hidráulica de 10 h. En experimentos con efluente secundario y misma concentración de BPA, la eliminación del contaminante se mantuvo alta, mientras que la composición de la matriz afectó a la estabilidad de la enzima e hizo necesaria la adición periódica de lacasa.
4. Lacasas de *Trametes versicolor* y *Myceliophthora thermophila* se inmovilizaron y coinmovilizaron con éxito en nanopartículas de sílice ahumada (fsNP). El biocatalizador resultante pudo reutilizarse

y exhibió una mayor estabilidad que las lacasas libres en efluentes de EDAR (estación depuradora de aguas residuales) y a pH ácido.

5. La velocidad de transformación de BPA en el efluente secundario contaminado fue inferior para las enzimas inmovilizadas y, para lograr la misma velocidad de degradación que con lacasa libre, se necesitaron actividades de lacasa inmovilizada superiores a las de la enzima libre. No se logró la transformación de diclofenaco en las condiciones reales de efluente de EDAR y deben considerarse mejoras para expandir el rango de substrato (ajustar el pH, adición de mediadores naturales, etc.).
6. Se desarrolló un proceso rápido para la producción de CLEAs magnéticos y robustos (MCLEAs) de lacasa de *T. versicolor*, utilizando microesferas magnéticas de sílice mesoporosa como soporte.
7. Los MCLEAs mostraron mayor estabilidad ante inhibidores, pH ácido y agua residual que la lacasa libre. Además, pudieron reutilizarse tras su fácil recuperación mediante un campo magnético.
8. Cuando se aplicaron a la eliminación de una mezcla de compuestos farmacéuticos de un efluente secundario real, los MCLEAs fueron capaces de transformar el compuesto fenólico acetaminofeno, y ciertos compuestos no fenólicos como el ácido mefenámico, indometacina y fenofibrato, con una eficacia similar o incluso mayor que la lacasa libre; mientras que otros farmacéuticos no sufrieron transformación alguna. Por lo tanto, más que para el tratamiento de caudales elevados de efluentes de EDARs, el empleo de MCLEAs podría ser una alternativa interesante para eliminar concentraciones notables de ciertos microcontaminantes presentes en determinados efluentes.

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CONCLUSIÓNS XERAIS

Esta Tese contribúe ó desenvolvemento de tecnoloxías en continuo ou semicontinuo, aproveitando o potencial oxidativo das lacasas para a eliminación de contaminantes de matrices reais.

O traballo desenrolado na presente Tese aborda a eliminación enzimática de dous tipos de contaminantes. Por unha banda, os hidrocarburos aromáticos policíclicos (HAPs), e máis especificamente, antraceno, compostos de carácter tóxico e altamente persistentes en solos debido a súa alta hidrofobicidade. O segundo tipo estivo constituído polos contaminantes orgánicos emerxentes, tales como compostos disruptores endocrinos ou compostos con actividade farmacéutica, liberados ó ambiente principalmente a través das augas residuais.

Dos dous temas principais da tese, extráense as seguintes conclusións específicas:

<u>Eliminación enzimática do composto de baixa solubilidade antraceno:</u>

1. Tres configuracións de reactores: micelar, bifásico e a combinación de ambos, permitiron o tratamento de concentracións de antraceno, o composto modelo pola súa alta hidrofobicidade, considerablemente superiores ás limitadas pola solubilidade na fase acuosa.
2. O reactor bifásico coa adición dun tensioactivo (Triton X-100) en concentracións superiores á concentración micelar crítica (CMC) demostrou ser a mellor configuración para eliminar o antraceno pola lacasa de *T. versicolor* e o mediador 1-hidroxibenzotriazol (HBT).

3. A adición de Triton X-100 mellorou a transferencia de masa do antraceno desde a fase orgánica, consistente nun 10% (v:v) de aceite de silicona saturado en antraceno (1900 µM), á fase acuosa. Ademais, o tensioactivo aumentou a solubilidade do antraceno na fase acuosa (de 0,08 a 9,4 mg/L), o que favoreceu a cinética de degradación. Adicionalmente, o Triton X-100 incrementou a estabilidade da lacasa, protexéndoa contra a desactivación, causada maioritariamente polos radicais do mediador. A combinación destes efectos conduciu á eliminación de altas cargas de antraceno a unha velocidade de oxidación elevada (16 µmol/ L_R·h), 1,5 veces superior á do sistema micelar con só Triton X-100, e 10 veces superior á acadada no reactor bifásico sen tensioactivo.

4. O principal produto da oxidación do antraceno polo sistema lacasa-HBT foi a antraquinona.
5. Un modelo baseado na premisa de que o HAP debe primeiro disolverse na fase acuosa para estar dispoñible á oxidación enzimática, previu de xeito satisfactorio a degradación do antraceno no reactor bifásico co tensioactivo. O devandito modelo consistiu en balances de masa na fase orgánica, considerando a velocidade de reparto do antraceno na fase acuosa; e na fase acuosa, tendo en conta a velocidade de transferencia do antraceno desde a fase orgánica, e unha cinética de oxidación enzimática de primeira orde con respecto ó antraceno.
6. A fase acuosa que contiña o sistema lacasa-mediador, púidose reutilizar en dous ciclos adicionais de oxidación no reactor bifásico co tensioactivo, sen adición extra de lacasa ou HBT.
7. Do mesmo xeito, demostrouse a viabilidade de reutilizar o aceite de silicona para disolver antraceno e levar a cabo ciclos consecutivos de degradación.
8. Os aceites vexetais de xirasol e de bagazo de oliva puideron utilizarse para disolver o antraceno no reactor bifásico con tensioactivo, debido á alta solubilidade do antraceno no aceite de xirasol (15000 µM) e de bagazo (27350 µM).

9. O aceite de bagazo de oliva resultou ser a mellor fase orgánica no reactor bifásico para levar a cabo a degradación do antraceno polo sistema lacasa-HBT. O aceite de bagazo demostrou unha maior estabilidade enzimática en comparación co aceite de silicona. Ademais, os oxi-radicais xerados durante a peroxidación dos ácidos grasos insaturados do aceite de bagazo de oliva polo sistema lacasa-HBT contribuíron á rápida degradación do antraceno no sistema.
10. O osíxeno xogou un papel importante no sistema e a aireación ou osixenación aumentaron a velocidade de oxidación do antraceno. O consumo do HBT durante o proceso requiriu a adición periódica do devandito mediador para manter unha velocidade de oxidación de antraceno elevada. As condicións optimizadas conduciron a unha velocidad de eliminación de 30,3 $\mu mol/L_R \cdot h$.
11. Os datos experimentais modeláronse con éxito mediante a aplicación de balances de masa en ambas fases. O maior valor da constante de velocidade (170,7 h^{-1}) en comparación co traballo anterior usando aceite de silicona (52,2 h^{-1}) podería atribuírse á maior concentración de mediador no medio de reacción así como ó proceso de oxidación lipídica. O coeficiente global de transferencia de masa K_La (1573.1 h^{-1}) foi o dobre do obtido para o mesmo reactor de dúas fases con aceite de silicona (788,1 h^{-1}) debido á menor tensión interfacial do sistema aceite de bagazo - 1% de Triton X-100 en comparación co aceite de silicona-1% de Triton X-100.
12. Desenvolveuse un proceso novidoso para a remediación de solos contaminados. Este baseouse na combinación da extracción do solo co aceite de bagazo e a catálise enzimática nun reactor bifásico con tensioactivo. O aceite vexetal, ben fresco ou rexenerado, mostrou unha elevada eficacia de extracción (superior ó 84,0%) do antraceno do solo contaminado (1004 mg/ kg). O envellecemento do solo provocou unha lixeira redución na extractabilidade do antraceno polo aceite en relación ó solo recén contaminado.

13. O reactor bifásico con tensioactivo operado co sistema lacasa-mediador permitiu a rexeneración de aceite de bagazo contaminado (eliminación do antraceno) en 48 h.
14. Demostrouse a viabilidade de reutilizar tanto a fase acuosa como a fase orgánica en sucesivos ciclos de operación do reactor bifásico, o que é desexable desde o punto de vista económico. Ademais, a reutilización da fase acuosa constituíu unha estratexia para minimizar o volume de residuos a ser xestionado, xa que a devandita fase acaba por constituír un residuo do proceso global.

Eliminación enzimática de compostos orgánicos emerxentes de efluentes secundarios por lacasa:

1. O potencial oxidativo da lacasa, o pH e a actividade enzimática afectaron de xeito significativo á transformación do bisfenol A (BPA), pola lacasa, como se demostrou en experimentos en discontinuo con disolucións modelo. As mellores condicións avaliadas foron pH 6 e 1000 U/L de lacasa de *T. versicolor*. A polimerización e a biodegradación foron os mecanismos de eliminación do BPA pola lacasa.
2. A eliminación en continuo do BPA (75 µg/L) pola lacasa libre foi posible mediante o uso dun reactor enzimático de membrana (REM), baseado nun reactor en continuo de tanque axitado acoplado a unha membrana cerámica de nanofiltración para reter o biocatalizador no sistema.
3. En solución tamponada (pH 6) logrouse a eliminación completa do BPA para un tempo de residencia hidráulico de 10 h. En experimentos con efluente secundario e a mesma concentración de BPA, a eliminación do contaminante mantívose alta, mentres que a composición da matriz afectou á estabilidade da enzima e fixo necesaria a adición periódica da lacasa.
4. Lacasas de *Trametes versicolor* e *Myceliophthora thermophila* inmobilizáronse e coinmobilizáronse con éxito en nanopartículas de sílice afumada (fsNP). O biocatalizador resultante foi reutilizable e exhibiu unha maior estabilidade que as lacasas libres en efluentes de EDAR (estación depuradora de augas residuais) e a pH ácido.

5. A velocidade de transformación de BPA do efluente secundario contaminado foi inferior para as enzimas inmobilizadas e, para lograr a mesma velocidade de transformación cá da lacasa libre, precisáronse actividades de lacasa inmobilizada superiores ás da enzima libre. Non se logrou a transformación do diclofenaco nas condicións reais do efluente da EDAR e deben considerarse melloras para expandir o rango de substratos (axustar o pH, adición de mediadores naturais, etc.).
6. Desenvolveuse un proceso rápido para a produción de CLEAs magnéticos e robustos (MCLEAs) de lacasa de *T. versicolor*, utilizando microesferas magnéticas de sílice mesoporosa como soporte.
7. Os MCLEAs mostraron maior estabilidade contra inhibidores, pH ácido e auga residual cá lacasa libre. Ademais, puideron reutilizarse tras a súa fácil recuperación mediante un campo magnético.
8. Cando se aplicaron á eliminación dunha mestura de compostos farmacéuticos dun efluente secundario real, os MCLEAs foron capaces de transformar o composto fenólico acetaminofeno, e determinados compostos non fenólicos como ácido mefenámico, indometacina e fenofibrato, cunha eficiencia semellante ou mesmo maior que a lacasa libre; mentres que outros compostos farmacéuticos non sufriron transformación ningunha. Polo tanto, mais que para o tratamento de caudais elevados de EDARs, o emprego de MCLEAs podería ser unha alternativa interesante para eliminar concentracións notables de certos microcontaminantes presentes en determinados efluentes.

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LIST OF ACRONYMS

A	Activity
ABTS	2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AC	Activated carbon
ANT	Anthracene
AOPs	Advanced oxidation processes
APTES	3-aminopropyltriethoxysilane
BAEE	N-benzoyl-l-arginine ethyl ester
BCA	Bicinchoninic acid
BDD	Boron-doped diamond
BOD	Biological oxygen demand
BPA	Bisphenol A
BSA	Bovine serum albumin
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
BTX	Benzene, toluene, xylene
CLEAs	Cross-linked enzyme aggregates
CMC	Critical micellar concentration
COD	Chemical oxygen demand
d	Sauter mean droplet diameter
DCF	Diclofenac
D_i	Impeller diameter and
DOC	Dissolved organic carbon
E_0	Redox potential
EDC	Endocrine disrupting compounds
EDG	Electron donating groups
EI	Electron impact
EMR	Enzymatic membrane reactor

EOCs	Emerging organic contaminants
ESI	Electrospray ionization
ESI-TOF MS	Electrospray ionization time of flight mass spectrometry
EWG	Electron withdrawing groups
FAME	Fatty acid methyl esters
FDA	Food and drug administration
FIA	Flow injection mode
FsNP	Fumed silica nanoparticles
FTIR	Fourier transform infrared
GAC	Granular activated carbon
GC	Gas chromatography
HBT	1-Hydroxybenzotriazole
H_{ci}	Coercive fields
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
IC	Inorganic carbon
IP	Isoelectric point
IY	Immobilization yield
k	First order kinetic constant
Ka	Dissociation constant
K_{cat}	Turnover number
K_La	Overall mass transfer coefficient
K_M	Michaelis constant
K_{mc}	Micelle phase/aqueous phase partition coefficient
K_{OW}	Octanol-water coefficients
K_{SW}	Anthracene partitioning coefficient
Lac	Laccase
LCEFM	Laccase-carrying electrospun fibrous membranes
LiP	Lignin peroxidase
LMEs	Lignin-modifying enzymes
LSC	Liquid scintillation counter
MCLEAs	Magnetically-separable cross-linked enzyme aggregates
MF	Microfiltration
MMSMB	Magnetic mesoporous silica microbeads
MnP	Manganese peroxidase

MPS	Mesoporous silica
M_r	Remnant magnetization
M_s	Saturation magnetization
MS	MS mass spectrometry
MSR	Molar solubilization ratio
MTBSTFA	N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide
MtL	Laccase from *Myceliophthora thermophila*
MW	Molecular weight
N	Impeller speed
NaOAc	Sodium acetate
NAPL	Non-aqueous phase liquid
NF	Nanofiltration
NOEC	Non observed effect concentration
NSAID	Non-steroidal anti-inflammatory drug
NTP	National toxicology program
PAC	Powdered activated carbon
PAHs	Polycyclic aromatic hydrocarbons
PCP	Pentachlorophenol
PhAC	Pharmaceutical active compound
PNEC	Predicted non effect concentration
PPCPs	Pharmaceuticals and personal care products
PROD	Products
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
RO	Reverse osmosis
S	Substrate molecule
$S^{\bullet}$	Oxidized substrate radicals
SAI	Sorption-assisted immobilization
S_{aq}	Anthracene concentrations in the aqueous phase
S_{or}	Anthracene concentrations in the organic phase
SPE	Solid phase extraction
T1, T2, T3	Type 1, Type 2,Type 3 copper site in laccase
TC	Total carbon
TMP	Trans-membrane pressure
TOC	Total organic carbon
TPPB	Two phase partitioning bioreactor

TvL	Laccase from *Trametes versicolor*
U	Unit of enzymatic activity
UF	Ultrafiltration
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
V_{aq}	Volume of aqueous phase
VIS	Visible
V_{org}	Volume of organic phase
VP	Versatile peroxidase
VSM	Vibrating sample magnetometer
We	Weber number
WL	Washing loss
WRF	White rot fungi
WWTP	Wastewater treatment plant
α, β	First order inactivation constants
ε	Molar extinction coefficient
ρ_d	Dispersion density
σ	Interfacial tension
Φ	Fraction of the dispersed phase

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AGRADECIMIENTOS

La presente Tesis Doctoral, realizada en el Grupo de Ingeniería Ambiental y Bioprocesos del Departamento de Ingeniería Química, USC, ha sido cofinanciada por una beca FPU (AP2010-2086) del Ministerio de Educación, Cultura y Deporte; y por los proyectos CTQ2013-44762-R y CTQ2010-20258 del Ministerio de Economía y Competitividad.

Para terminar, quisiera dedicar unas líneas para expresar mis agradecimientos a todos los que me habéis ayudado de un modo u otro a hacer posible la realización de este trabajo.

En primer lugar, a mis directoras de Tesis, la Dra. Gemma Eibes y la Prof. Maite Moreira, por su esfuerzo y dedicación durante estos cinco años. Maite, muchas gracias por darme la oportunidad de entrar en el Biogrup y por el tiempo dedicado al desarrollo de este trabajo. Gemma, además de por el aporte científico, gracias por tu paciencia, por estar siempre disponible para resolver mis dudas inmediatas y por tu optimismo para impulsar los proyectos de la Tesis. Asimismo, quiero expresar mi gratitud al Prof. Juan Lema, que también dirigió este trabajo pese a no firmar la primera página, por preocuparse por el desarrollo de la tesis y por sus siempre brillantes ideas y sugerencias en las intensas reuniones de equipo. Al Prof. Gumersindo Feijoo le agradezco el esfuerzo realizado para seguir muy de cerca este trabajo, haciendo huecos en su apretada agenda para las reuniones. Al Dr. Thelmo, le estoy muy agradecida por estar siempre dispuesto a ayudar y resolver los mil y un problemas técnicos de los reactores, así como por las interesantes ideas aportadas.

Gracias al personal técnico y administrativo del Biogrup, Carmen, Miriam, Pili, Sonia, Rosa y especialmente, Mar y Mónica. Además de por el tiempo dedicado al análisis de cada muestra y cada cromatograma, gracias por atender todas mis dudas y peticiones, siempre con una sonrisa.

I would also like to acknowledge Prof. Corvini, for giving me the opportunity to do a research stay at the Institute for Ecopreneurship of the FHNNW, in Basel; to Drs. Erik Ammann and Christoph A. Gasser, for the supervision of my work there, and Dr. Peter Nastold, who patiently helped me with the analysis of samples. I am also very grateful to Prof. Hubert Cabana, for giving me the chance

to do a research stay in the Université de Sherbrooke, for helping me to enter Canada in the longest day of my life, and for the supervision of my work, along with Dr. V.V Kumar.

Me gustaría agradecer de modo muy especial a mis primeros compañeros de fermentación, Angélica, Lucía Lloret, Paula D. y Rober, por sus consejos, ideas y discusiones sobre enzimas, y porque fueron como hermanos mayores en mis inicios en el Biogrup. También a Carlos, ¡qué tiempos aquellos donde compartíamos la cámara de flujo laminar!, y a todos los que pasaron de estancia por el laboratorio 2.3: Inés, Tatjana, Ángela, Enrique, Jazmín, Andrea, Luca… Gracias también a María y Yolanda, por estos meses compartiendo el laboratorio, y por las charlas transoceánicas vía skype. Del mismo modo, me gustaría dar las gracias a TOD@S los compañeros que forman o formaron parte del Biogrup: líneas de aguas, codigestión, y ACV, tanto de la escuela como del instituto; siempre dispuestos a echar un cable, y artífices del ambiente de compañerismo que se respira en el grupo: Leti, Edu, Rubén, Daga, Isaac, Rebe, Álvaro, Tere, Tania, María G., Ángeles, Santi C., Iván, Santi G., Sara, Andrea, Pedro, Lidia, Lorena, Lucía, Tomás, Antón, Carmen, Stefano, Alba, Xela, Andrés, Alberte, Laura, Chiara, Dafne, Natalia, Isa, Carolina, Pedro, Yago… Gracias Paula P., por una década de amistad. Muchas gracias Nico, porque un día te decidiste a retarme a un partido de tenis, en el que ganamos los dos, por aguantar todas mis conversaciones sobre el recurrente tema de la Tesis y por tu ayuda con la edición del documento. Asimismo, muchas gracias a todos aquellos que contribuyeron a que mis estancias en Sherbrooke, y especialmente en Basel, con el Rin, fuesen geniales: Marc, Arantxa, Malin, Michael, Arielle…

Me gustaría agradecer a todos mis amigos que me han estado apoyando y animando durante estos años. Gracias Alba, por las amenas conversaciones en el piso tras la jornada laboral. A Mª Teresa, por todas las tardes de sol junto al mar.

Por último, muchísimas gracias a mi hermano y a mis padres, que constituyen un pilar fundamental, por su apoyo incondicional, sus consejos, por los valores que me inculcaron y por sus palabras de ánimo, fundamentales para llegar hasta aquí.