UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Enxeñaría Química

Enzymatic bioreactors for the oxidation of estrogenic and anti-inflammatory compounds by laccases

Memoria presentada por

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Para optar ao grao de Doutor pola

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Que a presente memoria, titulada “Enzymatic bioreactors for the oxidation of estrogenic and anti-inflammatory compounds by laccases” presentada por Dª Lucía Lloret Caulonga para optar ao grao de Doutor en Enxeñería Química, Programa de Doutoramento en Enxeñería Química e Ambiental, realizouse baixo a nosa inmediata dirección no Departamento de Enxeñaría Química da Universidade de Santiago de Compostela.

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Resumo

Durante a última década, o interese arredor do impacto medioambiental dos estróxenos presentes en augas residuais urbanas creceu debido á súa capacidade de modificación do sistema endócrino de animais e humanos. Así mesmo, a descarga ao medioambiente de compostos antiinflamatorios supón tamén un foco de interese a causa do impacto negativo destes compostos, por exemplo, citotoxicidade para o fígado e o ril de certas especies. A degradación destes tipos de compostos supón un reto medioambiental debido ás súas complexas estruturas e limitada biodispoñibilidade. De feito, estes compostos foron detectados nos efluentes de plantas de tratamento de augas a diferentes concentracións que varían de nanogramos a microgramos por litro, xa que certos tratamentos convencionais só proporcionan eficacias parciais de transformación. Por tanto, investigáronse métodos alternativos coa fin de superar ese reto medioambiental, como métodos físicos (por exemplo sorción e separación con membranas), a eliminación con bacterias e algas e os procesos de oxidación avanzada (AOPs, polas súas siglas en inglés) (por exemplo ozonización, UV/H_2O_2, Fenton, etc). En xeral, os AOPs proporcionan altas taxas de eliminación pero baixa selectividade; non obstante, estes métodos implican nalgúns casos considerables costes asociados aos reactivos e/ou investimento, a formación de subproductos nocivos ou incluso a xeración de compostos con estroxenicidade maior que a do substrato inicial e/ou non biodegradables.

Unha alternativa avanzada podería basearse no uso de cultivos de fungos de podredume branca. Demostrouse que estes microorganismos son capaces de eliminar unha ampla gama de xenobióticos pola acción das súas enzimas oxidativas fúnxicas , como manganeso peroxidasa, lignina peroxidasa e lacasa; así pois, proponse neste traballo o uso de enzimas para levar a cabo a eliminación de estróxenos e antiinflamatorios. O uso de enzimas en procesos industriais está comúnmente ligado a redución de consumo de enerxía e reactivos e ademais, as enzimas poden normalmente reaccionar en condicións moderadas. No caso das lacasas (oxidasas con contido en cobre, EC 1.10.3.2), o uso de osíxeno como aceptor de electróns representaría unha vantaxe adicional para a aplicación destas enzimas en comparación con peroxidasas. En concreto, investigouse neste traballo o uso de lacasas de *Myceliophthora thermophila* e *Trametes versicolor* para a oxidación dos estróxenos estrona (E1), 17β-estradiol (E2) e 17α-etinilestradiol (EE2) e dos antiinflamatorios naproxeno (NPX) e diclofenaco (DCF). O obxectivo principal desta investigación é o desenvolvemento de tecnoloxía para

levar a cabo a oxidación catalizada por lacasas dos compostos anteriomente mencionados.

Nunha primeira etapa do traballo investigouse o uso de lacasas así como dos sistemas lacasa-mediador en reactores operados en modo discontinuo (Capítulo 2). Avaliáronse dúas lacasas e estudáronse os efectos do pH (que afecta non só á actividade e á estabilidade da enzima, senón tamén aos substratos obxectivo e á acción do mediador) e mediador (tipo e concentración) tanto sobre a eficacia de eliminación como na estabilidade da enzima, co obxectivo de establecer as bases para a óptima transformación deste tipo de compostos catalizada por lacasas. No caso dos estróxenos, estes compostos eliminároнse de maneira eficiente mediante lacasa de *Myceliophthora thermophila* incluso en ausencia de mediador e a pH neutro, o cal facilitará a aplicación do tratamento enzimático na descontaminación das augas residuais. Os antiinflamatorios foron transformados por lacasas de *Myceliophthora thermophila* e *Trametes versicolor* con rendementos variables. Ademais, avaliouse a toxicidade dos produtos de biotransformación xerados a partir das reaccións enzimáticas: demostrouse que as reaccións catalizadas por lacasa permiten a redución da estroxenicidade do medio que contén os compostos estroxénicos mediante análise LYES; demostrouse tamén que os subproductos de DCF presentan unha considerable maior biodegradabilidade aeróbica e a eliminación de toxicidade comprobouse tamén mediante ensaios Microtox®.

En vista dos resultados prometedores obtidos, levouse a cabo a operación en continuo de diferentes configuracións de biorreactor para a transformación dos compostos de interese: para este propósito, é común a inmobilización das enzimas para así permitir a separación do biocatalizador ou ben as enzimas son separadas do efluente mediante sistemas de membrana. Neste traballo de investigación ámbalas dúas alternativas foron evaluadas, desenvolvendo tecnoloxías factibles para tal fin.

Lacasa de *Myceliophthora thermophila* foi inmobilizada mediante diferentes procedementos para a aplicación dos biocatalizadores en reactores de leito fixo e de leito fluidizado (PBRs e FBRs, polas súas siglas en inglés) para a eliminación de E1, E2 e E2 (Capítulo 3). A enzima inmobilizouse mediante a súa encapsulación en matrices *sol-gel* mediante reaccións de hidrólise e polimerización de compostos silanos o que deu como resultado a formación dun hidroxel contendo a lacasa no seu interior; levouse tamén a cabo a inmobilización mediante unión covalente a soportes comerciais acrílicos que conteñen grupos epóxido activados: Eupergit C e Eupergit C 250L. Obtivéronse altas eficacias de inmobilización da proteína de 44-99 e 59-83% e actividades entre 1-80 e 5-17 U/g

para a enzima inmobilizada covalentemente e encapsulada, respectivamente. A menor eficacia catalítica da enzima inmobilizada mediante ámbolos dous métodos en comparación coa enzima libre compensouse co aumento da estabilidade e capacidade catalítica nun maior rango de temperatura, pH e en presenza de axentes inactivantes. Avaliouse a operabilidade dos PBRs mediante a súa aplicación na eliminación dun tinte sintético usado como composto modelo e ademais, os biorreactores foron operados en ciclos continuos consecutivos para verificar o potencial reúso dos biocatalizadores. Os PBRs foron aplicados nun seguinte paso da investigación na eliminación en continuo de E1, E2 e EE2, obtendo rendementos de 55-75 e 65-80% para a lacasa encapsulada e inmobilizada covalentemente, respectivamente; aproximadamente un 6-14 e 11-12% da eliminación atribuíuse á adsorción dos substratos. Non obstante, propoñeuse un FBR debido ás desvantaxes relacionadas co uso dos PBRs (aireación pobre ou pasiva, deficiente transferencia de materia e a formación de camiños preferenciais, etc.). Este sistema resultou ser factible na eliminación de substratos estroxénicos a concentracións no intervalo de 10-100 µg/L, e tanto o biorreactor como o biocatalizador foron estables durante máis de 10 días de operación; logrouse ademais unha redución da estroxenicidade do 90%.

Posteriormente, desenvolveuse e avaliouse o uso dun reactor enzimático de membrana (EMR, polas súas siglas en inglés) para a eliminación en continuo de estróxenos mediante lacasa libre de *Myceliophthora thermophila* (Capítulo 4). Nunha primeira etapa, leváronse a cabo experimentos en operación semi-continua (*fed-batch*) co obxectivo de investigar o efecto da aireación/osixenación, a velocidade de adición dos substratos e a actividade da lacasa, para a posterior realización de ensaios preliminares en continuo nun EMR de 370 mL. Con todo, o método convencional baseado no estudo individual de cada variable para a optimización do proceso non é o procedemento máis axeitado, e ademais consume moito tempo e é laborioso e incompleto. Polo tanto, a metodoloxía de superficie de resposta (RSM, polas súas siglas en inglés) úsase como alternativa adecuada. A aplicación de RSM permitiu a avaliación dos efectos individuais e cruzados das variables estudadas: osixenación, tempo de residencia hidráulico (TRH) e a actividade enzimática, na transformación en continuo dos substratos; por outra banda, investigáronse e optimizáronse diferentes variables de resposta: i) velocidade de eliminación, ii) velocidade de eliminación por unidades de enzima utilizadas (co obxectivo de minimizar os custos da tecnoloxía), e iii) a porcentaxe de redución da actividade estroxénica (coa fin de optimizar non só a eliminación dos compostos senón tamén da redución de toxicidade). Atopouse

unha actividade óptima de tan so 100 U/L para maximizar a eficacia da enzima: E1 oxidouse a una taxa de 0,06 mg/(L·h·U), aínda que a eliminación de estroxenicidade foi do 60%. Así mesmo, os valores máis elevados ensaiados (1000 U/L, TRH 4 h e 60 mg O_2/(L·h)) permitiron unha completa descontaminación. Para rematar, operouse un EMR de 2 L, durante 100 h e con 100 U/L de enzima, para corroborar a aplicabilidade real da tecnoloxía desenvolvida: o biorreactor foi probado para o tratamento enzimático de efluentes secundarios, recollidos nunha planta municipal de tratamento de augas, contendo E1, E2 e EE2 a unha concentración de 100 µg/L así como a niveis ambientais (0,29-1,52 ng/L). Acadáronse rendementos altos de eliminación (80-100 %) malia a inactivación parcial da enzima (ao redor de 20 % dentro das primeiras horas).

Por outro lado, existe en bibliografía unha falta significativa de coñecemento con respecto á identificación dos produtos de reacción dos compostos de interese porén da crecente investigación sobre a aplicación de enzimas fúnxicas para a oxidación de fármacos e compostos disruptores endócrinos. Así pois, traballouse nesta investigación para levar a cabo a identificación dos produtos de reacción de E1, E2 e EE2, así como os de DCF, resultantes da súa transformación mediante lacasas, como un primeiro paso para dilucidar as principais vías de reacción; con esta fin, aplicáronse diferentes técnicas analíticas como GC-MS, LC-APCI e LC-ESI-TOF (Capítulo 5). Demostrouse a formación de dímeros e trímeros de estróxenos a partir da reacción catalizada por lacasa de *Myceliophthora thermophila*, así como a transformación de E2 en E1, mediante a análise dos espectros obtidos e os posibles patróns de fragmentación, e foi corroborada pola determinación de masas exactas. Identificáronse ademais varios produtos de transformación de DCF como resultado de reaccións de descarboxilación catalizadas por lacasa de *Trametes versicolor*.

Na última etapa da investigación explorouse unha tecnoloxía emerxente: desenvolvéronse, caracterizáronse e aplicáronse microrreactores con lacasa inmobilizada co obxectivo de mellorar a eficiencia dos procesos catalizados por lacasas e ampliar o campo de aplicación destas enzimas (Capítulo 6). Demostráronse con anterioridade as vantaxes asociadas aos microrreactores: diminución dos volumes de reacción, alta eficacia e repetibilidade, aumento da eficacia de intercambio de calor e transferencia de materia, o control estrito das condicións de reacción grazas ao seu característico fluxo laminar, etc. Con todo, tan só uns poucos estudos previos consideraron estes sistemas para a aplicación de lacasas. Nesta investigación desenvolveuse un método novedoso, sinxelo, versátil e de baixo custo para a fabricación de microrreactores enzimáticos,

baseados na formación dunha membrana de lacasa de *Trametes versicolor* inmobilizada nas paredes internas de microtubos como resultado de reaccións de *cross-linking* e polimerización. Estes microrreactores empregáronse para a oxidación dos compostos de interese, obtendo elevados rendementos para tempos de residencia reducidos. Ademais, deseñouse un sistema de microrreactor en dúas etapas para a prevención da inactivación do biocatalizador cando se opera baixo condicións adversas para a enzima.

Resumen

Durante la última década, el interés acerca de impacto medioambiental de los estrógenos presentes en aguas residuales urbanas ha crecido debido a su capacidad de modificación del sistema endocrino de animales y humanos. Asimismo, la descarga al ambiente de compuestos antiinflamatorios supone también un foco de interés a causa del impacto negativo de estos compuestos, por ejemplo, citotoxicidad para el hígado y riñón de ciertas especies. La degradación de estos tipos de compuestos supone un reto medioambiental debido a sus complejas estructuras y limitada biodisponibilidad. De hecho, estos compuestos han sido detectados en los efluentes de plantas de tratamiento de aguas a diferentes concentraciones que varían de nanogramos a microgramos por litro, ya que ciertos tratamientos convencionales solo proporcionan eficacias parciales de transformación. Por tanto, se han investigado métodos alternativos con el fin de superar ese reto medioambiental, tales como métodos físicos (por ejemplo mediante sorción y separación con membranas), la eliminación con bacterias y algas y los procesos de oxidación avanzada (AOPs, por sus siglas en inglés) (por ejemplo ozonización, UV/H_2O_2, Fenton, etc). En general, los AOPs proporcionan altas tasas de eliminación pero baja selectividad; sin embargo, estos métodos implican en algunos casos considerables costes asociados a los reactivos y/o inversión, la formación de subproductos nocivos e incluso la generación de compuestos con estrogenicidad más alta que el sustrato inicial y/o no biodegradables.

Una alternativa avanzada podría estar basada en el uso de hongos de podredumbre blanca. Se ha demostrado que estos microorganismos son capaces de eliminar una amplia gama de xenobióticos por la acción de sus enzimas oxidativas fúngicas, como manganeso peroxidasa, lignina peroxidasa y lacasa; así pues, se propone en este trabajo el uso de enzimas para llevar a cabo la eliminación de estrógenos y antiinflamatorios. El uso de enzimas en procesos industriales está comúnmente ligado a una reducción en el consumo de energía y reactivos y además, las enzimas puede reaccionar normalmente en condiciones moderadas. En el caso de las lacasas (oxidasas con contenido en cobre, EC 1.10.3.2), el uso de oxígeno como aceptor de electrones representaría una ventaja adicional para la aplicación de estas enzimas en comparación con peroxidasas. Específicamente, en este trabajo se investigó el uso de lacasas de *Myceliophthora thermophila* y *Trametes versicolor* para la oxidación de los estrógenos estrona (E1), 17β-estradiol (E2) y 17α-etinilestradiol (EE2) y los antiinflamatorios naproxeno (NPX) y diclofenaco (DCF). El objetivo principal de esta investigación

es el desarrollo de tecnología para llevar a cabo la oxidación catalizada por lacasa de los compuestos mencionados anteriormente.

En la primera etapa del trabajo se investigó el uso de las lacasas así como de los sistemas lacasa-mediador en reactores operados en modo discontinuo (Capítulo 2). Se evaluaron dos lacasas y se estudiaron los efectos del pH (que afecta no sólo la actividad y la estabilidad de la enzima, sino también los sustratos objetivo y a la acción del mediador) y mediador (tipo y concentración) tanto sobre la eficiencia de eliminación como sobre la estabilidad de la enzima, con el objetivo de establecer las bases para la óptima transformación de este tipo de compuestos por lacasas. En el caso de los estrógenos, estos compuestos se eliminaron de forma eficiente mediante lacasa de *Myceliophthora thermophila* incluso en ausencia de mediador y a pH neutro, lo cual facilitará la aplicación del tratamiento enzimático en la descontaminación de las aguas residuales. Los antiinflamatorios fueron transformados por lacasas de *Myceliophthora thermophila* y *Trametes versicolor* con rendimientos variables. Además, se evaluó la toxicidad de los productos de biotransformación generados a partir de las reacciones enzimáticas: se demostró que las reacción catalizada por lacasa permite la reducción de la estrogenicidad del medio que contiene los compuestos estrogénicos mediante análisis LYES; se demostró también que los subproductos de DCF presentan una considerable mayor biodegradabilidad aeróbica y la eliminación de toxicidad se comprobó también mediante ensayos Microtox®.

En vista de los resultados prometedores obtenidos, se llevó a cabo la operación en continuo de diferentes configuraciones de biorreactor para la transformación de los compuestos de interés: para este propósito, es común la inmovilización de las enzimas para así permitir la separación del biocatalizador, o bien se separan del efluente mediante sistemas de membrana. En el presente trabajo de investigación ambas alternativas fueron evaluadas, desarrollando tecnologías factibles para tal fin.

Lacasa de *Myceliophthora thermophila* fue inmovilizada mediante diferentes procedimientos para la aplicación de los biocatalizadores en reactores de lecho fijo y de lecho fluidizado (PBRs y FBRs, por sus siglas en inglés) para la eliminación de E1, E2 y EE2 (Capítulo 3). La enzima se inmovilizó mediante su encapsulación en matrices *sol-gel* mediante reacciones de hidrólisis y polimerización de compuestos silanos dando como resultado la formación de un hidrogel conteniendo la lacasa en su interior; se llevó también a cabo la inmovilización mediante unión covalente a soportes comerciales acrílicos que contiene grupos epóxido activados: Eupergit C and Eupergit C 250L. Se

obtuvieron eficacias de inmovilización de proteína de hasta 44-99 y 59-83% y actividades entre 1-80 y 5-17 U/g para la enzima inmovilizada covalentemente y encapsulada, respectivamente. La menor eficacia catalítica de la enzima inmovilizada mediante ambos métodos en comparación con la enzima libre se compensó con el aumento de la estabilidad y capacidad catalítica en un mayor rango de temperatura, pH y en presencia de agentes inactivantes. Se evaluó la operabilidad de los PBRs mediante su aplicación en la eliminación de un tinte sintético usado como compuesto modelo y además, los biorreactores fueron operados en ciclos continuos consecutivos para verificar el potencial reúso de los biocatalizadores. Los PBRs fueron aplicados en un siguiente paso de la investigación en la eliminación en continuo de E1, E2 y EE2, obteniendo rendimientos de 55-75 y 65-80% para la lacasa encapsulada e inmovilizada covalentemente, respectivamente; aproximadamente un 6-14 y 11-12% de la eliminación se atribuyó a adsorción de los sustratos. No obstante, se propuso el uso de un FBR debido a las desventajas relacionadas con el uso de los PBRs (aireación pobre o pasiva, deficiente transferencia de materia, la formación de caminos preferenciales, etc.). Este sistema resultó ser factible en la eliminación de sustratos estrogénicos a concentraciones en el intervalo 10-100 µg/L, y tanto el biorreactor como el biocatalizador fueron estables durante más de 10 días de operación; se logró además una reducción de estrogenicidad del 90%.

Posteriormente, se desarrolló y evaluó el uso de un reactor enzimático de membrana (EMR, por sus siglas en inglés) para la eliminación en continuo de estrógenos mediante lacasa libre de *Myceliophthora thermophila* (Capítulo 4). En una primera etapa, se llevaron a cabo experimentos en operación semi-continua (*fed-batch*) con el objetivo de investigar el efecto de la aireación/oxigenación, la velocidad de adición de los sustratos y la actividad de la lacasa, para la posterior realización de ensayos preliminares en continuo en un EMR de 370 mL. Sin embargo, el método convencional basado en estudio individual de cada variable para la optimización del proceso no es el procedimiento más adecuado, y además consume mucho tiempo y es laborioso e incompleto. Por lo tanto, la metodología de superficie de respuesta (RSM, por sus siglas en inglés) se emplea como alternativa adecuada. La aplicación de RSM permitió la evaluación de los efectos individuales y cruzados de las variables estudiadas: oxigenación, tiempo de residencia hidráulica (TRH) y la actividad de lacasa, en la transformación en continuo de los sustratos; por otra parte, se investigaron y optimizaron diferentes variables de respuesta: i) velocidad de eliminación, ii) velocidad de eliminación por unidades de enzima empleadas (con el objetivo de minimizar los costes de la

tecnología), y iii) el porcentaje de reducción de la actividad estrogénica (con el fin de optimizar no sólo la eliminación de los compuestos sino también la reducción de toxicidad). Se encontró una actividad óptima de tan solo 100 U/L para maximizar la eficacia de la enzima: E1 se oxidó a una tasa de 0,06 mg/(L·h·U), aunque la eliminación de estrogenicidad fue del 60%. Asimismo, los valores más elevados ensayados (1000 U/L, TRH 4 h y 60 mg O_2/(L·h)) permitieron una completa descontaminación. Por último, se operó un EMR de 2 L, durante 100 h y con solo 100 U/L de enzima, para corroborar la aplicabilidad real de la tecnología desarrollada: el biorreactor fue probado para el tratamiento enzimático de efluentes secundarios, recogidos en una planta municipal de tratamiento de aguas, conteniendo E1, E2 y EE2 a una concentración de 100 µg/L así como a niveles ambientales (0,29-1,52 ng/L). Se alcanzaron rendimientos altos de eliminación (80-100%) a pesar de la inactivación parcial de la enzima (alrededor de 20% dentro de las primeras horas).

Por otro lado, existe en bibliografía una falta significativa de conocimiento con respecto a la identificación de los productos de reacción de los compuestos de interés, a pesar de la creciente investigación sobre la aplicación de enzimas fúngicas para la oxidación de fármacos y compuestos disruptores endocrinos. Así pues, se ha trabajado en esta investigación para llevar a cabo la identificación de los productos de reacción de E1, E2 y EE2, así como los de DCF, resultantes de la transformación mediante lacasas, como un primer paso para dilucidar las principales vías de reacción; para este propósito, se aplicaron diferentes técnicas analíticas como GC-MS, LC-APCI y LC-ESI-TOF (Capítulo 5). Se demostró la formación de dímeros y trímeros de estrógenos a partir de la reacción catalizada por *Myceliophthora thermophila*, así como la transformación de E2 en E1, mediante el análisis de los espectros obtenidos y los posibles patrones de fragmentación, y fue corroborada por la determinación de masas exactas. Se identificaron además varios productos de transformación de DCF como resultado de reacciones de descarboxilación catalizadas por lacasa de *Trametes versicolor.*

En la última etapa de la investigación se exploró una tecnología emergente: se desarrollaron, caracterizaron y aplicaron microrreactores con lacasa inmovilizada con el objetivo de mejorar la eficiencia de los procesos catalizados por lacasas y ampliar el campo de aplicación de estas enzimas (Capítulo 6). Se han demostrado con anterioridad las ventajas asociadas a los microrreactores: disminución de los volúmenes de reacción, alta eficacia y repetibilidad, aumento de la eficacia de intercambio de calor y transferencia de materia, el control estricto de las condiciones de reacción gracias a su característico flujo laminar,

etc. Sin embargo, tan sólo unos pocos estudios previos consideraron estos sistemas para la aplicación de lacasas. En esta investigación se desarrolló un método novedoso, sencillo, versátil y de bajo coste para la fabricación de microrreactores enzimáticos, basados en la formación de una membrana de lacasa de *Trametes versicolor* inmovilizada en las paredes internas de microtubos como resultado de reacciones de *cross-linking* y polimerización. Estos microrreactores se emplearon para la oxidación de los compuestos de interés, obteniendo elevados rendimientos para tiempos de residencia reducidos. Además, se diseñó un sistema de microrreactor en dos etapas para la prevención de la inactivación del biocatalizador cuando se opera bajo condiciones adversas para la enzima.

Summary

Over the past decade, public concern about the environmental impact of steroid estrogens present in municipal wastewaters has grown due to their potential for disturbing endocrine systems of animals and humans. Besides, the release of anti-inflammatory drugs to the environment also implies a great concern because of their negative effects, e.g. potential cytotoxicity to liver and kidney of some species. The degradation of these types of compounds implies an important ecological challenge as they have complex structures and low bioavailability. In fact, they have been detected in wastewater treatment plant effluents at different concentrations ranging from nanograms to micrograms per liter, once certain conventional processes can only render partial transformation yields. Hence, alternative treatment methods have been investigate in order to overcome that environmental issue, such as physical methods (e.g. sorption and membrane separation), microbial removal by bacteria and algae and advanced oxidation processes (AOPs) (e.g. ozonation, UV/H_2O_2, Fenton, etc.). In general, AOPs provide high removal rates but low selectivity; however, these methods imply in some cases considerable high costs associated to reagents and/or investment, the formation of harmful byproducts and even the generation of compounds with estrogenicity higher than that of the parent substrate and/or non-biodegradable.

An advanced treatment alternative may be based on the use of white rot fungi cultures. These microorganisms were reported to remove a wide range of xenobiotics by the action of fungal oxidative enzymes such as manganese peroxidase, lignin peroxidise and laccase; thereby, the use of enzyme-catalyzed transformation of estrogens and anti-inflammatories was proposed in the current work. The use of enzymes in industrial processes is commonly linked to a reduce consumption of energy and chemicals and moreover, they can usually react under moderate conditions. In the case of laccases (copper-containing oxidases, EC 1.10.3.2), the use of oxygen as electron acceptor would represent an additional advantage for the application of these enzymes in comparison with peroxidases. Specifically, the use of laccases from *Myceliophthora thermophila* and *Trametes versicolor* for the oxidation of the estrogens estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) and the anti-inflammatories naproxen (NPX) and diclofenac (DCF) was investigated in this research. The main goal of this work is the development of technology to perform the successful laccase-catalyzed oxidation of the compounds mentioned above.

In the first stage of the research the use of laccases as well as laccase-mediator systems was investigated in batch reactors (Chapter 2). Two different

laccases were evaluated and effects of pH (affecting not only the activity and stability of the enzyme but also the target substrates and the mediator action) and mediator (type and concentration) on both removal efficiency and enzyme stability were investigated aiming to establish the groundwork for the optimum laccase-catalyzed transformation of this type of compounds. In the case of estrogens, these compounds were successfully removed by *Myceliophthora thermophila* laccase even in the absence of mediator and at neutral pH, which was expected to facilitate the application of the enzymatic treatment on the detoxification of wastewaters. The target anti-inflammatories were transformed by *Myceliophthora thermophila* and *Trametes versicolor* laccase with variable yields. Furthermore, the toxicity of the biotransformation products generated from the enzymatic reactions was evaluated: laccase-catalyzed transformation was proved to be effective in reducing the estrogencity of the medium containing the estrogenic compounds by the application of LYES analysis; also, DCF byproducts presented considerable higher aerobic biodegradability and detoxification was also proved by Microtox® assays.

In view of the promising obtained results, continuous bioreactors were operated for the successful transformation of the target compounds: for this purpose, enzymes need to be immobilized to enable the recovery of the biocatalyst, or separated from the effluent by membrane modules. Here, both routes were applied and potential technologies were found to be feasible.

Laccase from *Myceliophthora thermophila* was immobilized by different procedures for the application of the biocatalysts in packed bed and fluidized bed reactors (PBRs and FBRs) for the removal of E1, E2 and E2 (Chapter 3). The enzyme was immobilized by its encapsulation in a sol-gel matrix based on silane compounds which hydrolyze and polymerize in the presence of the enzyme, resulting in a hydrogel with the laccase encapsulated inside; also, laccase immobilization was conducted by covalent bonding to commercial solid epoxy-activated acrylic supports, Eupergit C and Eupergit C 250L. Laccase was successfully immobilized by both procedures yielding bound protein percentages of up to 44-99 and 59-83% and activities 1-80 and 5-17 U/g for covalently immobilized and encapsulated laccase, respectively. The somewhat lower catalytic efficiency of laccase immobilized by both studied methods in comparison to that of free form was balanced by its increased stability and broader operational window related to temperature, pH and chemical inhibitors. The PBRs were evaluated by their application on the removal of a synthetic dye used as model compound; moreover, the bioreactors were operated in consecutive

continuous cycles aiming to verify the reusability of the biocatalysts. The proposed PBRs were applied in a next step of the research for the continuous removal of E1, E2 and EE2, providing removal yields of 55-75 and 65-80% for the encapsulated and the covalently immobilized laccase, respectively; 6-14 and 11-22% of elimination was attributed to adsorption of the substrates. Nonetheless, a FBR was developed due to the drawbacks related to the use of PBRs (poor or passive aeration, slow mass transfer and formation of preferential paths, etc.). This system was proved to be feasible on the elimination of estrogenic substrates at concentrations in the range of 10-100 µg/L, and the bioreactor and biocatalyst were stable for more than 10 days of operation; moreover, an estrogenicity reduction of 90% was achieved.

Afterwards, an enzymatic membrane reactor (EMR) was evaluated for the continuous removal of estrogens by free laccase from *Myceliophthora thermophila* (Chapter 4). In a first step, fed-batch experiments were carried out with the goal of investigating the effect of aeration/oxygenation, addition rate of the substrates and laccase activity, for conducting preliminary continuous assays in a 370-mL EMR. Nonetheless, the conventional method based on "one factor at a time" approach for the optimization of the process is not the most adequate procedure and moreover, it is time-consuming, laborious and incomplete. Hence, response surface methodology (RSM) is used as proper alternative. The application of RSM allowed the evaluation of individual and interrelated effects of oxygenation rate, hydraulic residence time (HRT) and laccase activity on the continuous transformation of the substrates; moreover, different response variables were investigated and optimized: i) removal rate, ii) removal rate per units of enzyme used (aiming to minimize the costs of the technology), and iii) the percentage of estrogenic activity reduction (in order to optimize not only the elimination of the parent compounds but also de detoxification of the effluent). Only 100 U/L were found as optimal to maximize the efficacy of the enzyme: E1 was oxidized by 0.06 mg/(L·h·U), although the removal of estrogenicity was 60%. On the other hand, the highest values assayed (1,000 U/L, HRT 4 h and 60 mg O_2/(L·h)) provided nearly complete detoxification. Finally, a 2-L EMR was operated, for 100 h and with minimal enzyme requirements (100 U/L), to corroborate the real applicability of the developed technology: the bioreactor was proved for the enzymatic treatment of secondary wastewater effluents, collected in a municipal wastewater treatment plant, containing E1, E2 and EE2 at only100 µg/L as well as at real environmental levels (0.29-1.52 ng/L). High removal yields

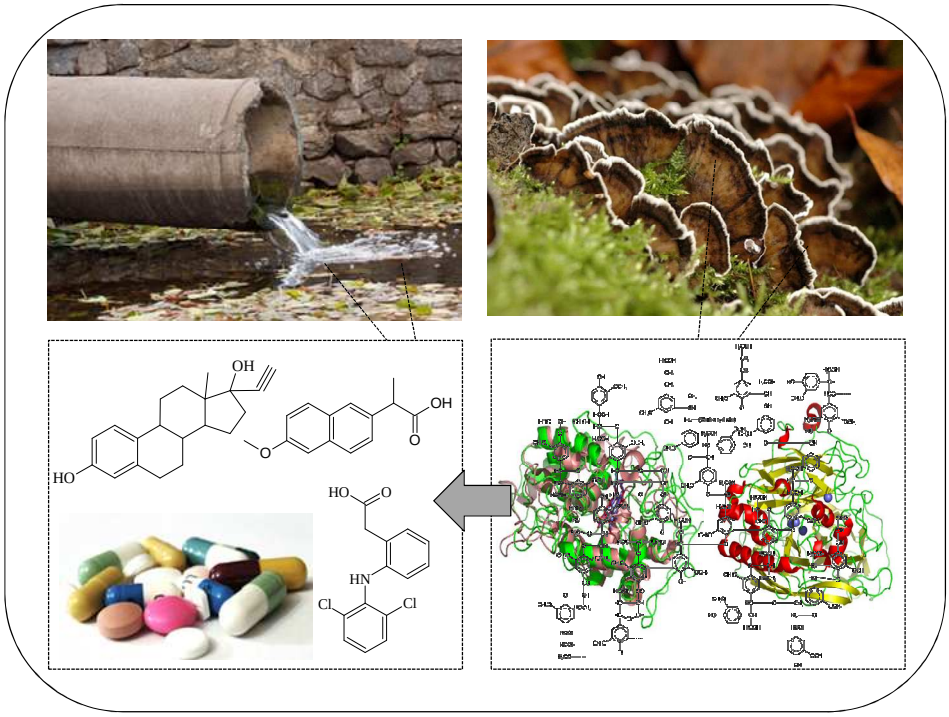
(80-100%) were attained despite partial inactivation of the enzyme (about 20% within the first hours).

On the other hand, there is a significant lack of knowledge in literature regarding the identification of reaction products of the target compounds despite the increasing research on the application of fungal enzymes for the oxidation of pharmaceuticals and endocrine disrupting compounds. Effort was paid in this work to perform the identification of the reaction products of E1, E2 and EE2 as well as those of DCF resulted from laccase-catalyzed transformation, as a first step to elucidate the main reaction pathways; for this purpose, different analytical techniques such as GC-MS, LC-APCI and LC-ESI-TOF were applied (Chapter 5). The formation of dimers and trimers of estrogens from the reaction catalyzed by *Myceliophthora thermophila* laccase, as well as the transformation of E2 into E1, was proved by analyzing the obtained spectra and the possible fragmentation patters, and corroborated by the determination of accurate masses. Furthermore, DCF transformation products resulted from decarboxylation reactions catalyzed by *Trametes versicolor* laccase were assessed.

In the last stage of the research an emerging technology was explored: laccase-immobilized microreactors were developed, characterized and applied with the objective of improving the efficiency of laccase-catalyzed processes and to broaden the range of application of these enzymes (Chapter 6). Microreactor systems have been demonstrated to present numerous advantages: the use of drastically reduced volumes of reactant solutions, high efficiency and repeatability, increased heat exchange and mass transfer, strict control of the reaction conditions by means of the characteristic laminar flow, etc. Nonetheless, only few previous studies considered these systems for the application of laccases. In the current research, a simple, versatile and inexpensive method was developed for the fabrication of enzymatic microreactors, based on the formation of a *Trametes versicolor* laccase-immobilized membrane on the inner wall of microtubes as a result of cross-linking polymerization reactions. These microreactors were used for the oxidation of the target compounds, obtaining great transformation yields with reduced residence times. Furthermore, it was designed a two-stage microreactor system for the prevention of the biocatalyst inactivation when operating under adverse conditions for the enzyme.

Chapter 1

General Introduction



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1. General Introduction

1.1. Target pollutants

The contamination of water by recalcitrant organic substances significantly affects the viability of water reuse of treated municipal or industrial water effluents. Historically, compounds such as substituted phenols, non-biodegradable chlorinated solvents, pesticides and surfactants were considered the main examples of relevant compounds difficult to be removed from wastewater. During the last years, pharmaceuticals and personal care products (PPCPs) and especially endocrine disrupting chemicals (EDCs) have been considered as emerging contaminants and have received much attention due their incomplete elimination by conventional wastewater treatment processes and their consequent release to the environment (Esplugas et al. 2007, Nakada et al. 2006, Rodríguez-Rodríguez et al. 2010, Suárez et al. 2008, Zhang et al. 2007).

1.1.1. Endocrine disrupting compounds

Over the last decades there has been a growing concern about the exposure to substances which are suspected to interfere with the endocrine system and therefore, they may cause health effect such as behavioral changes and reproductive abnormalities in human beings and wildlife.

Indeed, according to an European Union study, 118 substances were classified as potential EDCs; priority was assigned to carbon disulfide, o-phenylphenol, tetrabrominated diphenyl ether, 4-chloro-3-methylphenol, 2,4-dichlorophenol, 2,20-bis(4-(2,3-epoxypropoxy)phenyl)propane, resorcinol, 4-nitrotoluene and 4-octylphenol, as well as to the natural hormones estrone (E1) and 17β-estradiol (E2), and the synthetic estrogen 17α-ethinylestradiol (EE2) (CEE 2001).

EDCs can disperse quickly in the environment: some are released to the atmosphere as a result of combustion and incineration activities (e.g. dioxins) (Ying et al. 2002), but the principal sinks for EDCs in the environment are sewage treatment systems, before reaching receiving bodies (Liu et al. 2009). In fact, this type of compounds has been detected in wastewaters, sediments, groundwater and even drinking water (Esperanza et al. 2004, Liu et al. 2009). The main distribution of EDCs in the environment is represented in Figure 1.1:

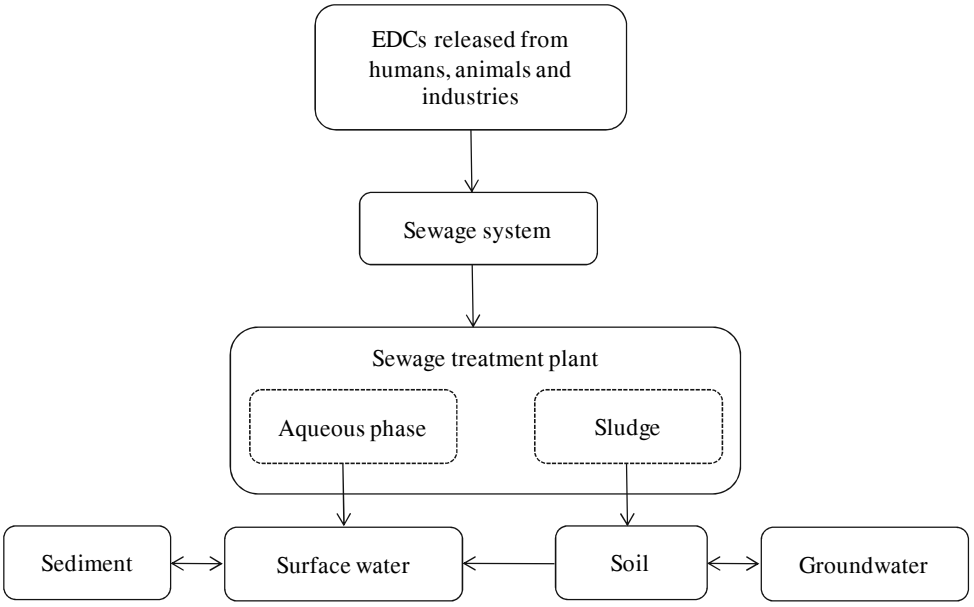


Figure 1.1. Scheme of the EDCs distribution in the environment.

The main classes of EDCs (natural steroidal estrogens, synthetic estrogens, phytoestrogens and various industrial chemicals) are generally represented with respect to their estrogenic potency (Servos et al. 1999). In this way, although the concentrations of phyto- and xenoestrogens in the aquatic environment are usually higher, natural and synthetic estrogens generally display much stronger estrogenic effects (Spengler et al. 2001).

1.1.1.1. Natural and synthetic estrogenic compounds

The list of contaminants EDCs resulting from human activities and found in wastewater is long; nonetheless, the current research is paying attention on the synthetic estrogen EE2 and the natural ones E1 and E2, once these compounds have been demonstrated to be the major contributors to the estrogenic activity detected in sewage effluents (Aerni et al. 2004, Desbrow et al. 1998, Ridgers-Gray et al. 2000). Moreover, different studies based on in vitro bioassay-directed chemical fractionation involved E2 and EE2 as the most potent estrogens in complex mixtures (Desbrow et al. 1998, Synder et al. 2001).

The structures of these selected estrogenic compounds are represented in Figure 1.2.

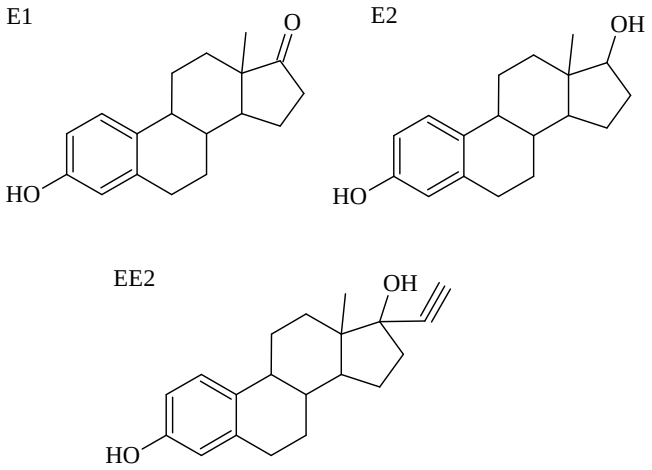


Figure 1.2. Structures of the target estrogenic compounds evaluated in this work.

E1

E1, also known as 3-hydroxyestra-1,3,5(10)- triene-17-one, is one of the naturally occurring estrogens, the others being E2 and E3. E1 concentrations in premenopausal mammals fluctuate according to the menstrual cycle. In premenopausal women, more than 50% of E1 is secreted by the ovaries. In prepubertal children, men and non-supplemented postmenopausal women the major portion of E1 is derived from the peripheral tissue conversion of androstenedione.

E2

Like other steroids, E2 is derived from cholesterol, being androstenedione the key intermediary. Androstenedione is converted to testosterone and then to E2 by the enzyme aromatase. In premenopausal women, E2 is produced by granulose cells of the ovaries, smaller amounts of E2 are also produced by the adrenal cortex, by the testes (in men) and fat cells are active precursors to E2 and continue even after menopause.

EE2

EE2, a synthetic estrogen used in birth control pills is mainly used in oral contraceptives and thus, it is also primarily released into the environment through human excretion (Kolpin et al. 2002, Sun et al. 2010).

1.1.1.2. Environmental risks of estrogens: occurrence and effects

All humans as well animals excrete hormones from their bodies which end up in the environment through the sewage discharge and animal waste disposal. Based on the daily excretion of estrogens from humans (Table 1.1) and the dilution effect, estrogens concentration levels in the range of ng/L are expected in the aqueous environmental samples (Johnson et al. 2000). The occurrence of these compounds in the aquatic environment is a result of their discharge in effluents from wastewater treatment plants (WWTPs) once sewage treatment facilities have shown very variable elimination efficiency of this kind of EDCs (Cabana et al 2007b, Fernández et al. 2007), with the consequent negative effect of potential endocrine disruption.

Table 1.1. Human daily excretion (µg) of estrogens (Johnson et al. 2000).

	E1	E2	EE2
Males	3.9	1.6	-
Menstruating females	8.0	3.5	-
Menopausal females	4.0	2.3	-
Pregnant women	600	259	-
Women	-	-	35

The main mechanisms through which they interfere with the endocrine system are: i) the simulation of the activities of physiological hormones, thereby participating in the same reactions and causing the same effects; ii) the inactivation, with competitive action, of hormone receptors and, consequently, the neutralization of their activity; iii) the interference with the synthesis, transport, metabolism and secretion of natural hormones, altering their physiological concentrations and therefore their corresponding endocrine functions (Diano and Mita 2011).

In this way, effects of estrogenicity in the environment on animals have been widely investigated and demonstrated few years ago. The deleterious effects observed in aquatic life and wildlife that may be caused by endocrine-disrupting mechanisms include the following: abnormal thyroid function in birds and fish (Moccia et al. 1981, 1986; Leatherland 1992), decreased fertility in birds, fish, shellfish, and mammals (Shugart 1980, Leatherland 1992, Gibbs et al. 1988), decreased hatching success in fish, birds, and reptiles (Kubiak et al. 1989, Bishop et al. 1991), demasculinization and feminization of fish, birds, reptiles, and

mammals (Munkittrick et al. 1991, Guillette et al. 1994), defeminization and masculinization of fish and gastropods (Davis and Bartone 1992, Ellis and Pattisina 1990) and alteration of immune function in birds and mammals (Erdman 1988, Martineau et al. 1988).

With regard to the target estrogens evaluated in this research (E1, E2 and EE2), previous works reported that several WWTPs effluents and rivers contain significant amounts of these substances that potentially induce harmful effects on various species. Some examples of the presence of these estrogens in WWTP effluents as well as in river water are summarized in Tables 1.2 and 1.3.

Table 1.2. Examples of the natural and synthetic estrogens in WWTPs effluents.

Sample site	Concentration (ng/L)			References
	E1	E2	EE2	
France	6.2-7.2	4.5-8.6	2.7-4.5	Cargouet et al. 2004
Canada	3	6	9	Ternes et al. 1999a,b
Netherlands	<0.4-47	<0.6-12	<0.2-7.5	Belfroid et al. 1999
England	1.4-76	2.7-48	bdl (0.3)*	Xiao et al. 2001
Italy	3	1.4	0.6	Baronti et al. 2000
Spain	<2.5-8.1	<5-14.5	<5	Petrovic et al. 2002

*bdl: Below detection limits, value in parenthesis.

Table 1.3. Concentrations of natural and synthetic estrogens found in river water.

Sample site	Concentration (ng/L)			References
	E1	E2	EE2	
France	1.1-3.0	1.4-3.2	1.1-2.9	Cargouet et al. 2004
Netherlands	<0.1-3.4	<0.3-5.5	<0.1-4.3	Belfroid et al. 1999
England	bdl (0.5)*	bdl (0.5)	bdl (0.5)	Xiao et al. 2001
Italy	1.5	0.11	0.04	Baronti et al. 2000
Spain	4.3	6.3	-	Petrovic et al. 2002

*bdl: Below detection limits, value in parenthesis.

Field studies using caged trout (*Oncorhynchus mykiss*), wild cyprinid roach (*Rutilus rutilus*) (Jobling et al. 1998) and estuarine flounder (*Platichthys flessus*) (Allen 1999, Lye et al. 1997) showed that the estrogenicity associated to E1, E2 and EE2 persists in receiving water and that the concentration of these compounds present in rivers and estuaries are high enough to induce deleterious

reproductive consequences. Indeed, several studies showed that even low concentrations (ng/L) of E2 can induce vitellogenin in male species and rainbow trout experimentally exposed to these substances (Routledge et al. 1998, Sumpter et al . 1996); indeed, Tanaka et al. (2001) reported that the disrupting activity of E2 is 1000-10,000 times greater than that of nonylphenol. Purdom et al. (1994) and Hansen et al. (1998) noticed that concentrations of E2 as low as 1 ng/L induces vitellogenin in male trout. In addition, Routledge et al. (1998) and Larsson et al. (1999) noted that EE2 can be a potential danger to fish and other aquatic organisms, even present at concentrations of 0.1-10 ng/L.

Also, the effects on reproduction of environmentally relevant mixtures of estrogens on Japanese medaka were recently reported by Jukosky et al. (2008). The effects of EE2 on hormonal responses and xenobiotic biotransformation system of Atlantic salmon were studied by Mortensen and Arukwe (2007). Estrogenic exposure affected metamorphosis and altered sex ratios in the northern leopard frog and the identification of critically vulnerable periods of development was studied by Hogan et al. (2008). In that work, it was shown that tadpoles exposed to EE2 during midmetamorphosis were delayed in development immediately following exposure and took 2 weeks longer to reach metamorphic climax.

The unexpected impacts of trace concentrations of estrogens on wildlife raised concerns about the potential effects of these chemicals on humans, but these impacts are still quite controversial. Some researchers attributed decreases in human sperm quality to EDCs in the environment (Sharpe et al. 1993, Stone 1994, Carlsen et al. 1995). Likewise, it has been suggested that sharp increases in breast, testicular and prostate cancers are related to EDCs in the environment (Carlsen et al. 1995, Gillesby et al. 1998). But other scientists have produced data refuting these arguments. Estrogenic hormones in water are less likely to cause adverse effects in humans than they are in fish due to differences in exposures. Fish may be constantly exposed to EDCs present in the aquatic environment, while humans are exposed mainly through ingestion of limited quantities of water (Snyder et al. 2003).

1.1.2. Pharmaceutical compounds

In recent years pharmaceutical drugs, which belong to the emerging pollutant group namely PPCPs, have emerged as a novel class of water contaminants for which public and scientific concern is steadily increasing due to the potential impact on human health and the environment even at trace levels (Vogna et al. 2004). This type of compounds may be excreted both as parent forms and as active metabolites 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). The incomplete degradation in the WWTPs considerably contributes to their presence in the environment (Carballa et al. 2004, Hofmann et al. 2007, Vogna et al. 2004). Additionally, they may get adsorbed on sewage sludge, where they bioaccumulate and reach the environment when the sludge is used as agricultural fertilizer. Indeed, several studies about the occurrence of pharmaceuticals in the aquatic media have detected numerous compounds belonging to different therapeutic families (e.g. antibiotic, analgesic, anti-inflammatory, antiepileptic, etc.) in aquatic media (Heberer 2002a, b, Soufan et al. 2012). Specifically, their presence in the environment has been confirmed in various countries such as Germany (Ternes 1998), Brazil (Stumpf et al. 1999), Italy (Andreozzi et al. 2003b), Spain (Carballa et al. 2004, Suárez et al. 2009), Finland (Lindqvist et al. 2005), India and Pakistan (Larsson et al. 2007), and several other countries such as Australia, Austria, Croatia, France, Greece, Netherlands, Sweden, Switzerland, United Kingdom, where more than 80 pharmaceuticals have been detected at levels of μg/L in sewage, surface and groundwaters (Heberer 2002a, b, Castiglioni et al. 2006).

The regulation about pharmaceuticals started recently. A guide book for the assessment of human drugs in the environment has been published in 1998 by the Food and Drug Administration (FDA), which states that an environmental assessment report has to be provided if the expected concentration in the aquatic environment is higher than 1 μg/L (Fent et al. 2006). On the other hand, the European Union began to consider human drugs as potential toxic agents with the Directive 92/18 ECC, where an ecotoxicity test for registration of pharmaceutical was claimed. Finally, according to Directive 2001/83/EC, an application to register new medicinal product for human treatment must be accompanied by an environmental risk assessment (EMEA 2005, Fent et al., 2006, Nikolaou et al., 2007).

This type of compounds are considered as emerging micropollutants due to several reasons: (i) their extensive and still increasing use, providing continuous

release of pharmaceuticals into the environment, mainly via human or animal excretions; (ii) their low persistence (they may turn pseudo persistent when the replacement rate overcome the transformation/removal rate, resulting in a constant environmental level of the compounds); (iii) their potentially toxic effects on aquatic and terrestrial organisms, since they are deliberately designed to cause a biological effect even at very low concentrations; (iv) some of them may bioaccumulate in biota (Daughton and Ternes 1999, Kummerer 2007, Nikolaou et al. 2007, Reif et al. 2008).

1.1.2.1. Anti-inflammatory compounds

A large consumption of pharmaceuticals by the population is evidenced, contributing with the continuous release of the parent compound and/or their metabolites. Although the quantity of pharmaceuticals consumed depends on their therapeutic group, anti-inflammatory drugs along with antibiotics are the groups of medicines most commonly used, the non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac (DCF) and naproxen (NPX), are the type of pharmaceuticals most commonly found within the aquatic environment (Schwaigera et al. 2004).

Therefore, these compounds have been used as representative of that therapeutic class to be evaluated in the current research. The consumption per year of DCF and NPX reported for different countries is summarized in Table 1.4.

Table 1.4. Consumption of the selected anti-inflammatories in different countries.

Compound	Country	Consumption per year (t)	References
DCF	Austria	6.7	Clara et al. 2005b
	Germany	81.7	Fent et al. 2006
	Switzerland	4.5	Fent et al. 2006
	Finland	9.6	Lindqvist et al. 2005
	England	26	Ziylan and Ince 2011
	Australia	4.4	Ziiylan and Ince 2011
NPX	Switzerland	1.7	Fent et al. 2006
	Finland	6.7	Lindqvist et al. 2005
	England	35	Ziylan and Ince 2011
	Australia	22.8	Ziylan and Ince 2011

DCF

This compound, namely (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid), is commonly used in the treatment of arthritis, ankylosing spondylitis and acute muscle pain. This compound has been recently designated as a devastating environmental pollutant because of its bioaccumulation in the food chain (Ilic et al. 2011).

DCF

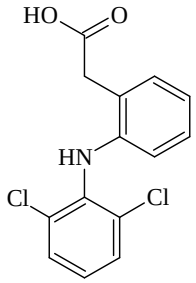


Figure 1.3. Structure of the target compound DCF.

NPX

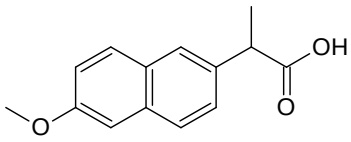


Figure 1.4. Structure of the target compound NPX.

NPX

NPX, (2-(6-methoxynaphthalen-2-yl)propionic acid), is an arylpropionic acid widely used for mild to moderate pain relief and in the treatment of osteoporosis and rheumatoid arthritis, menstruation and headaches; this drug is additionally used in veterinary medicine in appreciable quantities (Ziylan and Ince 2011).

1.1.2.2. Environmental risks of anti-inflammatories: occurrence and effects

A significant contribution of pharmaceuticals in the aquatic ecosystem is expected to be caused by manufacturing plants and effluents from hospitals. For instance, levels of 840 µg/L were found in WWTP influents close to drug manufacture plants in India (Fent et al. 2006, Ternes et al. 2006, Larsson et al. 2007). However, municipal wastewater is considered as the largest emission source of anti-inflammatory compounds once the main pathway of these chemicals is the human ingestion, excretion and disposal and due to the inefficiency of conventional treatment systems in WWTPs for their removal. In fact, several works dealing with the occurrence of NPX and DCF in the environment reported concentration of these compounds ranging from ng to µg/L in WWTPs effluents and even in surface waters; some examples are summarized in Tables 1.5 and 1.6.

Table 1.5. Examples of anti-inflammatory compounds in WWTPs effluents.

Compound	Concentration	References
DCF	1-49.1 ng/L	Esplugas et al. 2007, Gagnon et al. 2008
	0.17-2.5 µg/L	Daughton and Ternes 1999, Esplugas et al. 2007, Fent et al. 2006
NPX	bdl*-12.5 µg/L	Carballa et al. 2004, Daughton and Ternes 1999, Fent et al. 2006

*bdl. Below detection limits.

Table 1.6. Concentrations of anti-inflammatory compounds found in surface waters.

Compound	Concentration	References
DCF	ng/L	Vogna et al. 2004
	0.002-33.9 µg/L	Daughton and Ternes 1999, Ikehata et al. 2006, Jjemba 2006, Ziylan and Ince 2011
NPX	135.2 ng/L	Zhang et al. 2007
	0.002-33.9 µg/L	Daughton and Ternes 1999, Ziylan and Ince 2011

The ineffective elimination of pharmaceuticals, combined with the continuous input of such compounds, may lead to chronic low levels exposure and accumulation, resulting negative effects on life and environment, plus undesired collateral generation of microbial resistances (Martínez et al. 2011).

Oaks et al. (2004) published a significant work reporting that DCF has been identified as the cause of massive decline of vulture population in Pakistan and India making this species critically endangered, comparable to the disappearance of peregrine falcons and other predatory birds in the 1960s due to the pesticide DDT (dichlorodiphenyltrichloroethane) (Fent et al. 2006). Oaks et al. (2004) demonstrated that DCF was responsible of the high mortality (>95%) of the scavenging birds. Their death was mainly associated with visceral gout, caused by renal failure and subsequent deposition of uric acid on and within the internal

organs. The phenomenon is based on alimental habits of the vultures, which are fed on dead domestic livestock. In fact, in 1990s DCF was widely used in these regions as veterinary drug for its analgesic, anti-inflammatory and antipyretic properties.

Besides, cytological alterations in liver, kidneys, and gills were observed in rainbow trout following exposure to 1 µg/L for 28 days (Triebskorn et al. 2004). Renal lesions and alterations of the gills were also observed at 5 µg/L, as well as accumulation of DCF in the kidneys, gills, and liver (Schwaiger et al. 2004). Also, the potential adverse effects of NPX on biota such as reducing the lipid peroxidation system of bivalves were reported by Gagné et al. (2006). The ecotoxicity of NPX and its photoproducts was assayed on some aquatic organisms, such as bacteria, microcrustaceans and algae (Isidori et al. 2005, Della Greca et al. 2004); the reported results indicated the high ecotoxicity of NPX and that some photoderivatives are even more harmful than NPX.

Cleuvers (2004) investigated the impact of a mixture of several NSAIDs on *Daphnia magna* finding a considerable toxicity effect, even at concentrations at which the single substances showed no or only very slight effects. Bioaccumulation of this type of compounds along with gemfibrozil (a blood lipid regulating agent) into fish blood plasma (juvenile rainbow trout) was demonstrated by Brown et al. (2007).

1.1.3. Physicochemical properties of the selected estrogenic and anti-inflammatory compounds

The main physicochemical properties of the selected estrogens: E1, E2 and EE2, and anti-inflammatory compounds: NPX and DCF, are shown in Table 1.7.

Among the indicated parameters, water solubility (S_w) as well as the octanol-water partition coefficient (K_{ow}) are key values which determine the fate of the pollutants in the environment. In general, substances with $\log K_{ow}$ lower than 2.5 may be considered relatively hydrophilic, and those with values higher than 4 are expected to present high sorption potential. All the selected estrogens could be considered to yield medium sorption potential once they present $2.5 < \log K_{ow} < 4$, approximately (Jones-Lepp et al. 2007).

Table 1.7. Physicochemical properties of the selected estrogenic and anti-inflammatory compounds (Lai et al. 2000, Scheytt et al. 2005, Suárez et al. 2008, Ziylan and Ince 2011).

Compound	MW (g/mol)	S_w (mg/L)	P_v (Pa)	pKa	$logK_{ow}$
Estrogens					
E1	270.4	30	$3 \cdot 10^{-8}$	10.4	3.1-3.4
E2	272.4	3.6	$3 \cdot 10^{-8}$	10.4	3.9-4.0
EE2	296.4	11.3	$6 \cdot 10^{-9}$	10.5-10.7	2.8-4.2
Anti-inflammatories					
NPX	230.3	16	$2 \cdot 10^{-4}$	4.2-4.5	3.2-3.3
DCF	296.2	2.4	$8 \cdot 10^{-6}$	4.0-4.5	4.5-4.8

MW – Molecular weight
S_w – Solubility in water
P_v – Vapor pressure
pKa – Dissociation constant
K_{ow} – Octanol-water partition coefficient

The elimination of anti-inflammatories in the environment is not clear, although there is a consensus on process such as sorption, sedimentation and biotransformation. Sorption occurs via hydrophobic or electrostatic interactions between the drugs and the particulate matter or biomass (Carballa et al. 2005, Ziylan and Ince 2011). Nikolaou et al. (2007) reported ineffective adsorption process for acid drugs, such as DCF and NPX (pKa 4.0-4.5); for such chemicals, biodegradation is expected to be a more potent elimination pathway in aerobic or anaerobic mechanisms. However, sorption on soil or sediment could be an important factor in the case of DCF once it presents relatively high $logK_{ow}$ (4.5-4.8), a measure of hydrophobicity as well as low solubility; this would contribute to reduce its aqueous phase concentrations. Moreover, all the compounds have low vapor pressure (P_v), which suggests that negligible volatilization should be expected.

1.1.4. Removal of the selected estrogenic and anti-inflammatory compounds from wastewater

A number of researchers have reported the variable removal efficiencies attained by conventional processes in WWTPs, as those summarized in Table 1.8.

Table 1.8. Removal efficiencies of the target pollutants in WWTPs.

Comp.	Influent	Effluent	Removal (%)	References
Estrogens				
E1	44 ng/L	17 ng/L	61	D'Ascenzo et al. 2003
	31 ng/L	24 ng/L	23	Johnson et al. 2000
	43.1 ng/L	12.3 ng/L	69	Onda et al. 2003
	-	-	83	Ternes et al. 1999a,b
E2	5 ng/L	<1 ng/L	80	Behnish et al. 2001
	11 ng/L	1.6 ng/L	86	D'Ascenzo et al. 2003
	9.69 ng/L	4 ng/L	59	Johnson et al. 2000
	28.1 ng/L	1.2 ng/L	96	Onda et al. 2003
	35-125 ng/L	bdl*-30	44-100	Clara et al. 2005[a]
EE2	4.84 ng/L	1.40 ng/L	71	Johnson et al. 2000
	-	-	78	Ternes et al. 1999a,b
	0.40-13 ng/L	bdl-1.7 ng/L	52-100	Baronti et al. 2000
	4.9-7.1	2.7-4.5 ng/L	33-45	Cargouet et al. 2004
	3-70 ng/L	bdl-5 ng/L	33.3-100	Clara et al. 2005[a]
Anti-infl.				
NPX	-	-	55-98	Lindqvist et al. 2005
	0.4-0.6 µg/L	0.01-0.2 µg/L	78	Stumof et al. 1999
	-	-	50-80	Joss et al. 2005
DCF	3.02 µg/L	2.51 µg/L	17	Heverer et al. 2004
	0.6-0.8 µg/L	0.01-0.2 µg/L	75	Stumof et al. 1999
	-	-	23-60	Lindqvist et al. 2005

*bdl: Below detection limits.

Those processes in the WWTPs include: i) Pre-treatment: consisting of various 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 (in example solids, oils, grease and foam); sedimentation and flotation are the most common physical processes adopted; iii) Secondary treatment, in which biological reactors carry out the

decomposition of organic matter and removal of nutrients; among secondary treatments there are various techniques, such as lagooning, aerobic and anaerobic treatments and biofiltration processes; and 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 techniques such as chlorination or UV treatment. During these processes, the main removal mechanisms are based on solid sorption, volatilization, photo-oxidation and/or biological degradation. Among them, biodegradation and sorption were reported to be relevant for the elimination of the pollutants (Joss et al. 2005, Suárez et al. 2008).

Due to the ineffectiveness of these conventional treatments to completely remove these compounds, alternative post-treatments methods have been investigated:

- Physical methods such as sorption or membrane separation. For instance, Serrano et al. (2011) reported an elimination of NPX up to 64%, although DCF transformation was negligible, when using a sequential membrane bioreactor with the addition of powdered activated carbon. Also, novel sorbent materials were developed for the removal of this type of compounds, such as those based on carbon blacks (Cuerda-Correa et al. 2010) or mesoporous silicates (Rivera-Jiménez et al. 2008). Neira Ruiz et al. (2012) proposed the use of nanofiltration technology for the removal of conventional and emerging micropollutants.
- Microbial removal by bacteria, fungi and algae (Cajthaml et al. 2009a, b). For example, *Novophingobium tardaugens* bacteria isolated from activated sludge was reported to degrade E2 (Fujii et al. 2002), the removal of this compound and EE2 was assayed with *Trametes versicolor* fungus (Blánquez and Guieysse 2008), and various microalgae (*Selenastrum capricornutum*, *Scenedesmus quadricauda*, *Scenedesmus vacuolatus*, and *Ankistrodesmus braunii*) were utilized for the transformation of EE2 (Della Greca et al. 2008).
- Advanced treatments, which are receiving significant attention during the last years: i) chemical treatments, such as those based on oxidative catalysis, ozonation and other advanced oxidation processes (AOPs) (Ikehata et al. 2006, Ziylan and Ince 2011) and ii) enzyme-catalyzed transformation.

1.1.4.1. Elimination of the target pollutants by AOPs

AOPs have recently emerged as an important class of technologies for the oxidation and destruction of a wide range of organic pollutants in water and wastewater (Alvares et al. 2001, Zhou and Smith 2001). These methods have been proposed as an alternative to the physical removal of pollutants by membrane filtration or adsorption, once these methods are highly energy and material intensive and only suitable for the treatment of relatively clean wastewater (Larsen et al. 2004, Ikehata et al. 2006). The AOPs are characterized by a variety of radical reactions that involves combination of chemical agents: e.g. ozone (O_3), hydrogen peroxide (H_2O_2), transition metals and metal oxides, and auxiliary energy sources: e.g. ultraviolet-visible (UV-Vis) radiation, electronic current, ɤ-radiation and ultrasound (Ikehata et al. 2006).

The most common AOPs and examples of their application on the removal of the target pollutants evaluated in the current research are detailed below.

Ozonation

The reactivity of the substrates with ozone was found to be related to the functional groups in their structures as well the operating conditions. Ziylan and Ince (2011) reported 68-99% of NPX removal by lab-scale treatment with 3 mg/L O_3. Also, Nakada et al. (2007) proved the efficiency of ozonation in an operating WWTP: ozonation and sand filtration combined with activated sludge provided removal yields up to 80% of all the EDCs and pharmaceuticals assayed. Many other researchers reported the effective decomposition of estrogens by ozonation; for example, Huber et al. (2005) reported the successful elimination of 0.5 µg/L of E2, although a dose of ozone of 2 mg/L was required. The same authors showed that E1 is the least reactive towards ozonation among the three target estrogens.

The degradation of certain pharmaceuticals and EDCs was highly variable or poor, mainly because of the low concentration of the drugs and the unfavorable structural properties that lowered their reactivity with ozone (Ziylan and Ince 2011). Moreover, some authors reported that ozonation is not effective for toxicity reduction and mineralization, except with the presence of H_2O_2, which may slightly increase total organic carbon elimination, although not toxicity (Andreozzi et al. 2003a, Ziylan and Ince 2011).

Combined process UV/H_2O_2

The combined process UV/H_2O_2 is one of the most viable AOP technique by its potential for photolytic cleavage of all H_2O_2 to $OH^{\bullet}$ at a stoichiometric ratio of 1:2, provided that the light source has sufficient emission at 190–200 nm. Kim et al. (2009) reported removal of 90% of a large number of pharmaceuticals, including NPX and DCF, in lab-scale operations using 9.32 kJ/m^2 UV (medium pressure lamp), 7.8 mg/L H_2O_2 and drug concentration range of 3.0×10^{-6} to 120×10^{-6} mg/L. However, the use of low pressure halogen light sources at the same light intensity was found to be less efficient due to lower absorption capacity of H_2O_2 (Pereira et al. 2007).

Fenton and UV/Fenton

The process is based on the production of $OH^{\bullet}$ from Fenton's reagent (Fe^{2+}/H_2O_2) at acidic pH, Fe^{2+} acting as the homogeneous catalyst. Combined operation of Fenton oxidation with UV irradiation, namely photo-Fenton process, inherently produces more $OH^{\bullet}$ and is therefore more effective than the dark method. This technique was proved to properly eliminate E1, E2 and EE2 under acidic conditions (Feng et al. 2005). A photo-Fenton process was also employed in a parabolic collector solar pilot plant (30 W/m^2) and was found to provide partial degradation, mineralization and precipitation of DCF upon a considerable decrease in pH (Benitez et al. 2009). On the other hand, Ravina et al. (2002) reported the need of using a 400 W low pressure Hg lamp (254 nm) and an activation energy of 16 kJ/mol, which signifies the role of the energy-requiring reaction in the overall degradation process.

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. Hartman et al. (2008) found that irradiation of a 90 mg/L synthetic DCF solution successively by 216, 617 and 850 kHz at 90W for 1 h provides at least 87%, 90% and 24% degradation of the drug, respectively. Naddeo et al. (2009) proved the feasibility of this technology to remove that NSAID when is combined with ozonation.

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. Photocatalysts include titanium dioxide (TiO_2), zinc oxide, zinc sulfide, cadmium sulfide, among others. TiO_2 has been the most widely applied due to its considerable stability and activity (Augugliario et al. 2006). Ohko et al. (2002) demonstrated complete mineralization of 0.727 mg/L of E2 by using 1 g/L of TiO_2 and a 200 W Hg-Xe lamp, but an operation time of 3 h was needed. The process was also found highly effective for elimination of DCF; however, Rizzo et al. (2009) found that 20-min and 40-min treated samples were more toxic than untreated ones under certain conditions of TiO_2 loadings and DCF concentrations, as also observed by Méndez-Arriaga et al. (2008) during the treatment of DCF and NPX among other anti-inflammatories.

In general, AOPs provide high removal rates but low selectivity and, in some cases, imply considerable high costs associated to the reagents and/or investment. Furthermore, it should be highlighted that under certain conditions these processes may render harmful byproducts; even, transformation products might present higher estrogenicity in comparison to the parent compounds when eliminating estrogenic substrates and/or be non-biodegradable (Esplugas et al. 2002, Gogate et al. 2004, Kim et al. 2007, Krasner et al. 2006, Nakamura et al. 2006, Plewa et al. 2004, Shappell et al. 2008).

1.1.4.2. Laccases: an alternative for the oxidation of the target pollutants

In the current research, the use of oxidative enzymes, specifically laccases from different sources, is proposed as an alternative for the advanced oxidation of the selected estrogenic and anti-inflammatory compounds.

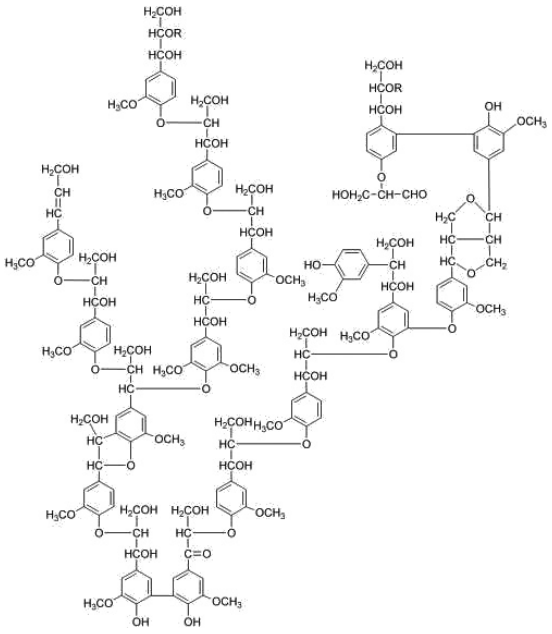
The use of enzymes in industrial processes is usually linked to a reduced consumption of energy as well as chemicals thus beneficial for the environment (Demarche et al. 2012). Moreover, the enzyme world market grew at a double-digit rate in the last years and was about $5.1 billion in 2009 (Sanchez and Demain 2010). Enzymes catalyze specific reactions and mostly act under moderate conditions (temperature, pH, solvents and ionic strength). Enzyme specificity also precludes undesired side-reactions, which would otherwise increase reactant consumption and correspondingly raise the cost of treatment, being a great advantage over conventional chemical treatment processes. Hence, enzymes represent a promising tool for the selective removal of pollutants from

waste streams. Oxidative enzymes and their potential use are deeper introduced in the following section of this chapter.

1.2. Oxidative enzymes

1.2.1. White rot fungi

Wood and other lignocellulosic materials are composed of three main constituents: cellulose, lignin and hemicellulose, which are strongly interconnected and chemically linked by non covalent forces and covalent bonds. Lignin is known to be highly recalcitrant due to its complex structure derived from the coupling of monolignols and three alcohols: p-coumaryl, coniferyl and sinapyl (Figure 1.5) (Kaneda et al. 2008).



Figue 1.5. Chemical structure of lignin.

White-rot fungi (WRF) belong to the class of basidiomycetes and certain ascomycetes; they constitute the most important rotting fungi, since they are the only known organisms able to mineralize lignin producing carbon dioxide and water. The term white-rot has been used to describe forms of wood decay where lignin is preferably degraded and thus leaving a light, white and rather fibrous residue due to the cellulose and hemicellulose accumulation, different from the brown powder observed after brown rot fungi action due to the color of lignin,

which remains intact (Figure 1.6) (Eriksson et al. 1990, Schwarze et al. 2000, Carlile et al. 2001). Within this group, *Phanerochaete chrysosporium*, *Bjerkandera adusta*, *Trametes versicolor*, *Pleurotus ostreatus* are the most extensively studied species (Schwarze et al. 2000).

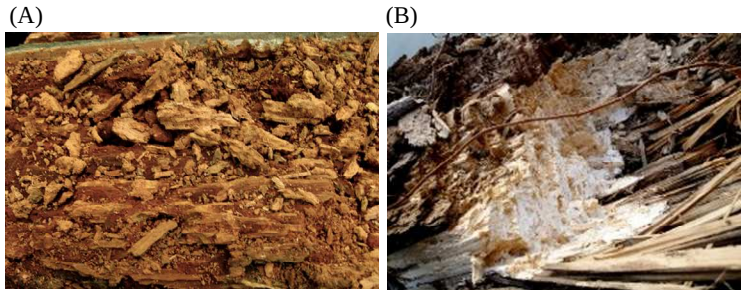


Figure 1.6. Material resulting from wood attacking by brown (A) and white (B) rot fungi.

The ability of fungi to degrade a compound as recalcitrant as lignin lead to consider the potential capability of this type of fungi to remove different recalcitrant and xenobiotic substances. Indeed, various fungal species has been successfully applied for the degradation of a wide range of pollutants, such as polycyclic aromatic hydrocarbons (PAHs), textile dyes, pesticides, and even pharmaceuticals and EDCs. Some examples are shown in Table 1.9.

However, low removal rates of the target compounds were reported when using these fungi; moreover, further research is needed aiming to establish a feasible technology for the application of WRF for bioremediation purpose once, for example, most works dealing with this treatment operated under sterile conditions, completely different to those which would be found in WWTP effluents.

Table 1.9. Examples of removal of different pollutants by fungi.

Pollutants		Fungal species	References
PAHs	Dibenzothiophene, fluoranthene, pyrene	*Bjerkandera* sp. BOS55	Valentín et al. 2007
	Benzo[a]pyrene	*Pleurotus ostreatus*	Egen and Majcherczyk, 1998
	Benzo[a]pyrene, pyrene, etc.	*Trametes versicolor* and *Irpex lacteus*	Borras et al. 2010
Dyes	Remazol Brilliant Blue R and Poly R-478	*Irpex lacteus* and *Pleurotus ostreatus*	Novotny et al. 2001
	Orange G, Amaranth, Remazol Brilliant Blue R, Cu-phthalocyanine and Poly R-478	*Bjerkandera adusta*	Eichlerova et al. 2007
	Indigo carmine	*Phanerochaete chrysosporium*	Podgornik et al., 2001
Pesticides	Diuron, atrazine and terbuthylazine	*Coriolus versicolor*	Bending et al. 2002
Pharmaceuticals	Carbamazepine	*Pleorotus ostreatus*	Golan-Rozen et al. 2011
	Diclofenac, naproxen and ibuprofen	*Phanerochaete chrysosporium*	Rodarte-Morales et al. 2012a,b
	Naproxen and carbamazepine	*Trametes versicolor*	Rodríguez-Rodríguez et al. 2010
	Naproxen	*Trametes versicolor*	Marco-Urrea et al. 2010[a]
EDCs	E2 and EE2	*Trametes versicolor*	Blánquez and Guieysse 2008
	Nonylphenol	*Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleorotus ostreatus*	Soares et al. 2005

1.2.2. The main oxidative enzymes

Delignification by WRF takes places thanks to the capacity of this type of fungi to produce extracellular enzymes, namely ligninolytic enzymes. WRF produce these enzymes during their secondary metabolism, since lignin oxidation provides no net energy to fungi (Wesenberg et al. 2003, Cabana et al. 2007b). In particular, the production of these enzymes has been reported to be caused by nitrogen starvation (Carlile et al. 2001). In addition, WRF secrete mediators of low molecular weight increasing the range of potentially biodegradable compounds.

There are two main types of oxidative enzymes: peroxidases and laccases (phenol oxidases). These enzymes act by generating highly reactive and non specific free radicals that randomly attack lignin molecule by breaking covalent bonds (Kersten and Cullen 2007). These biocatalysts have been successfully applied during the last years for the removal of recalcitrant compounds including pharmaceuticals and EDCs: some examples are shown in Table 1.10 for peroxidases, once the application of laccases will be deeper presented in a next section of this chapter.

Table 1.10. Removal of recalcitrant compounds by peroxidases.

Pollutants		References
PAHs	Anthracene	Karim et al. 2010
	Anthracene, dibenzothiophene and pyrene	Eibes et al. 2006
Dyes	Orange II	López et al. 2004, Mielgo et al. 2003
	Erichrome blue black R and fluorescein	Pirillo et al. 2010
	Poly R-478 and crystal violet	Moldes et al. 2003
Pharmaceuticals	Carbamazepine and diclofenac	Zhang and Geißen 2010
	Carbamazepine, diclofenac, naproxen, sulfamethoxazole, fluoxetine, citalopram	Ebes et al. 2011
EDCs	E1, E2 and EE2	Auriol et al. 2008
	Bisphenol A, triclosan, E1, E2 and EE2	Zheng and Colosi 2011

1.2.2.1. Peroxidases

Peroxidases are a large group of heme-containing enzymes which require the presence of H_2O_2 as the electron acceptor to oxidize lignin and lignin-related compounds (Mester and Tien 2000). Their mechanism of action entails mono-electronic processes and transforms substrates in radicals, which subsequently evolves through non-enzymatic reactions. Peroxidases have a typical enzymatic cycle (depicted in Figure 1.7) where 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, one electron is withdrawn from Fe^{3+} and one from porphyrin, generating Fe^{4+}. Compound I is then 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. 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 ($O_2^{\cdot-}$) (Mester and Tien 2000, Martinez 2002, Wong 2009).

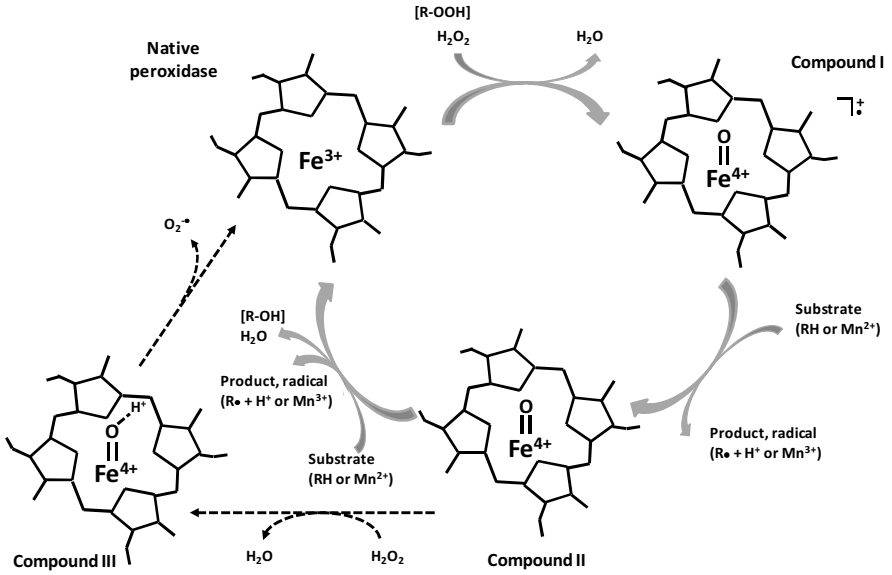


Figure 1.7. Catalytic cycle of peroxidases (adapted from Wesenberg et al. 2003).

Lignin peroxidase (LiP, EC 1.11.1.14)

It was the first ligninolytic enzyme isolated in 1980's decade from the fungus *Pycnoporous chrysosporium* (Glenn and Gold 1983, Tien and Kirk 1983). It is a glycoprotein able to catalyze the oxidation of phenolic and aromatic compounds with a similar structure to lignin. LiP shows a classical peroxidase mechanism, but it is unique in its ability to oxidize substrates of high redox potential (up to 1.4 V) (Wesenberg et al. 2003, Wong et al. 2009).

Manganese peroxidase (MnP, EC 1.11.1.13)

MnP is considered as the most common ligninolytic peroxidase produced by almost all white-rot basidiomycetes (Wesenberg et al. 2003). This enzyme has a similar specificity and catalytic cycle to other peroxidases; however, MnP is also able to oxidize Mn^{2+}, resulting in the formation of diffusible oxidants (Mn^{3+}) capable of penetrating the cell wall matrix and oxidizing phenolic substrates (Pérez et al. 2002, Wong et al. 2009). Like other peroxidases, MnP is sensitive to high concentrations of H_2O_2, but it can be rescued by Mn^{3+}. Ions of Mn^{3+} are quite unstable in aqueous media; to overcome this drawback, they form complexes with organic acids naturally secreted by the fungus, such as malonic or oxalic acid (Hofrichter 2002, Wong et al 2009).

Versatile peroxidase (VP, EC 1.11.1.16)

The enzyme VP is considered a hybrid between MnP and LiP (Wesenberg et al. 2003). It can oxidize not only Mn^{2+} but also veratryl alcohol and phenolic aromatic compounds with high molecular weight by manganese-independent reactions (Wong et al. 2009). VP has been found in cultures of several fungal strains such as *Pleurotus* and *Bjerkandera* species (Wesenberg et al. 2003, Asher et al. 2008).

1.2.2.2. Phenoloxidases

Laccases (EC 1.10.3.2)

Laccases are widely distributed multicopper oxidases with phenoloxidase activity that have been subject of increasing research since their discovery in 19th century, due to their high biotechnological applicability (Cañas and Camarero 2010). Yoshida first described laccase in 1883 from the exudates of the Japanese lacquer tree, *Rhus vernicifera.* However in 1896, for the first time, both Bertrand and

Laborde demonstrated laccase to be a fungal enzyme (Thurston 1994, Levine 1965).

These enzymes catalyze the oxidation of a wide variety of organic and inorganic substrates, including polyphenols, methoxy-substituted phenols and aromatic amines, with the concomitant four-electron reduction of oxygen to water. Laccases have broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups, and as such, the ability to react with the phenolic hydroxyl groups found in lignin (Kunamneni et al. 2007, Sinsabaug and Liptak 1997, Thurston et al. 2004, Wesenberg et al. 2003). The oxidation reaction of phenolic moieties is usually accompanied by demethylation, decarboxylation and can also result in ring cleavage (Wesenberg et al. 2003). Substrates oxidation by laccases occurs via a mechanism involving radicals, which can undergo further laccase-catalyzed reactions and/or nonenzymatic reactions such as hydration or hydrogen abstraction, and polymerization like in the case of phenolic substrates oxidation (Majeau et al. 2010). Other authors reported decarboxylation reactions of phenolic and methoxyphenolic acids (Agematu et al. 1993), as well as the attack to methoxyl groups through demethylation (Leonowicz et al. 1984). Dehalogenation of substituents located in *ortho* and *para* position may also take place in the case of substituted compounds (Schultz et al. 2001).

Laccases catalyzed-oxidation depends on the redox potential of the type-I copper, typically ranging between 500-800 mV. However, in presence of mediators, laccases are able to oxidize a wider range of substrates (Wong 2008); this fact will be further evaluated and discussed in Chapter 2.

The use of atmospheric oxygen as final electron acceptor represents a considerable advantage for the application of laccases compared with peroxidases and would facilitate the implementation of laccase-catalyzed processes (Wesenberg et al. 2003). Other advantage of using laccases is the fact reported by Auriol et al. (2008): these authors observed that laccases did not seem to be affected by the wastewater constituents whereas catalytic efficiency of horseradish peroxidase on the removal of estrogenic compounds was significantly affected by those components. Hence, these benefits besides the nonspecific nature and the extraordinary potential of these enzymes make them attractive for the development of advanced treatment for the oxidation of the target pollutants. Thereby, attention was paid in this Thesis research on the use of laccases.

Occurrence of laccases

Although laccases used in this work were produced by fungal species, this type of enzymes has been demonstrated to be also present in plants and bacteria (Benfield et al. 1964, Diamantidis et al. 2000). Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears and various other vegetables, as well as in various plant species, including lacquer, mango, mung bean, peach, pine, prune, sycamore, etc. (Levine 1965). Laccase activity has been reported only in few bacteria, including *Azospirillum lipoferum, Marinomonas mediterranea, Streptomyces griseus, and Bacillus subtilis*.

Most of the laccases described in literature were isolated from fungi, being the most common laccase producers the wood rotting fungi *Trametes versicolor*, *Trametes hirsuta*, *Trametes ochracea*, *Trametes villosa*, *Trametes gallica*, *Cerena maxima*, *Coriolopsis polyzona*, *Lentinus tigrinus* and *Pleurotus eryngii* (Madhavi and Lele 2009). Nonetheless, laccases are also found in saprophytic compost-inhabiting ascomycetes such as *Myceliophthora thermophila* and *Chaetomium thermophilum* (Morozova et al. 2007a). Fungal laccases are involved in the degradation of lignin and/or in the removal of potentially toxic phenols arising during lignin degradation; in addition, fungal laccases are hypothesized to take part in the synthesis of dihydroxynaphthalene melanins, darkly pigmented polymers that organisms produce against environmental stress or in fungal morphogenesis by catalyzing the formation of extracellular pigments (Kunamneni et al. 2007).

In the current research, fungal laccases from *Trametes versicolor* and *Myceliophthora thermophila* were assayed, as it will be detailed in the following chapters.

Molecular structure and catalytic mechanism

Laccases are glycoproteins with four copper atoms: type-I (T1, one Cu atom), that acts as the primary electron acceptor site where the enzyme catalyzes the oxidation of the substrate and which imparts the blue color to the enzyme; type-II (T2, one Cu atom) which forms a trinuclear copper-cluster with the type-III copper (T3, two Cu atoms), where the reduction of O_2 takes place (Majeau et al. 2010, Martínez et al. 2005, Wong 2008). The catalytic cycle of laccases is represented in Figure 1.8.

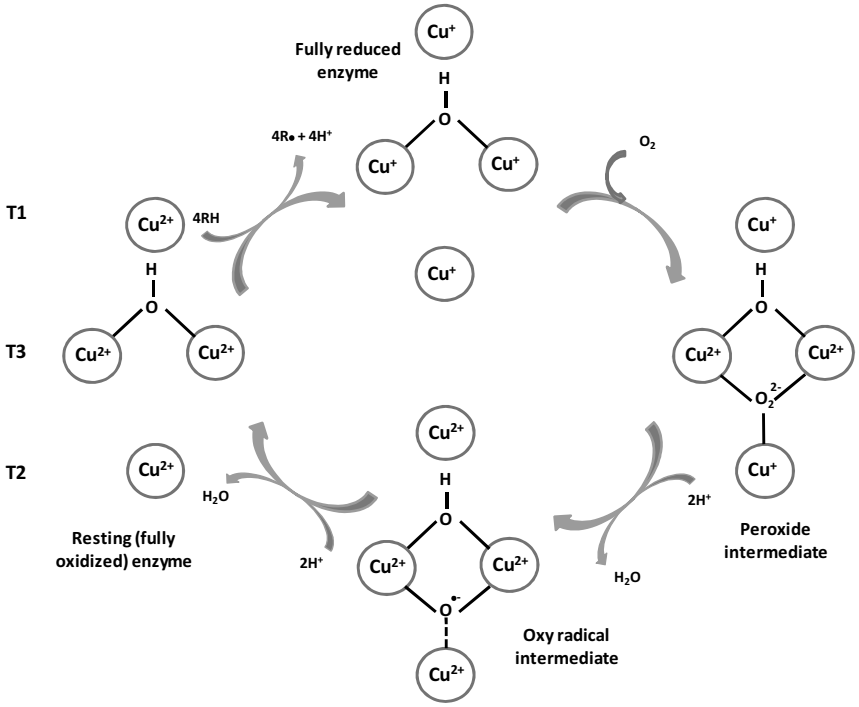


Figure 1.8. Catalytic cycle of laccases (adapted from Wong et al. 2009).

In the resting state the enzyme is completely oxidized; then, a total reduction of the metallic centers (Cu^{2+} to Cu^{+}) by the substrate occurred in 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 of the enzyme to a fully reduced state. Successive substrate oxidation is required to fully reduce the enzyme (Huang et al. 1999, Zoppellaro et al. 2001).

Reduction of dioxygen takes place in two steps via the formation of bound oxygen intermediates. The dioxygen molecule first 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 the oxygen has not been fully established, 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 (Lee et al. 2002, Palmer et al. 2001). 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 the T2 copper correlates with the decay of the intermediate in which the first water is released

and the second water molecule remains bound and slowly exchanged with the bulk solution (Wong et al. 2008).

The capture of four electrons by the substrate returns the enzyme to its native state; thus, 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 observed. Kurniawati and Nicell (2007a) reported that phenol concentrations higher than 10 mM caused the ratio to approach the limit of 4/1. Nevertheless, this ratio can vary between 4/1 and 1/1 depending on the nature and concentration of the substrate, and may even be lower in the case of polyphenol (Majeau et al. 2010). Anyhow, uncertainties remain about the electron transfer pathway and reduction of dioxygen to water and thus, there is no clear single mechanism for laccase-mediated reactions.

Other aspects related to laccases and non mentioned in this General Introduction will be addressed throughout the different chapters of this Thesis dissertation.

1.2.3. Applications of laccases

Two of the most intensively studied areas in the potential industrial application of fungal laccases are the delignification and pulp bleaching as well as the bioremediation of contaminating environmental pollutants (Kunamneni et al. 2007). However, many other applications have been investigated; some examples are listed below.

1.2.3.1. Laccases in the textile industry

The use of laccases in the textile industry is growing very fast since, apart from decolorizing textile effluents, laccases are used to bleach textiles, modify the surface of fabrics and synthetize dyes. Therefore, laccase-based processes might replace the traditionally high chemical, energy and water-consuming textile operations (Rodríguez-Couto and Toca-Herrera 2006). For instance, Campos et al. (2001) reported the degradation of indigo both in effluents and on fabrics using purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii* in combination with redox-mediators; they reported that bleaching of fabrics by the laccases correlated with the release of indigo degradation products. More recently, Pazarlogliu et al. (2005) showed that a phenol-induced laccase from *Trametes versicolor* was an effective agent for stone washing effects of denim fabric.

Tzanov et al. (2003) reported for the first time the enhancement of the bleaching effect achieved on cotton using laccases in low concentrations. As an alternative to the chemical scouring of rove, Ossola and Galante (2004) studied the effects of several enzymes including laccases under moderate conditions for the flax processing into yarn. Zille (2005) proved the ability of laccases for wool dyeing, and Yoon (1998) patented the application of laccase from *Trametes versicolor* plus a mediator to increase the shrink resistance of wool.

1.2.3.2. Medical and personal care application of laccases

Laccase can be used in the synthesis of complex medical compounds as anesthetics, antibiotics, sedatives, etc., including triazolo(benzo)cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins and dimerized vindoline. Also, a novel application field for laccases is in cosmetics. For example, laccase based hair dyes could be less irritant and easier to handle than current hair dyes; cosmetic and dermatological preparations containing proteins for skin lightening have also been developed (Kunamneni et al. 2008c).

1.2.3.3. Laccases in the food industry

Laccases have been used for the treatment of polyphenols in beer factory wastewater (Yague et al 2000), color in distillery wastewater (Gonzalez et al 2000) and aromatic compounds in olive mill wastewater (Jaouani et al. 2005). Moreover, the use of laccases was proposed for the oxidation and polymerization of polyphenolic substances for wine clarification (Minussi et al. 2002) as well as for the stabilization of fruit juices (Piacquadio et al. 1997).

1.2.3.4. Biosensor and diagnostic application of laccases

A number of biosensors containing laccase have been developed for immunoassays and for determination of glucose, aromatic amines and phenolic compounds (Kunamneni et al. 2008c). Laccase covalently conjugated to biobinding molecules (antibody, antigen, DNA, RNA, biotin, and streptavidin) can be used as a marker enzyme for immunochemical histochemical, cytochemical or nucleic acid detection assay (Schmid and Urlacher 2007). Bauer et al. (1999) developed an enzymatic method based on laccase to distinguish morphine from codeine simultaneously in drug samples injected into a flow detection system. Sabela et al. (2012) reported the use of a glassy carbon electrode modified with laccase for the detection of phenolic compounds in herbal teas.

1.2.3.5. Laccases for organic synthesis

Recently, increasing interest has been focused on the application of laccase as a new biocatalyst in organic synthesis. For example, laccase-catalyzed cross-linking reaction of new urushiol analogues for the preparation of "artificial urushi" polymeric films (Japanese traditional coating) was demonstrated by Ikeda et al. (2001). It was also mentioned that laccase induced radical polymerization of acrylamide with or without mediator (Ikeda et al. 1998). Laccase can also be used to synthetize various functional organic compounds including polymers with specific mechanical/electrical/optical properties, textile dyes, cosmetic pigments, flavor agents and pesticides (Kunamneni et al. 2008c).

1.2.3.6. Laccases in the pulp and paper industry

In the industrial preparation of paper the separation and degradation of lignin in wood pulp are conventionally obtained using chlorine- or oxygen-based chemical oxidants; it was proved that the pretreatment of wood pulp with laccase can provide milder and cleaner strategies of delignification (Barreca et al. 2003, Gamelas et al. 2005). Most of the studies which have been patented in this field are related to the use of laccase-mediator systems in the pulp-kraft bleaching processes (Kunamneni et al. 2008c). For example, Moldes and Vidal (2011) investigated the use of laccase and various sulfonated mediators in laccase-assisted bleaching of eucalypt kraft pulp. More recently, the potential of laccases for functionalizing lignocellulosic fibers has been studied. The laccase-based biografting of phenolic compounds for improving strength properties of kraft paper made from high-kappa pulps was evaluated by Chandra et al. (2002, 2004). Widsten et al. (2009) reported that laccase treatment combined with tannic acid enhances the adhesion of fibers in the environmentally friendly production of fiberboard. Also, the internal bond of particle boards is significantly enhanced by laccase-catalyzed functionalization with 4-hydroxy-3-methoxybenzylurea (Fackler et al. 2008). Recently, some researchers have focused on using laccase and some phenolic compounds for conferring antibacterial properties to lignocellulosic materials (Fillat et al. 2012a).

1.2.3.7. 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, including dyes and PAHs among others; some examples are summarized in Table 1.11.

Table 1.11. Removal of recalcitrant compounds by laccases.

Pollutants		Laccase source	Mediator	References
Dyes	Basic Green 4 and Acid Violet 17	*Cyathus bulleri*	-	Chhabra et al. 2009
	Reactive Black 5	*Trametes villosa*	-	Zille et al. 2003
	Reactive Blue, Reactive Black 5, Direct Blue 71, Basic Red 9 Base, Reactive Blue 19, Acid Blue 225, Acid Blue 74	*Trametes hirsuta*	-	Abadulla et al. 2000
	Reactive Yellow 15, Reactive Red 239 and Reactive Blue 114	Commercial laccase formulation - DeniLite IIS	-	Tavares et al. 2009
	Reactive Black 5	*Pleurotus sajor-caju*	1-hydroxybenzotriazole (HBT)	Murugesan et al. 2007
	Reactive Black 5, Acid Blue 25, Methyl Orange, Remazol Brilliant Blue B, Methyl Green and Acid Green 27	*Myceliophthora thermophila*	With and without HBT	Kunamneni et al 2008b
	Reactive Black 5	*Pycnoporus cinnabarinus* and *Trametes villosa*	Phenolic compounds used as natural mediators	Camarero et al. 2005
	Red FN-2BL, Red BWS, Remazol Blue RR and Blue 4BL	*Trametes versicolor*	Syringaldehyde	Mendoza et al. 2011
PAHs	Phenanthrece	*Trametes versicolor*	-	Han et al. 2004
	Acenaphthylene, anthracene, benzo[a]pyrene, etc.	*Trametes versicolor*	-	Majcherczyk et al. 1998
	Anthracene, pyrene, fluorene, fluoranthene, phenanthrene, and perylene	*Pleurotus ostreatus*	With and without HBT and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Pozdnyakova et al. 2006
	Benzo[a]pyrene	*Trametes versicolor*	ABTS	Dodor et al. 2004
	Benzo[a]pyrene	*Pycnoporus cinnabarinus*	p-hydroxicinnamic acids	Camarero et al. 2008

Table 1.11. Removal of recalcitrant compounds by laccases (Cont.).

Pollutants		Laccase source	Mediator	References
Pesticides	Phenylurea herbicide	*Trametes versicolor*	-	Jolivalt et al. 2000
	Chlorophenols	*Coriolus versicolor*	-	Zhang et al. 2008
	Halogenated pesticide	*Coriolopsis gallica*	Acetosyringone and syringaldehyde	Torres-Duarte et al. 2011
EDCs	Bisphenol A	*Trametes versicolor*	-	Kim and Nicell 2006
		Trametes villosa	-	Fukuda et al. 2004
		Coriolopsis polyzona	-	Cabana et al. 2007a,2009
		Commercial formulation (Daiwa)	With and without HBT, ABTS and natural mediators	Sei et al. 2008
	Triclosan	*Coriolopsis polyzona*	-	Cabana et al. 2007a,2009
		Ganoderma lucidum	Without and with HBT, ABTS and natural mediators	Murugesan e al. 2010
	E1, E2, EE2	*Phanorechaete sordida*	-	Tamagawa et al. 2006
		Commercial formulation (Daiwa)	-	Tanaka et al. 2009
		Commercial formulation (Daiwa)	With and without HBT, ABTS and natural mediators	Sei et al. 2008
		Trametes versicolor	With and without HBT	Auriol et al. 2007,2008
Pharmaceuticals	Oxybenzone	*Trametes versicolor*	Without and with HBT, ABTS and natural mediators	Garcia et al. 2011
	NPX	*Trametes versicolor*	HBT	Marco-Urrea et al. 2010a
	DCF	*Trametes versicolor*	-	Marco-Urrea et al. 2010b

Application of laccases for the removal of estrogens and anti-inflammatories

With respect to the target compounds assayed in this research, various works previously reported the efficiency of laccase and laccase-mediator systems on the removal of E1, E2 and EE2 (Table 1.11). However, important challenges were still pending once most of them were performed batchwise: the development of technology for the continuous application of enzymatic remediation was still a pending objective.

Regarding the target anti-inflammatory compounds (NPX and DCF), only few works dealing with the removal of these pollutants were published up to that the current research was initiated: Marco-Urrea et al. (2010a, b) performed *in vitro* experiments with *Trametes versicolor* laccase aiming to corroborate the role of this enzyme during the *in vivo* treatment. Nonetheless, the benefits of using laccases for bioremediation had not been exploited for the detoxification of these emerging contaminants and thus, further interesting investigation could be still conducted.

1.3. Objectives

In the present Thesis, the oxidation of estrogenic and pharmaceuticals compounds has been investigated, selecting the natural and synthetic hormones E1, E2 and EE2 and the anti-inflammatories NPX and DCF, due to their proved presence and negative effects in the environment. The use of laccases is proposed as an advanced treatment, which could be an alternative to conventional processes as well as to emerging AOPs.

The specific objectives can be described as follows:

i. Study and evaluation of laccase and laccase-mediator systems in batch reactors for the oxidation of the target compounds but also for their detoxification.

ii. Design and operation of continuous bioreactors to facilitate the implementation of the enzymatic treatment; different strategies were evaluated: enzyme immobilization by different techniques and enzyme retention by a membrane.

iii. Optimization of the continuous treatment by evaluating the main factors affecting the removal and detoxification efficiencies.

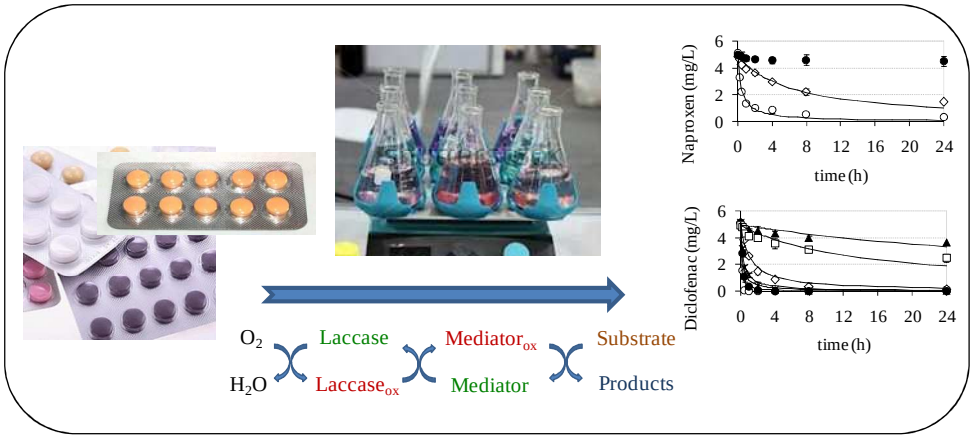
iv. Application of the enzymatic treatment under realistic conditions: pollutants at environmental concentrations and in real matrix.

v. Identification of the transformation products and main reaction mechanisms.

vi. Investigation of a novel technology based on enzymatic microreactors and evaluation of its potential application for laccases.

Chapter 2

Laccase and laccase-mediator systems: suitable tools for the removal of estrogens and anti-inflammatories in batch reactors*



* Part of this Chapter has been published as:

L. Lloret, G. Eibes, M.T. Moreira, G. Feijoo and J.M. Lema, On the use of a high-redox potential laccase as an alternative for the transformation of non-steroidal anti-inflammatory drugs (NSAIDs), Journal of Molecular Catalysis B: Enzymatic 97 (2013) 233-242.

L. Lloret, G. Eibes, T.A. Lú-Chau, M.T. Moreira, G. Feijoo and J.M. Lema, Laccase-catalyzed degradation of anti-inflammatories and estrogens, Biochemical Engineering Journal 51 (2010) 124-131.

Outline

2. Laccase and laccase-mediator systems: suitable tools for the removal of estrogens and anti-inflammatories in batch reactors

2.1. Introduction

In this research, the use of laccases is proposed as an alternative to perform the oxidation of estrogenic and anti-inflammmatory compounds. In a first step, the removal of the estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), and the anti-inflammatories: naproxen (NPX) and diclofenac (DCF), is evaluated in batch reactors aiming to establish the basis for further development of more complex bioremediation systems.

Laccase from the ascomycete *Myceliophthora thermophila* was initially selected as the biocatalyst of interest once this enzyme is produced at industrial scale with a price of $0.03 \cdot 10^{-3}$ € per Unit (U) of enzyme activity. Moreover, this low redox potential laccase can be heterologously expressed in industrial hosts (such as *Aspergillus oryzae*) with high yields, compared with the basidiomycete laccases whose heterologous expression provides reduced yields currently limiting their large-scale commercialization (Babot et al. 2011). Thus, *Myceliophthora thermophila* laccase has the advantage of being a commercially available cheap enzyme, which would enable the scale-up of a potential enzyme-based technology. Nevertheless, removal yields of recalcitrant substrates, such as NPX and DCF, could be limited due to the low redox potential of the enzyme (0.47 V) (Xu et al. 1999). Enhanced kinetics would be expected when using a high redox potential laccase, such as the one from *Trametes versicolor* (0.78 V) (Reinhammar 1972), which was, indeed, successfully utilized on the removal of pharmaceuticals, such as oxybenzone, from municipal primary effluent (Garcia et al. 2011); this enzyme is also commercially available but at a price of $3.8 \cdot 10^{-3}$ €/U.

On the other hand, besides from the oxidation capacity of laccase towards diphenols and aromatic amines the range of substrates potentially degraded may be wider by means of the action of mediators that boost the oxidation capacity of the enzyme (Bourbonnais and Paice 1990). These mediators are usually low molecular weight compounds that act as an ‘electron shuttle’ between the oxidized enzyme and the target compound (Fabbrini et al. 2002).

In case the mediator is present, it can be oxidized by laccase and subsequently oxidize the target compound which is either substrate or non-substrate of the enzyme, resulting in the formation of oxidation products and regeneration of the mediator (scheme in Figure 2.1). Therefore, the prospect of

using mediators offers important possibilities to either indirectly increase the range of compounds that can be oxidized through the action of laccase or to offer multiple modes of attack on a substrate, thereby leading to enhanced conversion of the target compound (Kurniawati and Nicell 2007b).

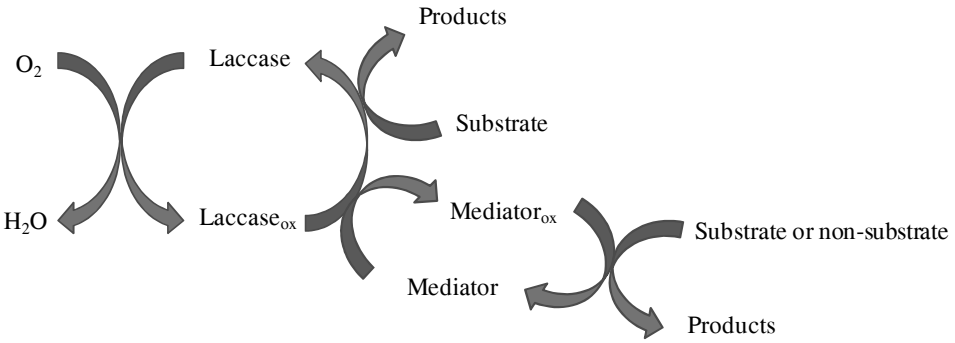


Figure 2.1. Scheme of the oxidation of compounds by laccase and laccase-mediator systems.

The most commonly used mediators are 1-hydroxybenzotriazole (HBT), 2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) and violuric acid (VA) (Fabbrini et al. 2002, Kurniawati and Nicell 2007b). However, the use of these synthetic substances could imply important costs, the potential generation of radical species with suspected toxicity and also, they have been suggested to be scarcely biodegradable (Camarero et al. 2004). In this way, transformation of nonphenolic aromatic structures was found when degrading lignin even by white-rot fungi that produce laccase as the sole ligninolytic oxidoreductase (Geng and Li 2002). Evidences suggest that free radicals of some fungal metabolites and lignin-derived products are involved in that phenomenon, acting as laccase mediators (Camarero et al 2007, 2008, Cañas et al. 2007). The use of these low cost and naturally occurring compounds may facilitate the application of laccase-catalyzed systems in biotechnological processes. Some of these compounds (e.g. p-hydroxycinnamic acids) are present in herbaceous plants as extractives forming lignin carbohydrates bridges; others (e.g. phenolic aldehydes, ketones and acids) are originated during lignin degradation and subsequently incorporated into the soil organic matter (Camarero et al. 2007).

Taking these considerations into account, different mediators were investigated in this work: i) synthetic: HBT and VA, and ii) natural mediators: syringaldehyde (SA), vanillin (V) and the p-hydroxycinnamic acids p-coumaric

acid (p-CA) and ferulic acid (FA); corresponding structures are depicted in Figure 2.2.

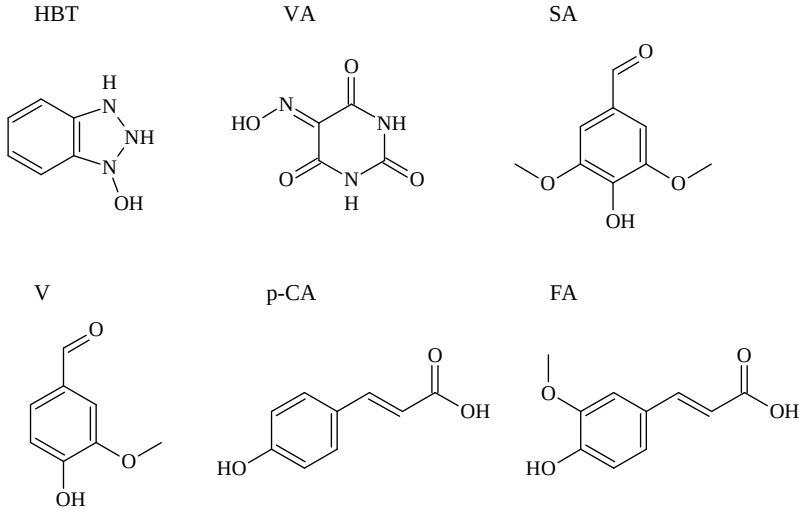


Figure 2.2. Structure of the laccase mediators used in this research.

Furthermore, the optimum pH for a certain laccase-catalyzed reaction is highly dependent on the substrate and moreover, pH could also limit the efficiency of the enzymatic treatment because of the pH-dependence of reactivity and stability of mediator radicals, redox potential of the substrate, etc. Overall, it is important to explore the effect of pH on the transformation of compounds of interest: estrogens and anti-inflammatories in this case, prior to further research, despite the pH influence on laccase activity and stability was also evaluated with the corresponding preliminary experiments, as it will be presented.

Summarizing, two different laccases were evaluated and effects of pH and synthetic and natural redox mediators on both removal efficiency and laccase stability were studied in this chapter, aiming to establish the groundwork for the successful laccase-catalyzed transformation of estrogenic and anti-inflammatory compounds. Furthermore, effort was paid to evaluate the toxicity of the biotransformation products from the laccase-catalyzed reactions, which could limit the applicability of this technology.

2.2. Materials and methods

2.2.1. Chemicals

The target compounds: estrogens (E1, E2, EE2) and anti-inflammatories (NPX and DCF), were purchased from Sigma-Aldrich. Stock solutions were prepared in methanol (J.T. Baker, HPLC grade, 99.8%). ABTS was provided from Fluka. The mediators evaluated: HBT, SA, VA, V, p-CA and FA, were purchased from Sigma-Aldrich. All other reagents used were of analytical grade.

The recombinant yeast *Saccharomyces cerevisae* was kindly provided by the Laboratory of Microbial Ecology and Technology (Labmet, Ghent University, Belgium). All Microtox® test reagents, including *Vibrium fischeri* bacteria, were purchased from SDIX.

2.2.2. Enzymes

Commercial laccase from *Myceliophthora thermophila* (56 kDa), namely Novozym 51003, was provided by Novozymes. This enzyme was produced by submerged fermentation of genetically modified *Aspergillus* sp. *Trametes versicolor* laccase was purchased by Sigma-Aldrich with an activity of ~13.6 U/mg, as indicated by the supplier. Three-dimensional structure of these enzymes can be found in literature and are shown in Figure 2.3.

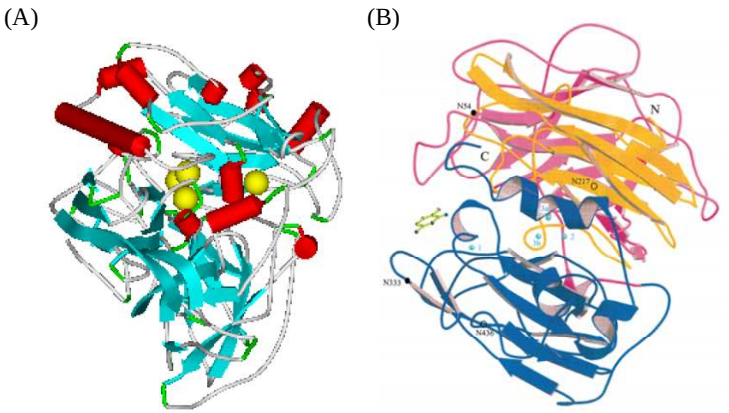


Figure 2.3. Three-dimensional representation of *Myceliophthora thermophila* (A) and *Trametes versicolor* (B) laccases (from López Cruz 2007 and Bertrand et al. 2002).

2.2.2.1. Determination of laccase activity

The activity of laccase was measured using a colorimetric assay with ABTS as a color-generating substrate, where the rate of color formation was proportional to enzyme activity.

The oxidation of 5 mM ABTS to its cation radical ($ABTS^{\cdot+}$) due to the catalytic action of laccase was monitored at a wavelength of 436 nm in 100 mM sodium acetate buffer, pH 5 and 30 ºC (ε_{436} = 29,300$M^{-1}\cdot cm^{-1}$) using a Shimadzu UV-1603 spectrophotometer. One Unit (U) of activity was defined as the amount of enzyme forming 1 µmol of $ABTS^{\cdot+}$ per min, and laccase activity was determined as U/L.

2.2.2.2. Evaluation of the catalytic activity and stability of the laccases

Effects of pH and temperature on laccase activity

The effect of pH on laccase activity was investigated by using ABTS as substrate as described above, but in 100 mM of citrate-phosphate-borate buffer once it is an effective buffer from pH 3 to 11 (Kurniawati and Nicell 2008, Xu 1997); pH values in the range 2-7 were assayed. Relative activities were determined as the ratio of the obtained activity at the corresponding pH and the maximum attained.

The effect of temperature (20-70ºC) on laccase activity was determined by measuring activity at the corresponding temperature and under standard conditions previously detailed. The relative activity was calculated as the ratio between the activity at each temperature and the maximum attained.

Effects of pH and temperature on laccase stability

The effect of the pH on the enzyme stability was studied by incubating laccases in 100 mM citrate-phosphate-borate buffer (pH 2-7) at room temperature during 24 h. Laccase activity was determined at different incubation times; for this purpose, appropriate volume of samples were taken and transferred to standard reaction conditions in order to measure laccase activity with 5 mM ABTS. The residual activity was calculated considering the laccase activity determined at the corresponding incubation time and pH and the theoretical initial value.

Thermal stability was determined by incubating enzymes in 100 mM phosphate buffer (pH 7) at selected temperatures in the range 20-70ºC. Samples were periodically withdrawn aiming to determine residual laccase activity as the ratio of measured value and the theoretical initial one.

Estimation of laccase affinity

Laccase affinity for the substrate ABTS was measured under the standard conditions previously indicated for the determination of laccase activity and at concentrations in the range 25-1500 µM. Experimental data was fitted to determine the Michaelis-Menten parameters by minimizing the sum of squared residuals

2.2.3. Removal of estrogens by *Myceliophthora thermophila* laccase

Laccase-catalyzed removal of E1, E2 and EE2 was assayed by using *Myceliophthora thermophila* laccase. The experiments were performed in 50-mL Erlenmeyer flasks sealed with Teflon under continuous magnetic stirring (about 150 rpm) at room temperature (about 25-26ºC). The reaction medium (20 mL) consisted of 5 mg/L of E1, E2 and EE2 (each and separately) and an initial laccase activity of 2,000 U/L. The effect of pH on the removal efficiency and laccase stability was evaluated at pH 4 (100 mM acetate buffer) and pH 7 (100 mM phosphate buffer) in the absence of mediator. Corresponding controls lacking laccase were run in parallel and demonstrated that removal of the compounds only occurs in the presence of enzyme.

Samples were withdrawn to measure laccase activity under standard conditions. Afterwards, samples were acidified to pH 2 by addition of HCl in order to stop the reaction by the enzyme inactivation and were frozen until further analysis. The residual concentration of E1, E2 and EE2 in the samples was quantified by high performance liquid chromatography (HPLC). All experiments were performed in duplicate and samples were analyzed twice. Removal yields (%) were calculated considering the residual concentration at each monitored time and the initial one (identical to that detected in the control experiment after the same period); residual activity (%) was calculated as the ratio between the measured laccase activity and the theoretical initial value.

2.2.4. Removal of anti-inflammatories

Removal of anti-inflammatory compounds, NPX and DCF, was conducted using laccase from different sources: *Myceliophthora thermophila* and *Trametes versicolor* laccase; moreover, both laccase and laccase-mediator systems were investigated in this case.

2.2.4.1. Removal of anti-inflammatories by *Myceliophthora thermophila* laccase

Effect of pH and HBT

Experiments were analogous to those for estrogens removal: NPX and DCF (5 mg/L each and separately) with 2,000 U/L of *Myceliophthora thermophila* laccase at pH 4 and 7 in batch reactors (Erlenmeyer flasks). The use of HBT at a concentration of 1 mM was also evaluated at both values of pH. Controls in the presence of mediator but lacking enzyme and those lacking enzyme and mediator demonstrated that the removal of the target compounds takes place only by enzymatic action.

Effect of natural mediators

The effect of various synthetic (HBT and VA) and natural mediators (SA, V, p-CA and FA), at a concentration of 1 mM, was assessed on the removal of the anti-inflammatories at pH 4 and with an initial laccase activity of 2,000 U/L.

Effect of mediator concentration

In order to determine the effect of the concentration of the mediators, experiments were carried out at pH 4, as described above, using different concentrations of HBT, VA and SA (mediators which provided the best results in the previous experiments) and an initial laccase activity of 2,000 U/L. Concentrations evaluated were: 0.1, 0.25, 0.5, 1 and 2 mM.

2.2.4.2. Removal of anti-inflammatories by *Trametes versicolor* laccase

Influence of pH and mediators in batch experiments

Enzymatic removal of NPX and DCF (5 mg/L each and separately) was also investigated with 2,000 U/L of *Trametes versicolor* laccase at pH 4 and 7. The effect of using a synthetic and a natural mediator, HBT and SA respectively, at a concentration of 1 mM was investigated at both values of pH. The effects of pH and mediators on both the removal efficiency and laccase stability were examined; kinetics of the oxidation reaction as well as enzyme inactivation were evaluated for the different experiments.

Fed-batch removal of DCF

An additional experiment was carried out with the aim of testing the transformation of DCF in fed-batch operation to evaluate the capability of the enzymatic system to remove consecutive loads of pollutant. The experiment was performed in a Biostat Q (B. Braun-Biotech International) reactor under continuous stirring at 26ºC controlled by circulating thermostated water through the reactor chamber. The reaction medium (250 mL) consisted of 5 mg/L of DCF in acetate buffer (100 mM, pH 4) and a single initial pulse of 2,000 U/L laccase. Fed-batch addition of DCF (5 mg/L each pulse) was carried out every hour during the course of the operation.

2.2.5. Toxicity evaluation

To investigate the potential toxicity of the medium resulted from the enzymatic treatment, various experiments were carried out: the reduction of estrogenic activity of the effluent from experiments of E1, E2 and EE2 removal was measured, whereas the toxicity of DCF transformation products was investigated using Microtox® test as well as by determining the aerobic biodegradability. Samples withdrawn from the experiments conducted under the best conditions evaluated were utilized for this purpose.

2.2.5.1. Determination of estrogenic activity

The estrogenic activity was measured by LYES (yeast estrogen screen-assay assisted by enzymatic digestion with lycticase); the protocol was adapted from that described by Schultis and Metzger (2004).

Yeast cultures, pre-incubated for 48 h, were resuspended in fresh yeast-peptone-dextrose medium (yeast extract 10 g/L, casein peptone 20 g/L, dextrose 20 g/L in distilled water). Samples of 50 µL were delivered into 1.5-mL eppendorfs; distilled water was used as control. Each eppendorf was inoculated with 450 µL of yeast suspension. The eppendorfs were sealed with parafilm and incubated at 37ºC. After 24 h, 200 µL of a stock lyticase solution (1 g/L; Sigma) diluted in Z-buffer (10 x; 60 mM $Na_2HPO_4 \cdot 7\ H_2O$, 40 mM NaCl, 1 mM $MgSO_4 \cdot 7\ H_2O$, 50 mM 2-mercaptoethanol) was added. The solution was incubated for 45 min at room temperature and then 175 µL Tween 80 (0.1 % v/v; Merck) was added. After 20 min of incubation (room temperature), 125 µL of a stock solution of chlorophenol red galactopyranoside (1 g/L; Sigma) was added. Finally, absorbances at 550 nm and 630 nm were measured after 2 h by using a Shimadzu

UV-1603 spectrophotometer. The estrogenic activity was calculated by the Equation (2.1):

$$\text{Response} = (\text{Abs}^{x}_{550nm} - \text{Abs}^{blk}_{550nm}) - (\text{Abs}^{x}_{630nm} - \text{Abs}^{blk}_{630nm}) \quad (2.1)$$

where x corresponds to the sample and blk to the control.

2.2.5.2. Aerobic biodegradability experiments

Aerobic toxicity was evaluated by the monitorization of cumulative oxygen consumption during aerobic biodegradation of the samples. For this, aerobic activated sludge collected from a membrane bioreactor operated for the postreatment of the effluent of an anaerobic UASB was used. Sludge, with concentrations 2.8 g VSS/L and 3.1 g TSS/L, was previously washed with phosphate buffer (10 mM, pH 7) and stored at 4ºC. Oxygen consumption was measured with an automated OxiTop device (WTW) consisting of bottles with manometric heads and a controller, and the determination was carried out according to the Standard Methods (1999). The technique was based on decreasing pressure inside the closed flasks containing the inoculated sample, being proportional to oxygen consumption. The assays were carried out in duplicate at 20ºC and lasted 13 days until values were maintained constant.

2.2.5.3. Microtox® test

Microtox® toxicity assays were performed by using a Microtox® model 500 Analyzer. The luminescent marine bacterium *Vibrium fischeri* was the bioassay microorganism used for these experiments. The standard manufacturer's procedure was followed to determine the toxicity. The results were expressed as $EC_{50,15min}$, which corresponds to the effective concentration of a sample that causes a reduction in the light output of the Microtox® test organism by 50% in 15 min of contact, and were estimated according to the "Basic Test" protocol of the software (Microbics Corporation, 1992).

2.2.6. Quantification of estrogens and anti-inflammatories

The determination of estrogens (E1, E2 and EE2) concentration was carried out through HPLC in a HP-1090 system (a Jasco XLC system was used for some analysis) equipped with a diode array detector at 210 nm. Chromatographic separation was performed on a Lichrocart 250-4 column packed with Lichrosphere 100 RP-18 5 µm (Merck) and 200 µL of injection volume was used. The mobile phase consisted of a binary mixture of solvents A (acetonitrile) and B

(50 mM phosphate buffer, pH 4.5) at a flow rate of 0.8 mL/min. The gradient method and running times were programmed as follows: initial conditions were 40% A for 1 min, then linearly programmed up to 80% until 20 min and held for 4 min, followed by a linear program at 40% for 5 min and held for 1 min.

Quantification of anti-inflammatory compounds (NPX and DCF) was carried out by HPLC by using the same system and monitoring the absorbance at 220 nm. The injection volume was 200 μL and the isocratic eluent (acetonitrile:50 mM phosphate, pH 4.5; 45:55) was pumped in at 0.8 mL/min.

2.3. Results and discussion

2.3.1. Evaluation of the catalytic activity and stability of the laccases

The potential use of an enzyme in a bioprocess will be favored by various factors, mainly: i) low costs of production; ii) inexpensive co-factors substrates; iii) good stability when stored under moderate conditions; iv) ability to exert its catalytic activity under a wide range of conditions (Kurniawati and Nicell 2008). As indicated, production costs of *Myceliophthora thermophila* laccase were much lower, although both commercial laccases were used in the case of the more recalcitrant compounds. Besides, laccases use oxygen as an inexpensive and readily available oxidant. Also, it has been extensively reported that laccases are relatively stable when stored under near neutral pH and below room temperatures (Bonomo et al. 2001, Xu et al. 1996); even, they have been also reported to show catalytic activity under different conditions (Call and Mucke 1997, Xu 1997). Anyhow, the catalytic activity and stability of the specific laccases used in the current work was evaluated under a wide range of reaction conditions; concretely, the effects of pH and temperature on both laccase activity and stabilities were investigated. The effectiveness of laccases in many industrial applications will depend on their potential inactivation over time under the operational conditions of the reaction environment, mainly pH and temperature (Kurniawati and Nicell 2008). Therefore, the study of the impacts of these factors will permit to elucidate the optimum conditions to ensure the robustness of the enzymes, as well as to decide the starting conditions for the next biotransformation experiments.

2.3.1.1. Effects of pH and temperature on laccase activity

Effects of pH and temperature on the laccase activity of both enzymes were investigated by assaying the activity at the corresponding values of pH and

temperature. Results found for *Myceliophthora thermophila* and *Trametes versicolor* laccases are shown in Figures 2.4 and 2.5, respectively.

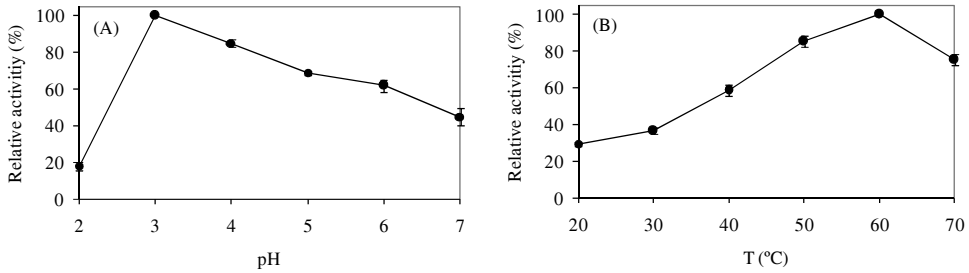


Figure 2.4. Effect of pH (A) and temperature (B) on the activity of *Myceliophthora thermophila* laccase.

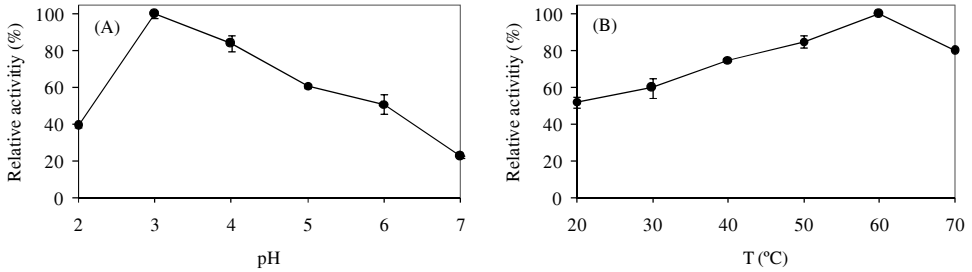


Figure 2.5. Effect of pH (A) and temperature (B) on the activity of *Trametes versicolor* laccase.

Both laccases presented their maximum activity at high values of temperature. This could be explained by the fact that, when the temperature is elevated, there is a tendency for the reaction rate to increase (Kurniawati and Nicell 2008). The decrease on laccase activity at temperatures higher than 60ºC could be due to the rapid laccase inactivation at those temperatures, as it will be detailed in a following section of this chapter, which would result in a diminished ABTS transformation; this is, the effect of the enzyme inactivation would be more significant than that of high initial reaction rate due to temperature.

On the other hand, it was observed that laccases exhibited maximum activity at pH 3; it means, optimal pH range for ABTS transformation is in the acidic region. The pH optima for fungal laccases was generally reported in the acidic region, with values which ranged from as low as 2.7 to as high as 7.5, but tipically in the range 3.5-6, depending on the substrate (Call and Mucke 1997, Gianfreda et al 1999).

Research on catalytic mechanisms have shown that the catalytic reaction of laccases involves: i) the sequential abstraction of electrons from the substrate to the T1 copper and subsequent sequential reduction of the T1 copper, ii) the sequential internal electron transfer from the T1 to the T2/T3 copper cluster, and iii) the binding and subsequent reduction of oxygen to water at the T2/T3 copper cluster, with the consequent oxidation of the target substrate (Gianfreda et al. 1999, Kurniawati and Nicell 2008, Xu 1997). Thereby, any pH-related structural or mechanistic changes in the substrate, oxygen or laccase would contribute to the pH-dependency of catalytic activity (Xu 1997). Besides, the pH may influence the redox potential of the reacting species, affecting the overall oxidation extent.

In this way, the oxidation of substrates by laccase is highly dependent on the redox potential difference between the substrate and the T1 copper of laccase (ΔE^0), which is the driving force for the electron transfer (Call and Mucke 1997, Xu 1997) and as shown in Equation (2.2):

$$\Delta E^0 = E^0(\text{T1 copper}) - E^0(\text{substrate}) \quad (2.2)$$

Due to oxidative deprotonation, the redox potential of phenolic substrates decreases as pH increases, whereas redox potential of fungal laccases was reported to slightly vary by 0.1 V in the range of pH 2.7-11 (Xu 1997). Thus, ΔE^0 would increase with the pH, which means a faster electron transfer from the substrate to T1 copper.

However, as the pH is higher there is also an increase in the hydroxide ion (OH^-) concentration, which were reported to bind to the T2/T3 cluster, and thus resulting in the disruption of the internal electron transfer between the T1 and T2/T3 centers (Kunamneni et al. 2007b); the value of ΔE^0 of this process can be expressed by Equation (2.3).

$$\Delta E^0 = E^0(\text{T2/T3 coppers}) - E^0(\text{T1 copper}) \quad (2.3)$$

The binding of anions reduces the E^0 value of T2/T3 coppers cluster (Kurniawati and Nicell 2008), and consequently the driving force for the internal electron transfer (Xu et al. 1998, Xu 2001). In this case, the driving force of this mechanism could be lower than that expressed by Equation (2.2), thus becoming the rate-limiting step (Xu et al. 1998).

Also, pH-dependence of oxygen reduction may also influence the pH oxidation profile; the value of ΔE^0 between oxygen and T2/T3 coppers is expressed by Equation (2.4):

$$\Delta E^0 = E^0(O_2/H_2O) - E^0(\text{T2/T3 coppers}) \quad (2.4)$$

E^0 (O_2/H_2O) tends to decrease as pH increases (Reinhamar 1984) and thus, ΔE^0 will be reduced at high values of pH. Even, that value could be negative, thus leading in thermodynamically unfavorable reaction (Kurniawati and Nicell 2008, Xu et al. 1999, Xu 1997, 2001). This effect would result in a decreased overall oxidation rate at high pH values.

A combination of all these factors might contribute to the creation of bell-shaped oxidation profiles, as those depicted in Figures 2.4 and 2.5.

2.3.1.2. Effect of pH and temperature on laccase stability

pH stability

Effect of pH on the laccase stability was investigated by incubating the enzymes in citrate-phosphate-borate buffer at the corresponding pH. Results for *Myceliophthora thermophila* and *Trametes versicolor* laccases are depicted in Figures 2.6.A and B, respectively.

Once the characteristics of ionizable side-chains and hence, the tertiary structure of the enzyme, depend on pH, laccases can be inactivated at extreme values (Kurniawati and Nicell 2008). In fact, it was observed that both laccases were particularly stable when incubated at neutral pH, whereas laccase activity markedly dropped under acidic conditions; for instance, *Myceliophthora thermophila* and *Trametes versicolor* laccases retained about 10-30% of its initial activity after 4 h of incubation at pH 4, but they were almost completely inactivate after only 1 h at pH 2.

In order to facilitate the comparison among stability results found at the different conditions evaluated as well as between both enzymes, experimental data was fitted to a first-order inactivation expression, as that shown in Equation (2.5) by minimizing the sum of the square residuals in order to determine the corresponding inactivation coefficients (k_D).

$$\text{Activity} = \text{Activity}_0 \cdot \exp(-k_D \cdot t) \quad (2.5)$$

The results found for both laccases are shown in Figure 2.7.

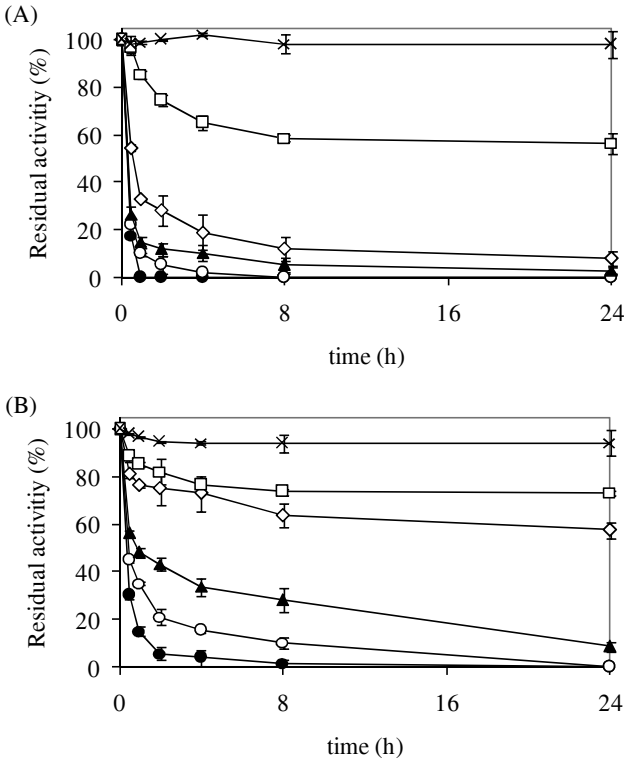


Figure 2.6. Effect of pH on the stability of *Myceliophthora thermophila* (A) and *Trametes versicolor* laccase (B). Values of pH evaluated: 2 (●), 3 (○), 4 (▲), 5 (◇), 6 (□) and 7 (×).

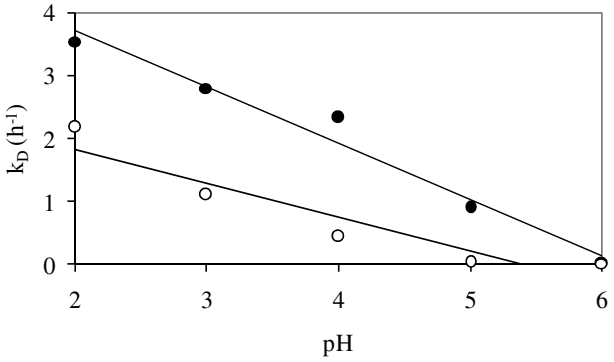


Figure 2.7. pH-dependent inactivation coefficients determined for *Myceliophthora thermophila* (●) and *Trametes versicolor* laccase (○). Correlation coefficients (R^2) between 0.840 and 0.999 were found.

Higher values of k_D were found for *Myceliophthora thermophila* laccase since, although the residual activities after 24-h incubation between both enzymes

were not extremely different, initial inactivation rates were greater for this enzyme as observed by the steeper slopes (Figure 2.6).

It is concluded from these results that a compromise solution should be achieved, especially if longer catalytic reactions are required, aiming to ensure proper catalytic activity as well as its maintenance over time. Nonetheless, pH impact could depend on the substrate to be transformed and also, it could affect other species present in the reaction medium, such as the reactivity and stability of other co-factors like laccase mediators. Thus, the influence of pH will be further evaluated during laccase-catalyzed transformation experiments of estrogens and anti-inflammatories.

Thermal stability

Thermal stability of the laccases was investigated by incubating both enzymes at temperature 20-70ºC in phosphate buffer (pH 7). Results for *Myceliophthora thermophila* and *Trametes versicolor* laccases are depicted in Figures 2.8.A and B, respectively.

Most enzymes are inactivated at elevated temperatures, which was reported to be caused by denaturation of tertiary structure through protein unfolding or disruption of the active site of the enzyme (D'Amico et al. 2003, Kurniawati and Nicell 2008). In this way, both laccases retained the total initial activity when incubated at 20 and 30ºC, whereas *Myceliophthora thermophila* and *Trametes versicolor* enzymes were completely inactivated after only 4 and 2 h at 70ºC, respectively.

Considering these results, it should be noted that if the enzyme is to be used in a reaction system at a pH close to alkaline values and at temperatures lower to 30ºC, it is expected that moderate portion of the catalytic activity would be lost due to pH and temperature. However, it could happen that the enzyme must be used under acidic conditions or high temperatures to favor its catalytic action because of the recalcitrant character of the target compound to be oxidized; in that case, a rapid decline of enzyme activity will be observed and the biocatalyst would be inefficient unless short reaction times or low levels of enzyme activity are required.

Also in this case, experimental data was fitted to first-order inactivation expression (Equation (2.5.)); results for both enzymes are shown in Figure 2.9.

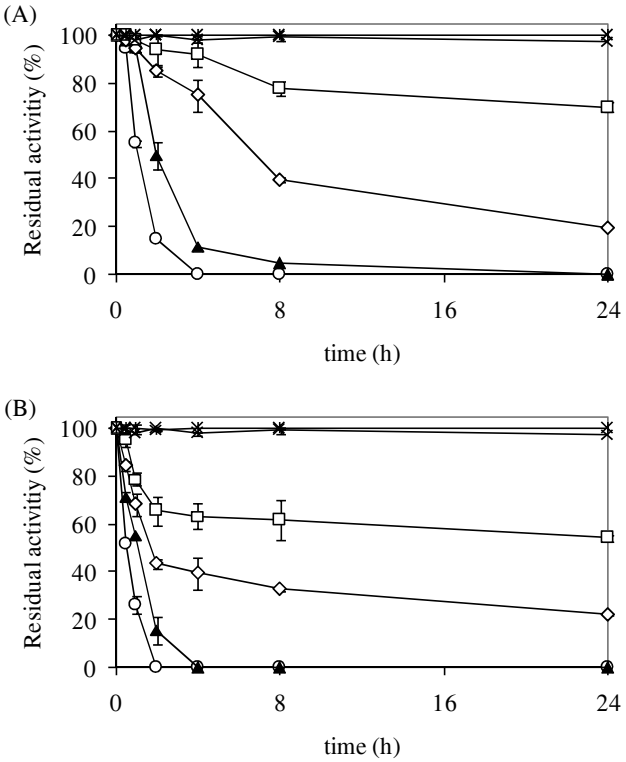


Figure 2.8. Effect of temperature on the stability *Myceliophthora thermophila* (A) and *Trametes versicolor* laccase (B). Values of temperature evaluated: 20 (∗), 30 (×), 40 (□), 50 (◇), 60 (▲) and 70 (○).

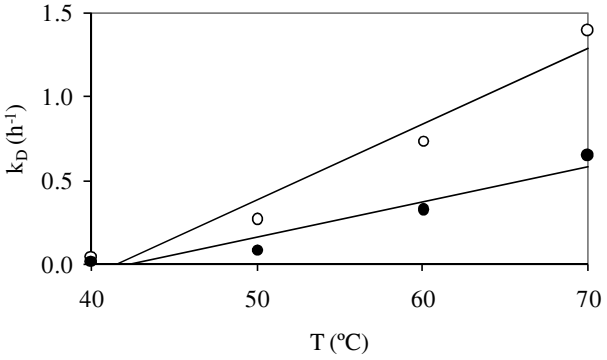


Figure 2.9. Temperature-dependent inactivation coefficients determined for *Myceliophthora thermophila* (●) and *Trametes versicolor* laccase (○). Correlation coefficients (R^2) between 0.890 and 0.996 were found.

In this case, higher k_D values, and thus greater temperature dependence, were found for *Trametes versicolor* laccase in comparison to that from *Myceliophthora thermophila.* This was expected once it is well documentated that thermophilic fungi, such as *Myceliophthora thermophila*, may comprise a rich source of thermostable industrial enzymes (Berka et al. 1997). Xu et al. (1996) reported that *Mycliophthora thermophila* laccase not only is more thermostable than laccases from the basidiomycetes *Trametes villosa* and *Rhizoctonia solani*, but also demonstrated a pronounced thermal activation such that preincubation at elevated temperatures gives higher activity. Thermal tolerance of the corresponding laccase could be an attractive feature for many biotechnological applications.

The effect of the temperature on the inactivation rate constant is described by the Arrhenius expression as shown in Equation (2.6):

$$k_D = A \cdot \exp\left(\frac{-E_a}{R \cdot T}\right) \tag{2.6}$$

where k_D is the inactivation coefficient, E_a is the activation energy, A is the pre-exponential factor, R is the universal gas constant and T is the temperatureThereby, E_a can be determined for both laccases by fitting the results of coefficients calculated for the different temperatures investigated to the previous expression; the results along with the correlation coefficients of the fitting are shown in Table 2.1. Values about 100-110 KJ/mol were found for both laccases.

Table 2.1. Activation energy determined for both laccases by fitting experimental data to Arrhenius equation.

Enzyme	E_a (KJ/mol)	R^2
Myceliophthora thermophila laccase	109	0.980
Trametes versicolor laccase	101	0.965

In view of these results, a temperature of 25-30ºC can be selected for further research once it still provided considerably initial reaction rate (about 40% of the maximum achieved at 60ºC) and the enzymes retained their total initial activity after incubation for 24 h. Furthermore, it would permit to reduce the energy requirements of the enzymatic treatment, which could be a limitation for the implementation of the developed technologies. In fact, Kurniawati and Nicell (2008) concluded that the optimal temperature for the application of *Trametes*

versicolor laccase was 30ºC after evaluating the effect of the temperature on the biotransformation of phenol: as the reaction progresses at elevated temperatures, the degree of thermal inactivation become more significant and the gains associated with high reaction rates at the early stage of the reaction are lost.

2.3.1.3. Estimation of laccase affinitiy

Although the electron transfer processes during the catalytic action of laccases were highlighted to discuss the effect of pH on the laccases activity profile, it is also noteworthy that the electron transfer is not the only important factor that governs the catalytic reaction: the binding of a substrate to the T1 copper besides the binding of oxygen to the T2/T3 cluster should occur before electron transfer can proceed. The binding of the substrate is commonly represented by the Michaelis-Menten constant, K_m, and play an important role in laccase-catalyzed oxidation: a substrate with a high K_m, which means a low affinity of the laccase for the substrate, might not be properly oxidized even though laccase presents a high oxidation potential (Kurniawati and Nicell 2008).

To explore this effect, K_m was calculated for both enzymes by using ABTS as model compound. The affinity of *Trametes versicolor* laccase towards ABTS appeared to be higher than that of *Myceliophthora thermophila*: K_m values of 34 and 56 µM were obtained, respectively. In view of these results, higher transformation rates would be expected when using *Trametes versicolor* laccase, although this might be dependent on the target substrate. Both laccases exhibited a v_{max} value in the range 2.7-3.1 mM/min.

2.3.2. Removal of estrogens by *Myceliophthora thermophila* laccase

The removal of estrogenic compounds (5 mg/L) in batch reactors was evaluated using a commercial enzyme from *Myceliophthora thermophila* (2,000 U/L). On the other hand, it was demonstrated that this laccase presents its higher activity at acid pH but low stability, whereas the stability is optimum at neutral and basic pH although the activity is reduced. Therefore, laccase-catalyzed removal of estrogens was assayed at two values of pH: 4 and 7, in order to study its effect on both enzyme stability and removal efficiency.

Removal yields and residual activity determined after 24-h treatment with laccase under both pH values are shown in Figure 2.10.

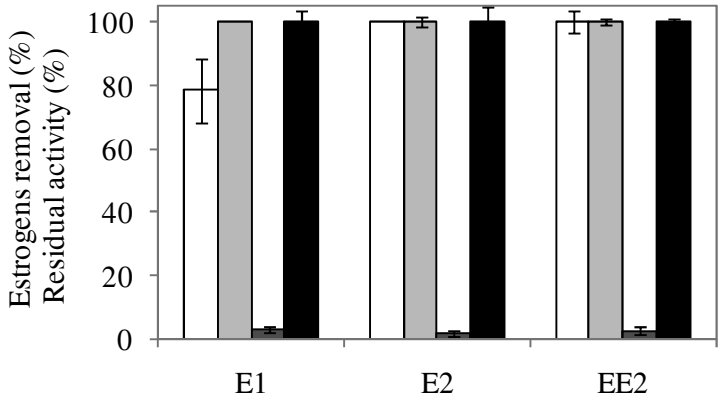


Figure 2.10. Removal percentages of estrogens attained after 24 h of treatment with *Myceliophthora thermophila* laccase at pH 4 (□) and 7 (■), and corresponding residual activity (■ and ■, respectively).

As observed, the three assayed estrogenic compounds were successfully removed by laccase at both pH 4 and 7 despite the observed enzyme inactivation at acid pH; thus, it can be stated that pH does not have a remarkable effect on estrogens elimination. However, a slight difference in the removal yield was found for E1 at pH 4 and 7: it seems that pH 7 favoured E1 elimination, increasing its transformation by 15-20%. Kim and Nicell (2006) also reported the maximum conversion of the substrate bisphenol A by laccase at pH 6, which corresponds to high stability but non-optimal pH in terms of maximum instantaneous activity. They explained this phenomenon due to a lower loss of activity by inactivation and/or an increased rate of interaction between the substrate and enzyme. In fact, the redox potential of phenolic substrates tends to decrease when pH increases (Xu 1997). Thus, the driving force which governs the electron transfer between the substrate and the T1 copper (ΔE^0, Equation (2.2)) would increase with the pH, and this translates into a faster electron transfer. This explanation implies that in this case that effect would be more important than the inhibition by OH^- anions binding and the pH-dependence of oxygen reduction mechanism (see Section 2.3.1.1).

The efficient removal of these compounds without the use of a mediator is probably caused by the fact that the selected estrogens contain phenolic groups and therefore, they are direct substrates of the enzyme (Bourbonnais and Paice 1990).

Regarding laccase stability, as expected, remarkable laccase activity decay at pH 4 was found; furthermore, laccase inactivation profile at pH 4 was fairly

similar to that found during stability assays (Figure 2.6.A). These findings indicate that estrogens present no significant impact on enzyme activity. This was corroborated by determining the inactivation coefficients (k_D) through the fitting of the experimental data to a first order inactivation model, as shown in Equation (2.5), by minimizing the sum of squared residuals. The results found for the data collected from the experiments of estrogens removal along with those of the stability experiments are shown in Table 2.2.

Table 2.2. Deactivation coefficients resulted from fitting experimental data obtained with *Myceliophthora thermophila* laccase at pH 4 to first order inactivation rates.

Target compound	k_D (h^{-1})	R^2
E1	2.378	0.983
E2	2.297	0.973
E2	2.358	0.969
Stability assay	2.322	0.981

Conversely, the enzyme retained its initial laccase activity after operation at pH 7, as occurred during the stability experiment in the absence of substrate. In view of these results, estrogens removal by laccase at pH 7 seems to be the optimum strategy in terms of removal efficiency and enzyme stability. In order to verify this fact, concentration profiles of the estrogenic compounds during their laccase-catalyzed removal at neutral pH were obtained; also, experimental data were fitted to second order kinetics. Obtained results are shown in Figure 2.11.

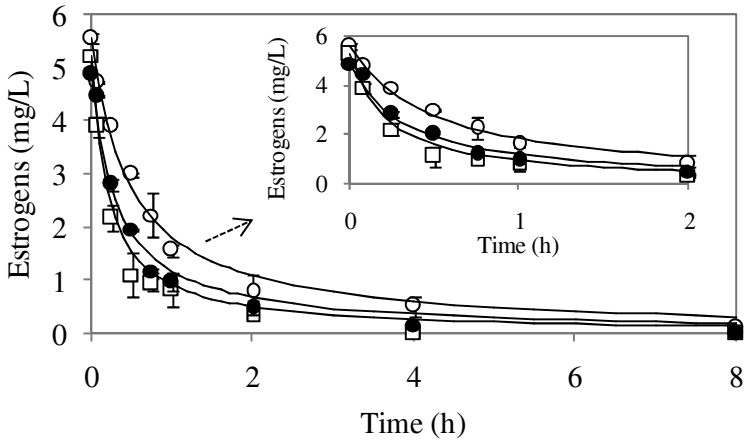


Figure 2.11. Concentrations of E1 (○), E2 (□) and EE2 (●) during the treatment with *Myceliophthora thermophila* laccase at pH 7. Solid lines represent the fitting of data to second order kinetics.

The results of kinetic coefficients obtained from the fitting are summarized in Table 2.3.

Table 2.3. Kinetic coefficients resulted from fitting experimental data of estrogens removal with *Myceliophthora thermophila* laccase at pH 7 to second order kinetics.

Target compound	k (L/(mg·h))	R^2
E1	0.372	0.992
E2	0.903	0.994
EE2	0.646	0.988

High kinetic coefficients and thus, fast removal reactions were observed. For instance, removal yields about 75-85% were achieved after only 1 h while nearly complete elimination was detected after 2 h of treatment with laccase.

Envisaging the operation of a continuous reactor, the stability of the enzyme is a key parameter. It was observed that the operation at pH 7 was beneficial not only for enzyme stability but also for removal percentages. Moreover, neutral values of pH would be favourable when working with real effluents, avoiding the need of acidification before laccase treatment. Similar conclusions were reported by Auriol et al. (2007), who selected pH 6-7 as the optimum for the enzymatic

removal of estrogens. Hence, further investigation dealing with laccase-catalyzed removal of estrogenic compounds was performed at pH 7.

2.3.3. Removal of anti-inflammatories

2.3.3.1. Removal of anti-inflammatories by *Myceliophthora thermophila* laccase

Effect of pH and HBT

In order to evaluate the capability of *Myceliophthora thermophila* laccase (2,000 U/L) to remove the selected anti-inflammatory compounds, NPX and DCF (5 mg/L), batch experiments were performed aiming to select the optimum pH, as previously conducted when dealing with estrogens removal. Moreover, the use of HBT (1 mM) was included during these preliminary assays once this type of compounds is known to be more recalcitrant. Removal yields attained after 24-h treatment under all the conditions evaluated are shown in Figure 2.12.

NPX was not eliminated in the absence of mediator and the use of HBT and pH 4 was required to attain removal yield above 65%, suggesting that NPX is not a direct substrate of the enzyme. On the other hand, DCF was removed by approximately 80% without the synthetic mediator, although pH 4 was required with the consequent laccase inactivation. The use of HBT clearly enhanced DCF transformation: complete removal was detected at pH 4 and elimination percentage higher than 50% was found at pH 7.

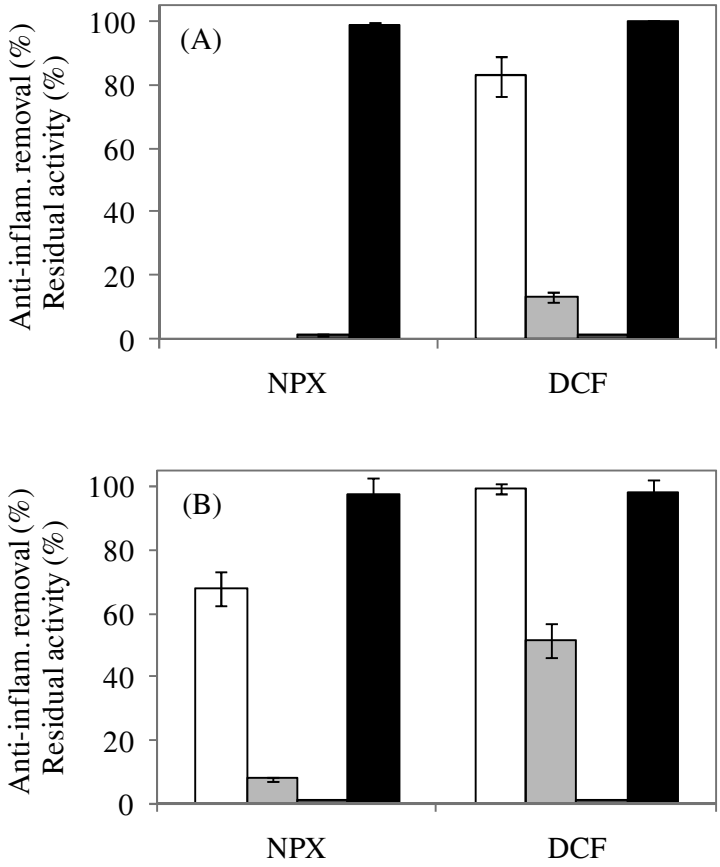


Figure 2.12. Removal percentages of anti-inflammatories attained after 24 h of treatment with *Myceliophthora thermophila* laccase in absence (A) and presence (B) of HBT, at pH 4 (□) and 7 (■), and corresponding residual activity (■ and ■, respectively).

In view of these results, pH 4 was selected for the following experiments and the use of mediators was further investigated.

Effect of natural mediators

In this stage, the capability of a number of compounds, either produced by fungi or present during the degradation of lignocellulose substrates, to mediate the transformation of anti-inflammatories with laccase, was evaluated. The mediated action of four natural mediators: SA, V, FA and p-CA, was compared with that of synthetic mediators such as HBT and VA, all at a concentration of 1 mM. The efficiencies of the mediators used are presented in Table 2.4.

Table 2.4. Removal percentages (%) of anti-inflammatories attained after 24 h of treatment with *Myceliophthora thermophila* laccase at pH 4 in absence and presence of natural and synthetic mediators.

Mediator	NPX	DCF
HBT	68	>99[a]
VA	36	>99
SA	<10	>99
V	-[b]	95
p-CA	-	92
FA	-	85
-	-	83

[a] Concentration below detection limits.
[b] No removal was observed.

Concentrations below detection limits, which imply removal efficiencies higher than 99%, were found for DCF when HBT, VA and SA were used. In the presence of V and p-CA high transformation efficiencies were also achieved, and a removal yield fairly similar to that in the absence of mediator was detected with FA. Contraringly, NPX was only removed when HBT and VA were used (68 and 36%), whereas no transformation was observed with natural mediators.

The greater efficiency of HBT and VA as mediators is probably caused by their high redox potential: 1.1 and 0.92 V, respectively (Fabbrini et al. 2002, Xu et al. 2000). The redox potential of SA was demonstrated to be lower, but this means that it is more easily oxidized by laccase and moreover, the lower reactivity of SA radicals towards the substrate would be compensated by their relatively higher stability (Arzola et al. 2009, Camarero et al. 2005). Among the natural mediators tested, SA provided the best results, probably because of the two methoxy groups of the molecule (see structures in Figure 2.2), which would facilitate its oxidation by laccase due to the corresponding electron donor effect; however, V presents a single methoxy substituent. On the other hand, FA and p-

CA provided the lowest enhancement in comparison with respect to the use of laccase alone. This could be explained by the fact that, although both compounds present hydroxyl groups (like V and SA) as well as unsaturated C-C double bond which can provide additional attack site, they also have a carboxyl group; hence, the redox potential of these molecules may increase and thus being oxidized by laccase to a lesser extent. Camarero et al. (2007) reported a lower efficiency of p-CA as a laccase mediator as a result of both higher pKa of its phenolate group and the lower stability of its radicals compared with those of SA that have lower pKa and form stable radicals.

Complete NPX and DCF concentration-time profiles were determined when using HBT, VA and SA to evaluate in more detail the effect of these mediators; the results are depicted in Figure 2.13.

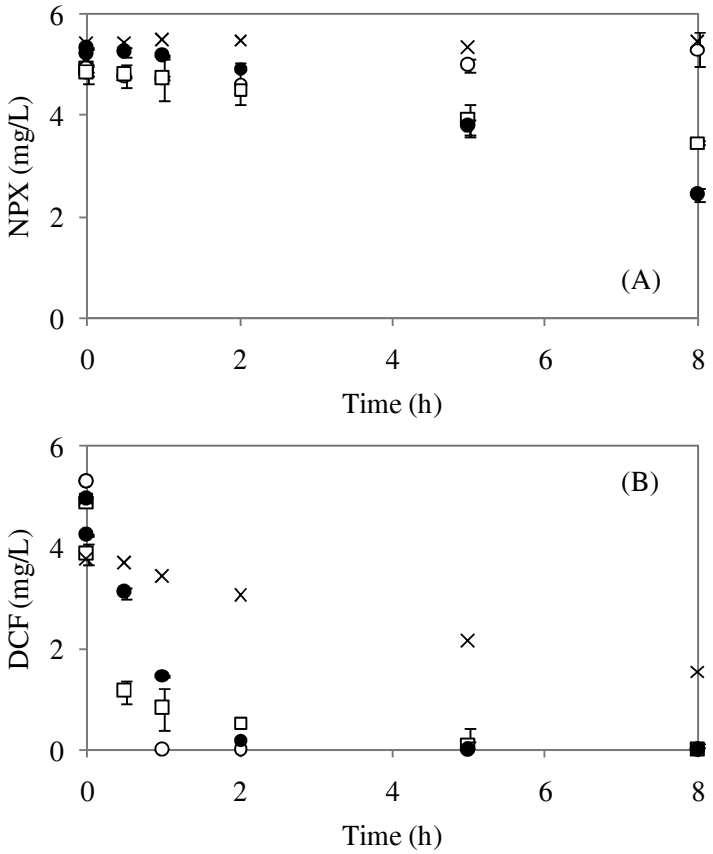


Figure 2.13. Concentrations of NPX (A) and DCF (B) during the treatment with *Myceliophthora thermophila* laccase at pH 4 in the absence of mediator (×) and in the presence of HBT (●), VA (□) and SA (○).

NPX was partially removed in the presence of HBT or VA: 50 and 30% after 8-h reaction, respectively, which means that only a slight improvement was attained after 24 h (Table 2.4). With regard to DCF, removal yields above 80% were already obtained in the presence of the mediators after only 2 h of incubation; even more, no DCF was detected after 1-h catalyzed reaction with SA.

Effect of mediator concentration

High concentrations of mediators could lead to an increased efficiency of the laccase-mediator system; however, excessive mediator concentration could result in undesirable reaction byproducts (Majeau et al. 2010). Besides, this could imply a limitation of the system in certain applications, for instance in wastewater treatments, due to the high cost of the mediators and the negative impacts on the effluent toxicity, especially when using synthetic mediators (Kurniawati and Nicell 2007b). The effect of the concentration of the mediators on the removal of the anti-inflammatories assayed was evaluated (Figure 2.14).

A concentration of 1 mM HBT was necessary to remove NPX by 50% after 8 h of treatment; lower concentrations of the mediator led to a NPX removal of only 10-20% for the same period of time. However, the increase of HBT concentration to 2 mM did not enhance the removal efficiency. On the other hand, no transformation was observed when SA was used as mediator, independently of the concentration assayed. VA mediated the elimination of NPX with a transformation percentage of 40% for the highest concentration of the mediator (2 mM). Regarding DCF transformation, it was proved that HBT concentration could be reduced to 0.5 mM without limiting its removal efficiency: this anti-inflammatory was completely eliminated after only 2 h with 0.5 or 1 mM of HBT, and the reaction was even faster with 2 mM HBT. Great removal yields were also achieved with SA; however, 2 mM VA was required to attain complete transformation of DCF in less than 4 h of batch operation.

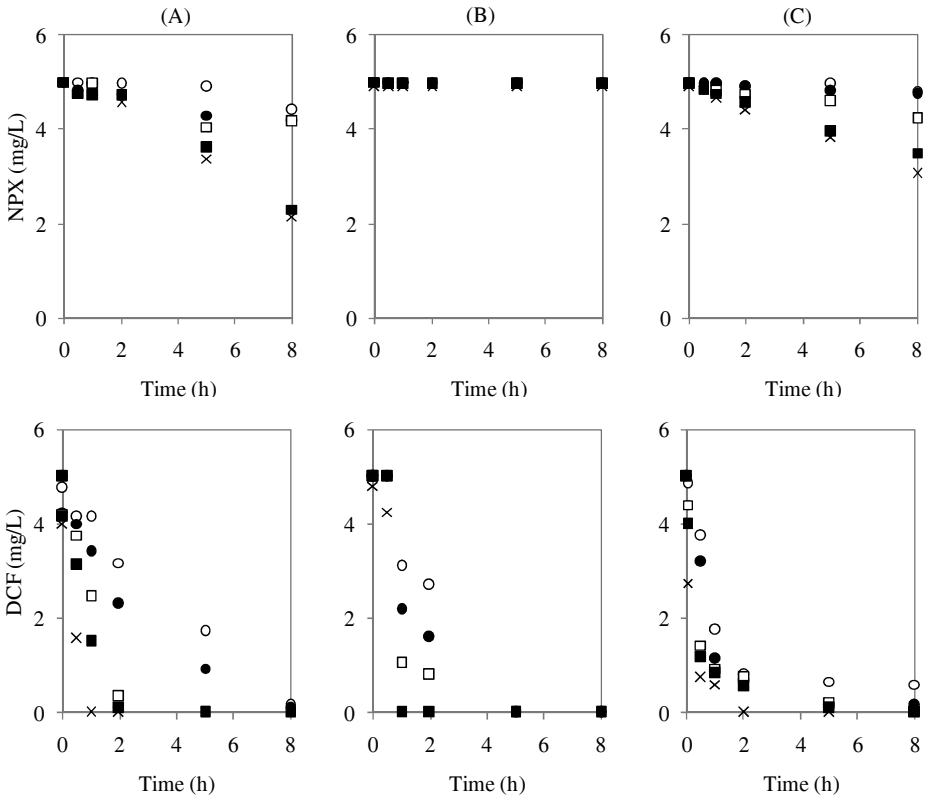


Figure 2.14. Concentrations of NPX and DCF during the treatment with *Myceliophthora thermophila* laccase at pH 4 in the presence of HBT (column A), SA (column B) and VA (column C). Symbols represent variable concentrations of the mediators: 0.1 (○), 0.25 (●), 0.5 (□), 1 (■) and 2 mM (×).

2.3.3.2. Removal of anti-inflammatories by *Trametes versicolor* laccase

Influence of pH and mediators

In the previous section, the enzymatic removal of anti-inflammatories was investigated by using *Myceliophthora thermophila* laccase with variable pH and mediators. Nonetheless, removal rates were not extensive, which might be due to the low redox potential of the enzyme and the important recalcitrant character of NPX and DCF. In fact, the use of mediators and acid pH were mandatory conditions to achieve significant efficiencies.

Therefore, a laccase with higher redox potential: *Trametes versicolor* laccase, was selected for further research. Furthermore, the influence of pH and mediators (HBT was used as synthetic mediator and SA as natural one) were

further investigated and discussed in this stage of the work. Batch experiments were conducted for the biotransformation of 5 mg/L of DCF and NPX (each and independently), with an initial enzyme activity of 2,000 U/L and in the absence or presence of 1 mM of HBT or SA; all conditions were evaluated at pH 4 and 7.

The influence of pH and mediators not only on the removal efficiency (Figures 2.15.A and B) but also on the enzyme stability (Figures 2.15.C and D) was explored. Besides, both transformation reaction and enzyme inactivation kinetics were studied and the corresponding parameters (k and k_D, respectively) were determined by minimizing the sum of squared residuals, as previously conducted with estrogens, for all the conditions assayed (Tables 2.5 and 2.6, respectively).

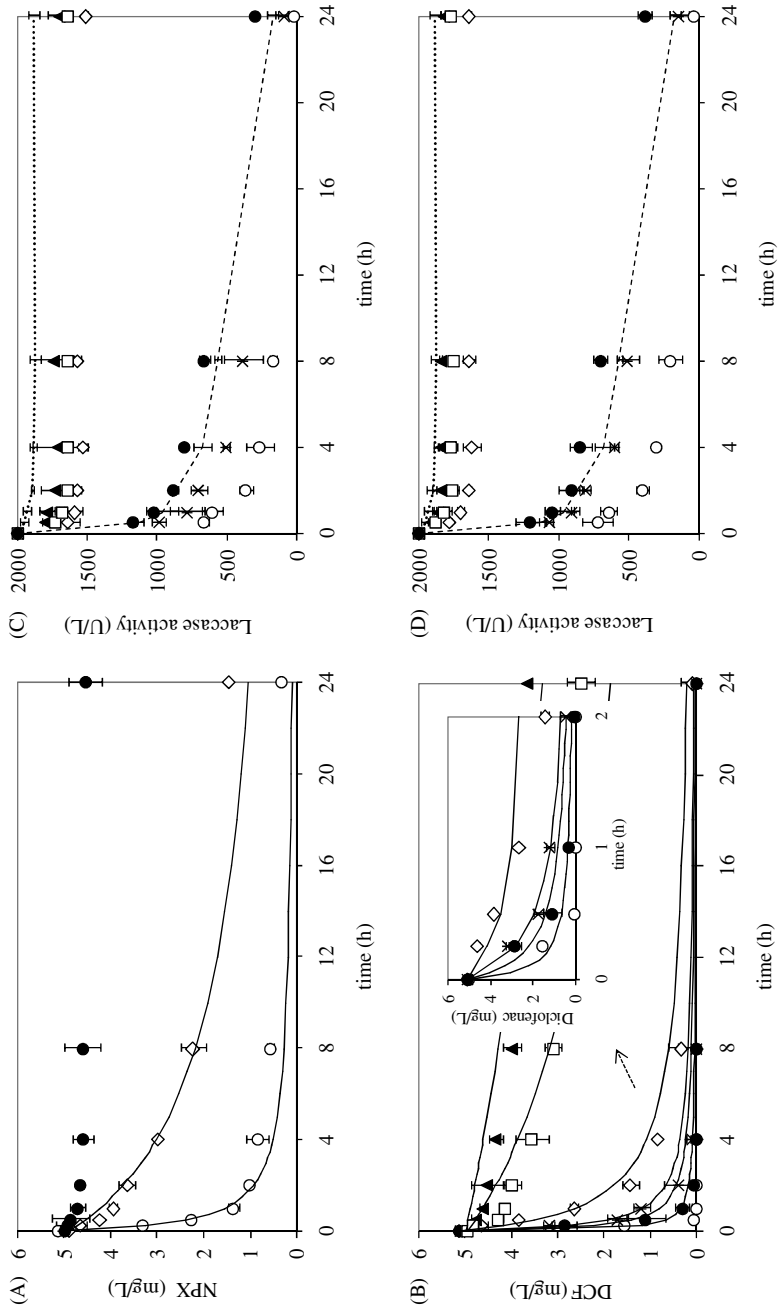


Figure 2.15. Concentrations of the target compounds and enzyme activity during the enzymatic treatment of NPX (A and C) and DCF (B and D) with *Trametes versicolor* laccase, at pH 4: without mediator (✳), with HBT (○), with SA (●), and 7: without mediator (▲), with HBT (◇), with SA (□). Solid lines represent the fitting of data to second order kinetics; enzyme stability data (without substrate) at pH 4 (----) and 7 (····) are also shown.

Effects on the removal yields

Among all the conditions tested, NPX was significantly removed at both pH 4 and 7 in the presence of HBT, while a slight removal was observed at pH 4 in the presence of SA (Figure 2.15.A). A removal percentage close to 95% was attained after 24 h by laccase-HBT system and pH 4, although high elimination yields of 80 and 89% were achieved after only 2 and 8 h, respectively. Lower removal rates were observed at pH 7: NPX was eliminated by 70% after 24-h treatment in the presence of HBT. It can be observed that NPX concentration was reduced more rapidly during the first hours of operation and decreased slower after 8 h. This is the typical tendency expected for second order kinetics, and thus, this model was applied to test the experimental data. This behavior could be promoted by the enzyme deactivation, which is more pronounced at the initial hours of the experiments. Also, it may be due to the instability of the nytroxyl radicals (N-O$^{\bullet}$) generated by laccase-catalyzed oxidation of HBT, which might decay into species that are no longer functional as mediators (Cantarella et al. 2003). The differences in treatment efficiency as function of pH can be easily compared by analyzing the kinetic coefficients (k) obained: laccase-HBT system depicted a constant of 0.032 L/(mg·h) at pH 7, which increased to 0.445 L/(mg·h)at pH 4 (Table 2.5).

Regarding DCF, promising results were obtained in both laccase and laccase-mediator systems (Figure 2.15.B). At neutral pH, partial removal of DCF (27%) was observed in the absence of mediator after 24 h, which corresponded to a low kinetic constant of 0.004 L/(mg·h) (Table 2.5). Nevertheless, the use of SA increased DCF removal up to 50% (k=0.014 L/(mg·h)) and was nearly complete when using HBT as evidenced by the higher kinetic constant: k=0.182 L/(mg·h); moreover, removal yield of 84% was already attained after only 4 h by using the synthetic mediator. Furthermore, the efficacy of the treatment was significantly improved at acid pH as indicated by the more pronounced slopes. Also at pH 4, the best results were found with the laccase-HBT system, followed by laccase-SA and the laccase system without mediator: DCF was below detection limits (removal percentage up to 99%) after only 30 min, 2 and 4 h, respectively (k=2.618, 1.028 and 0.643 L/(mg·h)).

Table 2.5. Removal percentages of NPX and DCF attained with *Trametes versicolor* laccase under all the conditions evaluated and kinetic coefficients resulted from the fitting of experimental data to second order kinetics.

Target compound	pH	Mediator	Time (h)	Removal (%)	k (L/(mg·h))	R^2
NPX	4	-	-[a]	-	-	-
		HBT	24	94	0.445	0.995
		SA	24	<10	-[b]	-
	7	-	-	-	-	-
		HBT	24	70	0.032	0.993
		SA	-	-	-	-
DCF	4	-	4	>99[c]	0.643	0.993
		HBT	0.5	>99	2.618	0.987
		SA	2	>99	1.028	0.980
	7	-	24	27	0.004	0.940
		HBT	24	98	0.182	0.994
		SA	24	50	0.014	0.956

[a] No removal was observed.

[b] Data were not fitted to the second-order kinetic equation, only slight removal was observed.

[c] Concentration below detection limits.

The rationale behind the higher efficacy observed for DCF in comparison to NPX may be explained on the different chemical structures. In general, electron donating groups (EDG) are prone to oxidative attack whereas electron withdrawing groups (EWG) generally diminish reactivity. Both NPX and DCF

contain a carboxylic acid group, which is a strong EWG; however, DCF is based on an aromatic amine, an EDG-type functional group, which may decrease the potential redox of the compound. Indeed, laccases are known to be able to attack amine groups of molecules (Hoff et al. 1985). Similar effect was observed by Almansa et al. (2004) who studied the influence of different dyes structure on their removal by laccase. These authors identified that the presence of carboxyl groups in tartrazine structure was responsible of the more recalcitrant behavior of the dyes, while hydroxy-substituted dyes were the ones most susceptible to the enzyme action.

As indicated above, poorer reaction rates were found at neutral pH, even though the redox potential of aniline compounds such as DCF was reported to decrease at high pH values (Daneshgar et al. 2009). On the other hand, higher removal yields of both anti-inflammatories were attained at pH 4 in spite of the enzyme inactivation, which corresponds to a pH close to the optimum value in terms of maximum activity. The recalcitrant character of NPX and DCF forced to operate at those conditions which provided the maximum catalytic activity of the biocatalyst to achieve extensive removal efficiency despite the poorer enzyme stability. Furthermore, Xu et al. (2000) reported that the redox potential of HBT does not significantly vary with pH in the range between 4 and 9, contrarily to other phenolic substrates of laccases. Thus, the higher efficiency of the laccase-HBT system on the removal of anti-inflammatories at pH 4 in comparison to pH 7 appeared to be caused by the higher relative activity of the enzyme at acid pH and consequently a higher initial oxidation rate. In the case of SA, it was reported that the stability of the phenoxy radicals ($C\text{-}O^{\bullet}$) formed by its laccase-catalyzed oxidation is higher in slightly acidic aqueous media (Camarero et al. 2005).

In addition, the lower removal of NPX and DCF at pH 7 in comparison to that at pH 4 could be also explained by the fact that these compounds are primarily deprotonated at neutral pH (pKa 4.0-4.5 (Suárez et al. 2008)). This could result in an electrostatic repulsion that hinders the surface-complex formation, necessary for electron transfer (Huber et al. 2003). Furthermore, the driving force of the reaction would be increased for higher H^+ concentrations as protons are also reactant in the redox reaction activated by laccase (Forrez et al. 2010).

With regard to the effectiveness of the mediators, the feasibility of the laccase-mediator system greatly depends on the balanced combination between stability and reactivity of the mediator radicals. It was observed that HBT allowed for the highest removal yields, probably due to the high redox potential of this

mediator, 1.1 V (Xu et al. 2000), which may favor the higher reactivity of the corresponding N-O$^{\bullet}$ radicals despite their short half-life and rapid decay to benzotriazole (Cantarella et al. 2003, Murugesan et al. 2010). However, both HBT and SA, which belong to N-OH and C-OH groups, respectively, were proposed to act via hydrogen atom transfer mechanism (Murugesan et al. 2010, Torres-Duarte et al. 2011). Therefore, the difference in redox potential between enzyme and mediator could limit the reaction but the enthalpic balance between the dissociated bond in the target substrate and the forming one in the mediator would be the driving force of the substrate transformation (Cañas and Camarero 2010). Although Kawai et al. (1989) detected that C-O$^{\bullet}$ radicals generated by the laccase-catalyzed oxidation of SA are converted into compounds which are not involved in the laccase-mediator system, Camarero et al. (2005) indicated that SA forms more stable radicals due to the presence of two methoxy groups on the aromatic ring that prevent the formation of biphenyl-type structures by radical condensation. Moreover, high oxidation capacity of SA is expected due to the electron donor effect of those methoxy substituents.

Overall, the results evidenced the important enhancement achieved by using laccase from *Trametes versicolor* in comparison to the previous results for that from *Myceliophthora thermophila*. For instance, *Trametes versicolor* enzyme allowed complete elimination of DCF after only 4 h and 30 min by laccase and laccase-HBT systems, respectively, at pH 4; however, operation times of 24 and 2 h were required to attain significant yields (83 and nearly 100%) when using *Myceliophthora thermophila* laccase. The improvement was more noticeable for NPX: no transformation of this compound was detected after 24-h treatment with *Myceliophthora thermophila* laccase at pH 7 in the presence of HBT and a removal yield of 68% was attained at pH 4, while *Trametes versicolor* laccase provided elimination efficiencies of 70 and 94% under the same conditions. These results are explained on the different redox potentials of the laccases T1 site: 0.78 V for *Trametes versicolor* laccase (Reinhammar et al. 1972) and 0.47 V for *Myceliophthora thermophila* laccase (Xu et al. 1999). The use of a laccase with high redox potential would be translated into enhanced reaction rates due to the higher rate of electron transfer from the substrate to the T1 copper in the active site of laccase (ΔE^0, Equation (2.2)), which is generally regarded as the rate-limiting step in the laccase-catalyzed reactions (Kurniawati and Nicell 2007b). Moreover, a higher affinity of *Trametes versicolor* laccase for the substrate was found (ABTS was used as model), as it was previously indicated.

Additionally, removal percentages achieved were higher than those of conventional biological systems in wastewater treatment plants: e.g. the degradability of DCF was reported to be low (about 30%) in the aerobic biological treatment and highly variable (between 0 and 75%) in the anaerobic treatment (Carballa et al. 2004). Advanced oxidation processes (AOPs) such as ozonation were successfully applied but different results were reported with these technologies and high doses of the reagents were required in some cases to attain high removal yields. Ikehata et al. (2006) described complete degradation of pharmaceuticals by several AOPs in high quality wastewater. However, Gagnon et al. (2008) obtained low removal efficiencies between 21 and 29% for DCF and NPX by UV treatment, and the same authors reported total removal of NPX but a high concentration of ozone of 15 mg/L was necessary.

The use of white rot fungi was also proposed by various researchers due to their demonstrated capacity to degrade and detoxify recalcitrant environmental pollutants. Marco-Urrea et al. (2010a) attained almost complete removal of NPX at 10 mg/L after 6 h by fungal cultures of *Trametes versicolor*, which was attributed to the action of laccase and the intracellular enzyme cytochrome P-450. These authors also achieved complete removal of both DCF and intermediates by *Trametes versicolor* pellets within 24 h (Marco-Urrea et al. 2010b). Rodarte-Morales et al. (2012) reported the operation of stirred tank and fixed-bed reactors for the removal of anti-inflammatories by free and immobilized *Phanerochaete chrysosporium*. However, the complexity of these *in vivo* systems and the difficulties of their application under non-sterile conditions make the use of the enzymes a more attractive alternative; however, only few researchers utilized *in vitro* systems for the removal of anti-inflammatory compounds prior to the current research. Eibes et al. (2011) reported removal yields of 55-80% within 7 h and 100% after only 20 min of 2.5 mg/L of NPX and DCF, respectively, by using versatile peroxidase, and Zhang and Geißen (2010) obtained complete elimination of DCF at 5 mg/L in 2 h with lignin peroxidase but with high amount of H_2O_2 above 3 mg/L. Nonetheless, the use of atmospheric oxygen as final electron acceptor represents a considerable advantage for the application of laccases compared with peroxidases and would facilitate the implementation of laccase-catalyzed processes.

Effects on the enzyme stability

Laccase activity was monitored during the enzymatic treatment under the different conditions evaluated aiming to study their influence of the biocatalyst stability. The activity profiles obtained for NPX and DCF treatment were fairly similar, as observed in Figures 2.15.C and D, respectively, where the profiles found for the stability experiments are also plotted as reference.

The biocatalyst was slightly inactivated over the first hour of operation at pH 7, as occurred when studying the stability at this pH, and thereafter the remaining activity was maintained constant. For instance, the residual laccase activity was 85 and 91% after 24 h of laccase-catalyzed removal of NPX and DCF, respectively, while a value of 94% was found during the corresponding stability experiment. These results suggested an additional inactivation due to the presence of the anti-inflammatories in the medium, more noticeable in the case of NPX and it was even more pronounced in the presence of mediators.

Greater impacts were detected during the experiments at pH 4 as observed in the activity profiles and once again, the presence of NPX and DCF led to a higher deactivation in comparison with experiments lacking substrate, although similar residual activities in the range 5-9% were found after 24-h operation. This fact was verified by the values of deactivation constants: k_D values were 0.757 and 0.552 h^{-1} in removal experiments of NPX and DCF respectively, whereas it was 0.449 h^{-1} for the stability assays (Table 2.6).

Table 2.6. Residual activity after 24 h of enzymatic treatment of NPX and DCF with *Trametes versicolor* laccase as well as after the enzyme stability assays, and inactivation coefficients resulted from the fitting of experimental data to first order inactivation kinetics.

Target compound	pH	Mediator	Residual activity (%)	k_D (h^{-1})	R^2
NPX	4	-	5	0.757	0.939
		HBT	1	1.510	0.970
		SA	15	0.356	0.892
	7	-	85	-[a]	-
		HBT	76	-	-
		SA	82	-	-
DCF	4	-	7	0.552	0.923
		HBT	2	1.347	0.967
		SA	19	0.317	0.887
	7	-	91	-	-
		HBT	82	-	-
		SA	89	-	-
Stability assays	4	-	9	0.449	0.916
	7	-	94	-	-

[a] Data were not fitted to the equation, only initial inactivation was observed.

Despite the excellent removal results achieved with HBT at pH 4, this mediator was proved to cause a detrimental effect on the enzyme since nearly complete inactivation was measured after 24 h of laccase-mediated treatment (k_D=

1.510 and 1.347 h^{-1} for NPX and DCF, respectively). Laccase inactivation induced by HBT was previously reported in other works focused on the removal of other type of substrates such as dyes (Chhabra et al. 2009) and triclosan (Murugesan et al. 2010), and it could be attributed to the attack of free radicals from the mediator which could oxidize the aromatic amino acid residues on the protein surface (Aracri et al. 2009, Li et al. 1999). It is expected that high concentrations of mediator entail faster reactions although at the expense of greater enzyme inactivation, which would imply a reduction of the global processing capacity (Kurniawati an Nicell 2007b).

Interestingly, SA seems to exhibit a stabilizing effect, which resulted in k_D values lower than that for the stability assay: constants between 0.356 and 0.317 h^{-1} were found. Indeed, residual activities of 15 and 19% were detected for NPX and DCF, whereas the enzyme retained only 5-7% of its initial activity in the absence of the mediator. The same stabilizing effect was observed by Fillat et al. (2010) when studying the stability of *Pycnoporous cinabarinus* laccase with and without the mediator in the absence of target substrate. Mai et al. (2000) found some phenolic compounds acting as enzyme agents to stabilize laccase by their binding to the active sites or suitable points of the enzyme protein chain. Additionally, SA is considered a natural mediator and consequently, it would facilitate the application of the laccase-mediator system in environmentally friendly biotechnological process. This makes this mediator an interesting choice for particular applications, such as the oxidation of non-phenolic compounds or when slow reaction rates are expected and/or long operation is required. Indeed, although HBT has been one of the most extensively used mediators (Johannes and Majcherczyk 2000), SA has been also successfully applied on the laccase-mediated removal of various compounds such as dyes (Camarero et al. 2005), triclosan (Murugesan et al. 2010) and pesticides (Torres-Duarte et al. 2011).

Fed-batch removal of DCF

Laccase-catalyzed removal of DCF was also evaluated in fed-batch operation by the addition of 5 mg/L of the target compound to the reaction vessel every hour. The profiles of DCF concentration and laccase activity are shown in Figure 2.16, as well as the kinetic coefficients obtained by the fitting of experimental data to a second order rate equation, considering the concentration measured after the DCF pulse as the initial concentration of the corresponding batch.

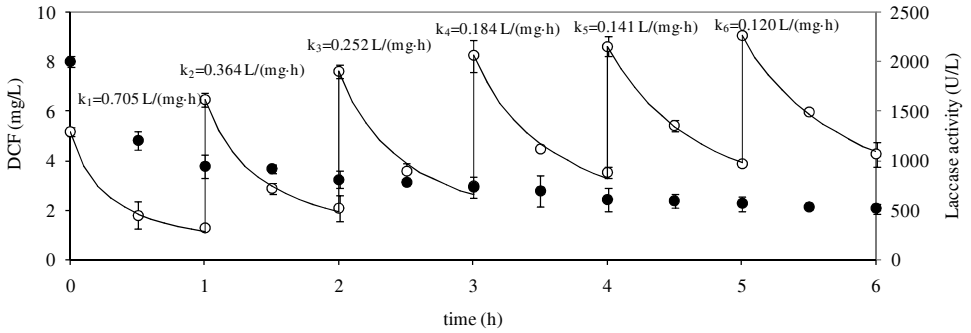


Figure 2.16. Concentration of DCF (○) and laccase activity (●) during the enzymatic treatment in fed-batch operation with *Trametes versicolor* laccase at pH 4. Solid lines represent fitting of removal profiles to second order kinetics; kinetic coefficient for each batch are also provided (correlation coefficients (R^2) up to 0.995 were found).

The results appeared to evidence the influence of the enzyme activity on the kinetics since the kinetic constant was progressively reduced from the first to the sixth batch in parallel to the decrease of laccase activity. For instance, kinetic coefficients were 0.705, 0.364 and 0.252 L/(mg·h) for initial activities of 2,000, 945 and 810 U/L, respectively; however, from the fourth to the sixth batch, the constants were fairly similar because the enzyme activity was maintained at similar levels between 720 and 550 U/L. Nevertheless, removal rates of DCF were progressively increased from 3.90 to 4.81 mg/(L·h) (data not shown) due to the accumulation of the target compound in the medium with increasing concentrations (5-9 mg/L) during the different batches.

Anyhow, these results demonstrated the potential capability of the enzymatic system to remove the selected anti-inflammatory compounds with low enzyme requirement. DCF was eliminated by 76% with 2,000 U/L and at initial concentration of 5.16 mg/L (first batch), while reductions of 8 and 15% on the removal percentage in the second and third batches were observed for a laccase activity about 2 and 2.5-times lower even though the initial concentration of DCF was considerably higher (6.45 and 7.60 mg/L, respectively).

2.3.4. Toxicity evaluation

Evaluation of toxicity of reaction medium after enzymatic treatment was performed with those samples taken from experiments run under the most suitable conditions evaluated: samples from experiments conducted at pH 7 for E1, E2 and EE2 removal with *Myceliophthora thermophila* laccase, and samples from

experiments at pH 4 in the case of DCF transformation with *Trametes versicolor* laccase without mediator.

2.3.4.1. Reduction of estrogenic activity after E1, E2 and EE2 removal by laccase

Although the catalyzed transformation process was demonstrated to be effective in eliminating the target compounds, the main focus concerning the removal of estrogenic substrates should be the residual estrogenicity as these compounds are the major contributors to the estrogenicity associated to sewage treatment plants effluents, and their biotransformation products could still present significant toxicity (Aerni et al. 2004, Leusch et al. 2005).

Therefore, further analyses were conducted aiming to evaluate the potential detoxification attained by using *Myceliophthora thermophila* laccase. For this purpose, samples taken after 1, 2, 8 and 24 h of laccase-catalyzed removal of E1, E2 and EE2 at pH 7 were analyzed by LYES assay. The reduction of estrogenic activity (%) was determined considering the estrogenicity measured for each monitored time and compound and that of time zero, it means, before laccase addition (identical to the level detected in the control experiment). The obtained results are shown in Figure 2.17.

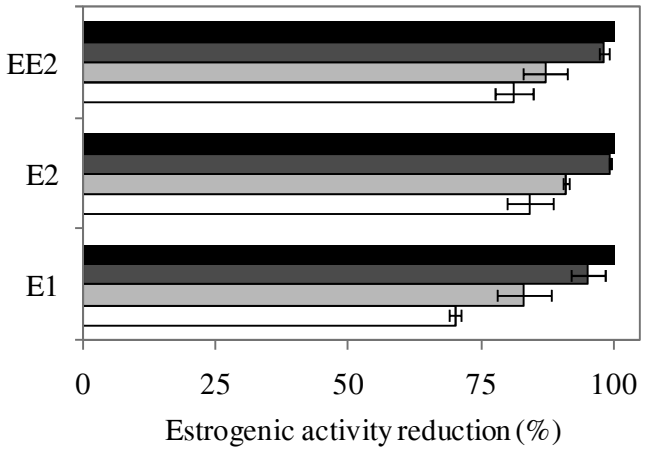


Figure 2.17. Estrogenic activity reduction after 1 (□), 2 (■), 8 (■) and 24 h (■) of removal of E1, E2 and EE2 at pH 7 with *Myceliophthora thermophila* laccase.

The data revealed the successful detoxification of the reaction mixture by using laccase: no estrogenic activity was detected in 24-h samples, estrogenicity reduction levels higher than 95% were found after 8 h and values between 83 and

91% were observed after only 2 h. However, detoxification efficiencies were slightly poorer after 1 h: estrogenic activity removal of up to 81-84% was determined for E2 and EE2, whereas 30% of residual estrogenicity was still found when removing E1. Anyhow, these results corroborated that the disappearance of the target compounds takes places in parallel with the elimination of their related estrogenic activity.

These findings are consistent with previous studies (Auriol et al. 2008, Tamagawa et al. 2006), which reported the correlation between estrogenicity and the removal of the parent compounds when using laccase or manganese peroxidase in batch experiments. Tsutsumi et al. (2000) also demonstrated the ability of these enzymes to reduce the estrogenic activity of different compounds, such as bisphenol A and nonylphenol. In that case, HBT was used as mediator to attain total removal after 6 h of batch operation. This strongly suggests that ligninolytic enzymes are promising tools to perform the effective detoxification of wastewaters contaminated by estrogenic substances.

2.3.4.2. Aerobic biodegradability and Microtox® evaluation after DCF removal by laccase

Aerobic toxicity of the medium resulted from the enzymatic treatment of DCF with *Trametes versicolor* laccase at pH 4 was evaluated by the monitorization of cumulative oxygen consumption. Experiments were performed with samples taken after 8 h of laccase-catalyzed transformation of DCF as well as with samples from the corresponding controls lacking enzyme, aiming to investigate the impact of the enzymatic treatment on the biodegradability. Results obtained for 8-h samples and untreated solution are shown in Figure 2.18.

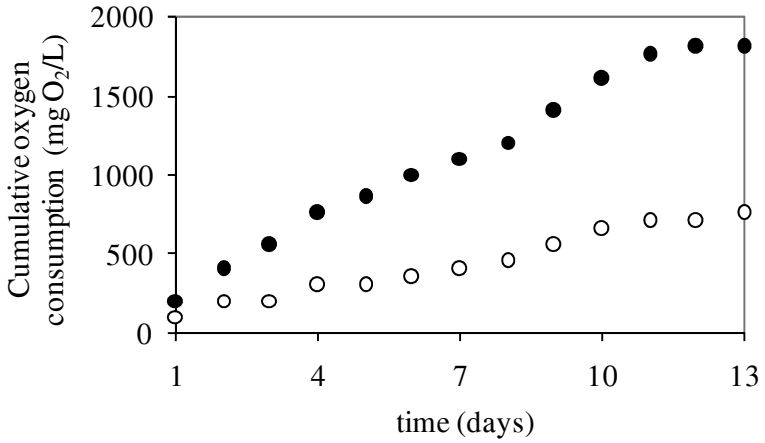


Figure 2.18. Cumulative oxygen consumption during aerobic biodegradation experiments with samples taken after 8 h of enzymatic transformation of DCF by *Trametes versicolor* laccase at pH 4 (●) and corresponding control lacking enzyme (○).

Low values of oxygen consumption were found for untreated DCF confirmed the persistence of this compound, as previously reported (Quintana et al. 2005). In contrast, the results clearly evidenced that treatment by laccase increased the biodegradability of the medium being the values of oxygen consumption significantly higher for the sample after enzymatic removal, especially for experiments intervals longer than 4 days. For instance, values of 300 and 650 mg O_2/L were found for the untreated samples after 4 and 10 days, whereas values 2.5-fold higher were found for the laccase-catalyzed treated solution. This improved aerobic biodegradability of the enzymatic treatment effluent suggests the generation of less toxic products and thus, it would favor the applicability of laccase-catalyzed removal system as an environmentally-friendly technology for the elimination of this type of compounds from aquatic media.

Microtox® test was also carried out to evaluate the toxicity of the laccase-catalyzed transformation products of DCF; for this purpose, different samples were analyzed and results are presented in Table 2.7.

Table 2.7. Microtox® test results of the different samples analyzed to evaluate the toxicity of DCF transformation products after treatment with *Trametes versicolor* laccase at pH 4.

Sample	$EC_{50,15\ min}$ (%)
Control	29
8-h treatment with laccase	8
8-h treatment with laccase + ultrafiltration	57
8-h treatment with laccase + aerobic biodegradation	97

First, control (untreated solution) and 8-h samples were analyzed, obtaining values of 29 and 8%, respectively. Since lower values EC_{50} mean higher toxicity to Microtox® test bacteria, results show that toxicity is increased after laccase treatment. This effect was thought to occur due to the presence of the protein in the solution which could limit the determination by interfering in the light output. Therefore, the sample was filtered by using an Amicon cell (Millipore) with a 10 kDa ultrafiltration membrane aiming to separate the enzyme from the medium. With this, the value of EC_{50} of the resulted sample would correspond to that of the DCF transformation products and moreover, a sample from a continuous treatment of DCF with laccase where the enzyme is retained by immobilization or by using a membrane would be simulated. It was observed that the toxicity of this sample was considerable lower than that of untreated solution: a value of 57% was determined, suggesting the generation of biotransformation products less toxic than the parent substrate. Furthermore, Microtox® test was also applied for the 8-h sample after the aerobic biodegradation experiment described above. In this case, the toxicity of the products was even lower: an EC_{50} close to 100% was detected.

2.4. Conclusions

The results presented in this chapter demonstrated the ability of laccases and laccase-mediator systems to remove estrogenic and anti-inflammatory compounds, as well as the feasibility for the successful reduction of toxicity of the treated medium. The results obtained from characterization of the enzymes in terms of pH and temperature impacts served as basis for the biotransformation experiments and discussion of the corresponding results.

In the case of estrogens, E1, E2 and EE2 were eliminated by *Myceliophthora thermophila* laccase at both pH 4 and 7. Thus, envisaging the application of a continuous bioreactor, neutral pH can be selected for the transformation of this type of compounds once it is beneficial not only for enzyme stability but also for removal percentages (significant transformation efficiencies were found within only 1-h operation), and moreover it was expected to favor the treatment of wastewaters, avoiding their acidification.

Furthermore, LYES analysis of samples resulted from the enzymatic treatment proved that the elimination of the target compounds leads to a parallel elimination of their estrogenicity. Hence, laccase-catalyzed treatment is presented as a promising tool for the detoxification of wastewaters containing estrogenic substrates. In view of these results, further research was directed towards developing different reactor configurations which allow an efficient utilization of the enzyme, thus conferring high productivity and low costs.

With regard to anti-inflammatories, two different enzymes were evaluated for the removal of NPX and DCF at different values of pH and with various laccase mediators. The identified as the optimum strategy to attain significant removal efficiencies of anti-inflammatories when *Myceliophthora thermophila* laccase is used, is the application of laccase-mediator systems under acidic conditions. In this way, the effect of synthetic mediators, HBT and VA, was evaluated, yielding degradation efficiencies of 68 and 36% for NPX, respectively, whereas almost complete elimination of DCF (below detection limits) was detected. Besides, various natural mediators (SA, V, p-CA and FA) were tested aiming to ensure an environmentally friendly and cost-effective technology. Among the compounds assayed, SA provided the best results: DCF was removed within only 1 h with 1 mM SA, although NPX transformation was not affected by the use of these mediators.

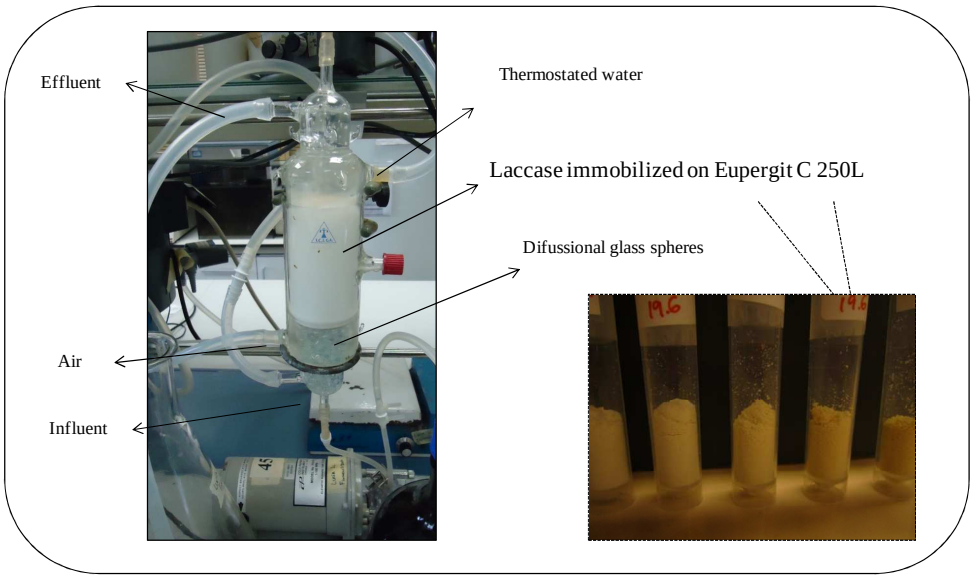
The use of a high-redox potential enzyme, *Trametes versicolor* laccase, leads to more favorable kinetics due to the increase of ΔE^0 between the substrate and the T1 copper of the biocatalyst. Results proved high removal yields for NPX (70-94%) after 24 h in the presence of HBT at neutral and acidic pH, while DCF was completely transformed at pH 4 within shorter periods, from 30 min to 4 h, both in the absence or presence of mediators. The operation at a higher pH implied the addition of mediators to remove DCF: 50 and 98% transformation yields were found for SA and HBT, respectively. Kinetic parameters of the transformation reactions and laccase inactivation coefficients were useful for the estimation and comparison of the results for the different experimental conditions assayed.

The improvement provided by the natural mediator SA was comparable to that found for the synthetic mediator HBT when removing DCF, despite its lower redox potential. This was probably caused by the significant higher stability of SA radicals in comparison to nytroxil radicals generated by HBT oxidation. Furthermore, it was proved that HBT provokes further inactivation of laccase under acidic conditions, probably because of the attack of its free radicals which could oxidize the aromatic residues on the protein surface.

Interestingly, laccase-catalyzed treatment of DCF was proved to result in biotransformation products less toxic than the parent substrate, with a considerable higher aerobic biodegradability. Hence, this technology might be considered as a basis for the development of enzymatic processes applicable to the environmental field for bioremediation of anti-inflammatories and even many other emerging contaminants.

Chapter 3

Laccase immobilization, characterization and application of the biocatalysts*



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L. Lloret, G. Eibes, G. Feijoo, M.T. Moreira and J.M. Lema, Continuous operation of a fluidized bed reactor for the removal of estrogens by immobilized laccase on Eupergit supports, Journal of Biotechnology 162 (2012) 404-406.

L. Lloret, F. Hollmann, G. Eibes, G. Feijoo, M.T. Moreira and J.M. Lema, Immobilisation of laccase on Eupergit supports and its application for the removal of endocrine disrupting chemicals in a packed bed reactor, Biodegradation 23 (2012) 373-386.

L. Lloret, G. Eibes, G. Feijoo, M.T. Moreira, J.M. Lema and F. Hollmann, Immobilization of laccase by encapsulation in sol-gel matrix and its characterization and use for the removal of estrogens, Biotechnology Progress 27 (2011) 1570-1579.

Outline

3. Immobilization of laccase, characterization and application of the biocatalysts

3.1. Introduction

In Chapter 2, the capability of soluble laccase from *Myceliopthora thermophila* for the removal of estrogenic compounds was demonstrated: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) were successfully eliminated within short treatment periods at neutral pH and even in the absence of mediator.

However, the use of free enzymes is usually limited by their potential inactivation by inhibitory compounds, extreme values of pH or high temperatures. Furthermore, technology must be applied aiming the recovery of the enzyme in order to avoid its release with the product as well as to facilitate the reuse of the biocatalyst and consequently to reduce the operational costs. With the goal of overcoming these challenges, immobilization is the most commonly used method for enzyme stabilization; additionally, immobilization of the enzymes allows for their simple physical separation from the reaction medium, enabling the enzymes to be applied in continuous systems (Bornscheuer 2003, Kunamneni et al. 2008b, Osma et al. 2010). In this sense, enzyme immobilization seems to be the most straightforward way to implement laccase-based treatment in continuous mode. Nonetheless, the immobilization procedures could result in conformational alterations of the enzyme and loss of catalytic activity (Fernández-Fernández 2012). Specifically, immobilized laccases have been used for various applications: e.g. decolorization of dyes (Kunamneni et al 2008b, Yamak et al. 2009, Russo et al. 2008), treatment of olive oil mill wastewaters (D'Annibale et al. 2000, Berrio et al. 2007) and even for the construction of biosensors (Vianello et al. 2004) and enzyme-based fuel cells (Nogala et al. 2006).

A classification of the main immobilization methods could be as follows: i) Immobilization on supports by physical, ionic or covalent binding mainly by 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 synthetized in the presence of the enzyme resulting in its physical retention; and iv) self-immobilization by the use of bifunctional cross-linkers (Bayramoglu and Arica 2009, Brady and Jordaan 2009, Dayaram and Dasgupta 2008, Fernández-Fernández 2012). A scheme of some of these methods is depicted in Figure 3.1.

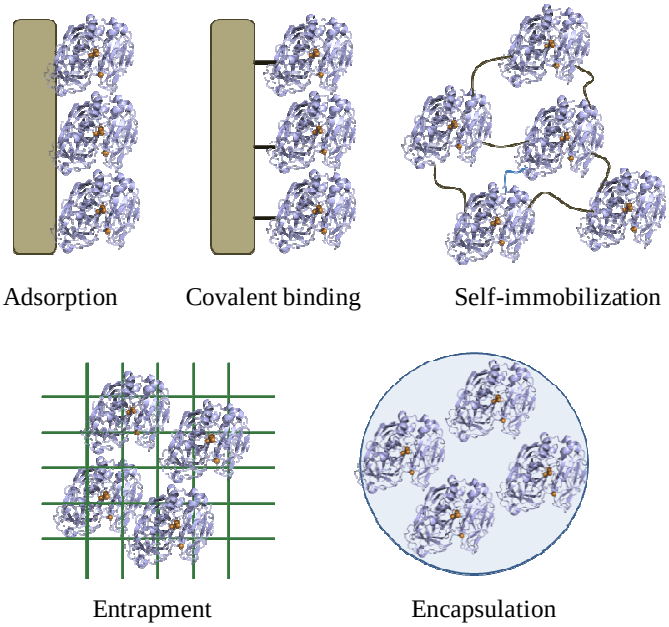

Figure 3.1. Scheme of the immobilization of enzymes by different methods (adapted from Fernández-Fernández et al. 2012).

In this research, attention was paid to two different immobilization methods: covalent immobilization of the laccase on commercial supports and immobilization by encapsulation in a sol-gel matrix.

Enzyme immobilization by covalent binding onto a support has the advantage of tight fixing of the enzyme, which minimizes its leaching from the carrier. Moreover, the formation of multiple covalent bonds between the enzyme and the support is expected to reduce conformational flexibility and thermal vibrations, thus preventing protein unfolding and denaturation (Hanefeld et al. 2009). The application of covalent immobilization techniques considers two different possibilities: either the use of inert carriers which can be properly activated or the use of commercially available active supports. Among the latter, Eupergit carriers have been reported to be effective supports for immobilization of laccases (Hublik and Schinner 2000, Katchalski-Katzir and Kraemer 2000, Knezevic et al. 2006, Russo et al. 2008). Eupergit supports (epoxy-activated acrylic polymers) have been developed between 1974 and 1980 by Röhm, Darmstadt, Germany. These carriers are very stable and present satisfactory chemical and mechanical properties, being considered suitable for covalent immobilization of enzymes for industrial applications (Kraemer et al. 1985).

On the other hand, the incorporation of enzymes in silica matrices has demonstrated to be a good strategy to immobilize enzymes with considerably high efficiency, which can be successfully accomplished by using the sol-gel technology (Reetz et al. 2000, Sangeetha et al. 2008). Sol-gels are a new class of materials that have been found to be suitable for the immobilization of various biological molecules (proteins, enzymes, antibodies) by their encapsulation into the sol-gel matrix during the formation of the hydrogel (Hanefeld et al. 2009, Sangeetha et al. 2008). Numerous advantages of this procedure have been indentified: e.g. improved resistance to thermal and chemical inactivation, remarkable stability of encapsulated enzymes during storage and operation, and the immobilization procedure takes place at mild conditions for the enzyme. Moreover, sol-gel polymers are non-toxic, they do not swell in aqueous or organic solvents, and present mesoporous structure and high pore volume which facilitate the diffusion of the substrates and their interaction with the enzyme (Gill 2001, Hanefeld et al. 2009). The sol-gel technique often consists of using the route with methyltrimethoxysilane (MTMS) and tetramethoxysilane (TMOS) as precursors, as performed in the current research. Their initial hydrolysis in aqueous medium brings about the formation of the sol solution; upon the crosslinking of this solution in the presence of the enzyme, self-organization into a porous three-dimensional network occurs, providing high yields of immobilization via enzyme encapsulation (Gill 2001, Jin and Brennan 2002, Sangeetha et al. 2008, Vidinha et al. 2006).

The main goal of the work presented in this chapter was to investigate the immobilization of *Myceliophthora thermophila* laccase for its application on the continuous removal of estrogenic compounds. With this aim, laccase was immobilized on Eupergit supports as well as by encapsulation in a sol-gel matrix: both procedures were optimized and the resulting biocatalysts were characterized regarding their stability and catalytic activity over a wide range of conditions. Furthermore, packed bed reactors (PBRs) were proposed and applied for bioremediation purposes. First, the bioreactors were used for the decolorization of a synthetic dye, Acid Green 27 (AG27), to demonstrate the feasibility of the technology as well as to evaluate the potential reusability of the biocatalysts; afterwards, the PBRs were utilized for the continuous removal of E1, E2 and EE2. In view of the results obtained and the operability of these bioreactors, a fluidized bed reactor (FBR) was applied aiming to improve the efficiency of estrogens removal by laccase immobilized on Eupergit supports. This bioreactor was used

for the elimination of estrogens at concentrations as low as 100 and 10 µg/L and it was operated for 15-16 days to investigate its stability and that of the biocatalyst.

3.2. Materials and methods

3.2.1. Chemicals and enzyme

E1, E2 and EE2 were purchased from Sigma-Aldrich. Stock solutions were prepared in methanol (J.T. Baker, HPLC grade, 99.8%). 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and the dye AG27 were purchased from Fluka. All other reagents used were of analytical grade. The precursors MTMS and TMOS used for the enzyme encapsulation were supplied by Sigma-Aldrich. Epoxy-activated acrylic beads, Eupergit C and Eupergit C 250L, were purchased from Rohm GmbH.

The recombinant yeast *Saccharomyces cerevisae* was kindly provided by the Laboratory of Microbial Ecology and Technology (Labmet, Ghent University, Belgium). Laccase from *Myceliophthora thermophila* was supplied by Novozymes.

3.2.2. Determination of enzyme activity

Laccase activity of free enzyme was determined by monitoring the oxidation of ABTS as described in Chapter 2. Immobilized laccase activity was assayed by incubating 20 mg/mL of biocatalyst preparation in a solution of 5 mM ABTS (in 100 mM sodium acetate, pH 5) at 30ºC. Immobilized laccase activity was expressed in U/g biocatalyst.

3.2.3. Protein estimation

Protein estimation was performed by a Pierce® 660 nm Protein Assay Kit (Thermo Scientific), using bovine serum albumin as standard.

3.2.4. Immobilization of laccase by encapsulation in sol-gel matrix

Laccase was encapsulated in a sol-gel matrix by means of the formation of a hydrogel. Prior to immobilization, the sol was prepared as described by Veum et al. (2004) by the hydrolysis of the precursors used (MTMS and TMOS). Acidic water (1.38 mL, pH adjusted by the addition of HCl) was added to a mixture of MTMS (2.10 g), TMOS (9.08 g) and distilled water (10.4 mL), being the final pH ~2.80, and stirred vigorously in a 100 mL round bottom flask to obtain a homogenous mixture; the formed methanol was then removed by using a rotary

evaporator. The mixture was then cooled at 0ºC and water was added to restore the same volume of the methanol removed. The solution prepared was used immediately for the encapsulation of the enzyme.

Laccase was dissolved in 100 mM phosphate buffer (pH 7), centrifuged and filtered. Then, the precursor solution (sol) and the diluted enzyme were mixed at a ratio of 50:50 (v/v, %). The concentration of the enzyme in the solution was the suitable according to the selected ratio of mg of protein per mL of sol precursor. The mixture was stirred for 20 s and maintained for 4-5 min; after that period of time the mixture gelled, obtaining a hydrogel with the enzyme encapsulated inside. The resulted hydrogel was cut in particles with diameter less than 3-4 mm, washed with 100 mM phosphate buffer (pH 7) and stored at 4ºC.

Different values of mg of protein/mL sol precursor were evaluated: 2.2, 4.4, 6.6, 11 and 22 mg/mL sol precursor. The immobilization efficiency was determined as the difference between the total protein loaded and the remaining protein in the supernatant.

3.2.5. Immobilization of laccase on Eupergit supports

Laccase was immobilized on Eupergit supports by the method described by Katchalski-Katzir and Kraemer (2000). First, the enzyme was diluted in 100 mM phosphate buffer (pH 7), centrifuged and filtered for its subsequent immobilization. The selected amount of laccase was mixed with 0.5 g of the carrier in 1 M phosphate buffer (pH 7) in a final volume of 5 mL. The mixture was vortexed for 2 min and then, the suspension was incubated at room temperature with gentle shaking for 24 h. The biocatalyst was then filtered using a glass filter (Whatman), washed with 100 mM phosphate buffer (pH 7), dried under vacuum and stored at 4ºC.

Different amounts of laccase: 11, 22, 55, 110 and 220 mg (22-880 mg of preotein/g of support) were evaluated to study the effect on the immobilization efficiency and biocatalyst activity, and two different supports were tested: Eupergit C and Eupergit C 250L. Controls with identical experimental conditions lacking support were performed in parallel. Bound protein was calculated from the difference between the protein content in the control and the protein remaining in solution of the immobilization assay. The recovered activity was calculated as the ratio of the measured activity of the solid biocatalyst and the theoretically immobilized one onto the support. This theoretical activity was defined as the difference between the laccase activity in the controls and the remaining in solution in the supernatant of the immobilization assay.

3.2.6. Characterization of the biocatalysts

Both encapsulated enzyme and covalently immobilized laccase on Eupergit C 250L were biochemically characterized by assaying their catalytic activity and stability over a wide range of conditions.

3.2.6.1. Optimum pH and temperature

The effect of pH on the activity of immobilized enzymes was investigated by determining laccase activity in 100 mM citrate–phosphate–borate buffer at different pHs in the range between 2 and 7. To study the effect of temperature, laccase activity was measured at 20-70ºC. Relative activities were calculated as the ratio between the activity at each evaluated pH or temperature and the corresponding maximum attained.

3.2.6.2. pH and thermal stability

The effect of pH and temperature on the immobilized enzymes stability was studied by incubating the biocatalyst in 100 mM citrate–phosphate–borate buffer (pH 2-8) at room temperature and in 100 mM phosphate buffer (pH 7) at selected temperatures (20-80ºC), respectively. Samples were periodically taken and transferred to standard conditions to determine the residual activity as the ratio between the measured activity and the theoretical initial one.

3.2.6.3. Stability against chemical inactivation

The stability of immobilized enzymes against different inactivating agents was tested by incubating the biocatalysts in 100 mM sodium acetate buffer (pH 5) containing different inhibitors, which were individually assayed: 30 µM NaN_3, 10 µM $ZnCl_2$, 10 µM $CoCl_2$, 10 µM $CaCl_2$, 25% (v/v) methanol and 25% (v/v) acetone; these experiments were also performed with free laccase.

3.2.6.4. Determination of kinetic parameters

Kinetic parameters of immobilized enzymes were determined by measuring the laccase activity under standard conditions using ABTS as substrate at concentrations in the range of 25-1500 µM. The parameters values were obtained by fitting experimental data to the Michaelis-Menten equation (Sigma Plot 7.0, SPSS Inc).

3.2.6.5. Storage stability

The storage stability of immobilized laccases was investigated at 4ºC and at room temperature; corresponding residual activities were measured after 1, 2 and 3 months of storage.

3.2.7. Application of encapsulated and immobilized laccase on Eupergit supports for the removal of pollutants in PBRs

Both encapsulated and immobilized enzyme on Eupergit C 250L were applied for the removal of the dye AG27 and for the transformation of E1, E2 and EE2 in the corresponding PBRs.

3.2.7.1. Description of the PBRs used

PBR with encapsulated enzyme in sol-gel matrix

The PBR with laccase encapsulated in a sol-gel matrix was constructed using a 50-mL closed beaker (Millipore Amicon cell) equipped with 0.45 µm polyethersulfone filter (Millipore), which was packed with 10 g of biocatalyst (10.7 U/g). The reactor system was operated at room temperature under air pressure to allow the influent to flow through the encapsulated enzyme bed. Due to the difficulties in maintaining a constant inlet flow rate, the HRT slightly fluctuated between 50 and 60 min. A scheme of the enzymatic reactor system is shown in Figure 3.2.

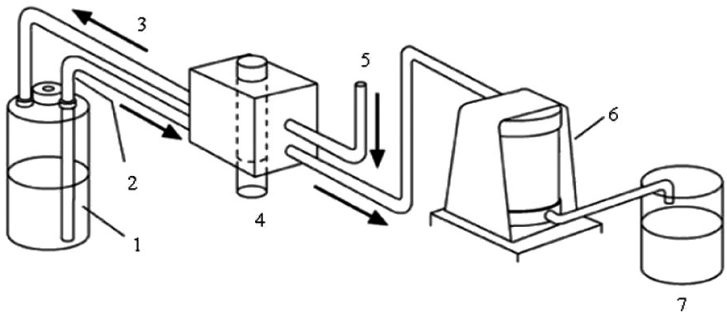

Figure 3.2. Scheme of the PBR used for the continuous removal of E1, E2 and EE2 as well as for the removal of AG27 in consecutive continuous cycles by encapsulated laccase. 1-Feed storage, 2-Feed inlet, 3-Air inlet, 4-Selector valve, 5-Compressed air supply, 6- Reaction cell containing the packed bed with encapsulated enzyme, 7-Products.

PBR with immobilized enzyme on Eupergit supports

The PBR used for the application of immobilized laccase on Eupergit supports was based on a 50 mL glass column (16 cm height, 2 cm internal diameter) packed with 10 g (9.7 U/g) of biocatalyst and operated at room temperature. The influent was fed to the bioreactor at a HRT of 50-60 min through the top of the column, which contained a ceramic filter in the bottom part to retain the biocatalyst.

3.2.7.2. Decolorization of the model synthetic dye AG27 by immobilized laccases in PBRs

The previously described PBRs were used for the decolorization of the synthetic dye AG27 used as a model compound in order to test the feasibility of the enzymatic system. Furthermore, the PBRs were operated in repeated continuous cycles of 4 and 5 h for covalently immobilized and encapsulated laccase, respectively, to investigate the potential reusability of the biocatalysts.

For this purpose, the bioreactors were continuously fed with a solution of 50 mg/L of AG27 in phosphate buffer (100 mM, pH 7) and at the operational conditions previously detailed. After one cycle of continuous operation (4 or 5 h), the operation was stopped and the solution was drained. Afterwards, the biocatalyst was washed with 20 mL of 100 mM phosphate buffer solution (pH 7) by its continuous circulation through the packed bed. Then, the operation was restarted for the following cycle.

Samples were periodically withdrawn from the bioreactor effluent to measure laccase activity in order to evaluate the potential leaching from the support. Also, samples from the influent and effluent were collected to measure the AG27 concentration by the determination of the absorbance and thus, calculating the percentage of decolorization attained (%). Moreover, an experimental control was performed under identical conditions but with the biocatalysts prepared with previously inactivated enzyme (at 105ºC for 4 h) to evaluate the percentage of dye eliminated by the adsorption.

Scanning electron microscope (SEM)

SEM pictures of laccase immobilized on Eupergit C 250L before and after AG27 decolorization in the corresponding PBR were taken by using a Field Emission JSM-7500F (JEOL) equipment. The samples, previously dried under vacuum at 105ºC, were mounted on a metal stub and sputtered with gold in order to make the sample conductive, and the images were taken at an accelerating voltage of 1 kV.

3.2.7.3. Continuous removal of E1, E2 and EE2 by immobilized laccases in PBRs

The previously described PBRs with encapsulated laccase and immobilized enzyme on Eupergit supports were applied for the continuous removal of E1, E2 and EE2 from a mixture of 5 mg/L (each) in 100 mM phosphate buffer (pH 7). In this case, the operation was continuously maintained for 8 h.

Samples were taken from the bioreactors influent and effluent to determine the concentration of estrogens by high performance liquid chromatography (HPLC) in order to calculate the level of removal achieved (%). Residual laccase activity of the biocatalyst was determined after 8 h, when the operation was stopped. Corresponding controls were conducted with previously inactivated enzyme to determine the potential elimination of the target compounds due to adsorption.

3.2.8. Continuous removal of E1, E2 and EE2 by immobilized laccase on Eupergit supports in a FBR

A different reactor configuration was proposed to improve the efficiency of the enzymatic system: the operation of a FBR with laccase immobilized on Eupergit C 250L was considered in this section.

3.2.8.1. Description of the FBR used

The FBR used for the continuous removal of E1, E2 and EE2 consisted of a glass jacketed column with internal diameter of 5 cm and height of 11 cm (working volume of 215 mL), filled with 4 g of biocatalyst (25 U/g). It comprised ceramic filters in the upper and lower ends allowing the retention of the biocatalyst. Glass spheres were used to fill the lower compartment and facilitate the homogenous diffusion of air and feed stream. Air was supplied to the bioreactor at a flow of 0.5 L/min to maintain the fluidization of the bed and to ensure the homogenous mixture of the biocatalyst in the whole reactor volume. Temperature was maintained at 26ºC by circulation of thermostated water. The influent was fed to the bioreactor through the bottom of the column. A scheme of the reactor is shown in Figure 3.3.

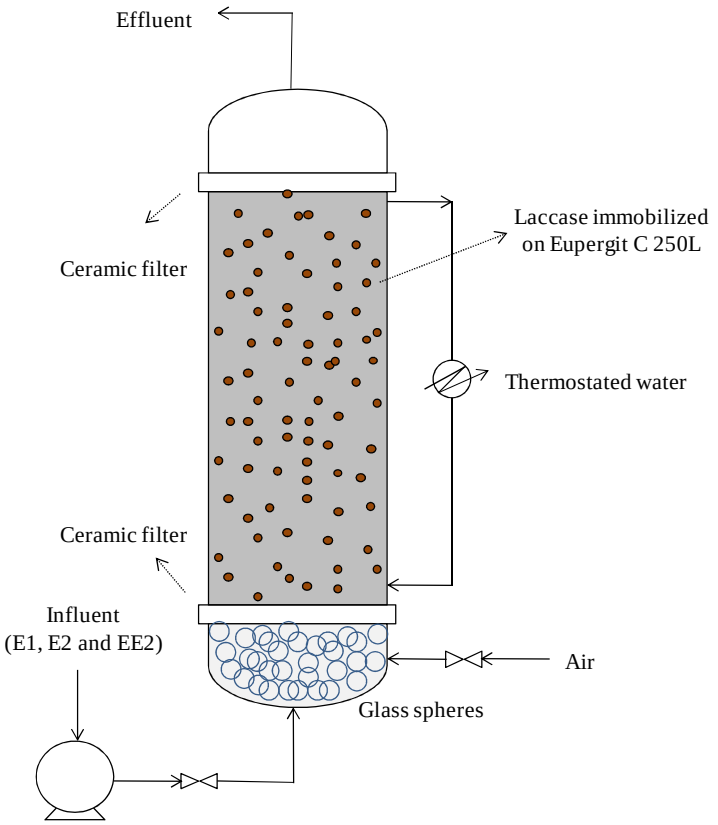

Figure 3.3. Scheme of the FBR used for the continuous removal of E1, E2 and EE2 by immobilized laccase on Eupergit supports.

Determination of the residence time distribution (RTD)

The determination of the RTD curve by stimulus-response experiments was carried out to characterize the hydraulic behavior of the FBR. With this goal, an aliquot of 0.1 mL of NaOH (20 M), which was used as tracer, was injected in the feeding during the operation of the bioreactor with distilled water. Samples were periodically withdrawn from the effluent to measure their conductivity, which would be correlated with NaOH concentration by the corresponding calibration curves. The experimental data obtained were adjusted to the tank-in-series model described by Levenspiel (1999) to obtain the RTD characteristic of the bioreactor. From this, the number of ideal tanks connected in series (N), which represent the EMR was calculated. A low number of N would mean that the behavior of the bioreactor is similar to that of a complete stirred tank reactor, whereas a high value of N means a behavior similar to a plug flow reactor (Fogler 2006).

3.2.8.2. Continuous removal of E1, E2 and EE2 by immobilized laccase in a FBR

Experiments performed at level of mg/L

In a first experiment, the continuous bioreactor was operated for 10 h. The reactor was fed with a mixture of E1, E2 and EE2 at a concentration of 5 mg/L (each) in phosphate buffer (100 mM, pH 7) at a HRT of 50 min (feed addition rate 6 mg/(L·h)) to ensure identical addition rate of estrogens per unit of enzyme to that in the PBRs (about 0.06 mg/(L·h·U)).

Removal yields (%) were calculated from the difference between the concentration in samples taken from the influent and effluent of the FBR, determined by HPLC; removal rates expressed in terms of mg removed/(L·h) were also calculated. Laccase activity of the biocatalyst was determined after 10 h, when the operation was stopped. Moreover, control experiments were conducted under identical conditions with the biocatalyst prepared with inactivated enzyme in an attempt to determine the potential removal by adsorption.

Experiments performed at level of µg/L

In a subsequent experiment, the reactor was fed with a stock solution of 100 µg/L of estrogens (each) in phosphate buffer (100 mM, pH 7) at a HRT of 50 min (feed addition rate of 120 µg/L·h) in order to evaluate the feasibility of the system to remove the compounds at much lower concentrations. Moreover, the bioreactor was operated for 15 days aiming to investigate its stability and that of the biocatalyst.

An additional experiment was carried out to study the potential application of the FBR to remove E1, E2 and EE2 at concentrations closer to environmental levels. For this purpose, the bioreactor was fed with phosphate buffer (100 mM, pH 7) containing the estrogens at a concentration of 10 µg/L; the system was operated for 16 days. Aiming to study the effect of the HRT, its value was varied between 50 and 150 min in periods of 3 days for each value of HRT assayed, with the consequent change in the feed addition rate. Then, the operation of the reactor was maintained until a significant biocatalyst inactivation was observed. These conditions are detailed in Table 3.1.

Table 3.1. Operational parameters of the long-term experiment performed in the FBR for the continuous removal of estrogens at 10 µg/L.

Stage	Operational periods (days)	HRT (min)	Feed addition rate (µg/L·h)
I	1 - 3	50	12
II	4 - 6	75	8
III	7 - 9	100	6
IV	10 - 16	150	4

For these experiments performed at initial concentrations at level of µg/L, quantification of estrogens was done by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS-MS) and estrogenic activity by yeast estrogen screen assay assisted by enzymatic digestion with lyticase (LYES). The removal yields of both target compounds and estrogenic activity (%) were calculated considering the concentrations detected in the influent and effluent of the bioreactor; removal rates, expressed in terms of µg/(L·h), were determined. Corresponding controls were carried out with biocatalyst prepared with inactivated enzyme to evaluate the potential adsorption.

Furthermore, laccase activity was monitored as follows: samples of biocatalysts were taken directly from the inside of the FBR by interrupting the continuous feed flow and opening the upper part of the column. After being filtered under vacuum, samples were analyzed under standard conditions to determine residual laccase activity. After washing with phosphate buffer (100 mM, pH 7), the biocatalyst was recycled back to the reactor and the operation was immediately restarted in order to restore the steady-state operation of the FBR.

3.2.9. Determination of the concentration of the AG27 dye

AG27 transformation was determined by measuring the corresponding absorbance at its maximum absorption wavelength (650 nm) with a Shimadzu UV-1603 spectrophotometer. The percentage of decolorization was calculated as the ratio between the concentration of the dye with respect to the initial value.

3.2.10. Determination of estrogens concentration

3.2.10.1. Quantification of estrogens at level of mg/L by HPLC

Determination of E1, E2 and EE2 concentrations by HPLC was conducted by applying the method described in Chapter 2.

3.2.10.2. Quantification of estrogens at level of µg/L by LC-APCI-MS-MS

Determination of E1, E2 and EE2 in samples taken from experiments performed at 10 and 100 µg/L of initial concentration was conducted by LC-APCI-MS-MS. The LC system used (Alginet 1100, Alginet Technologies) was equipped with Synergi 4u MAX-RP 80A column (4.60 mm × 250 mm, 4 µm, Phenomenex) for analyte separation. The injection volume was 10 µL and the mobile phase consisted of a binary mixture of 0.1% formic acid (v/v) in water (A) and 100% methanol (B) at a flow rate of 0.7 mL/min. The gradient was as follows: initial conditions were 70% B, increased linearly up to 88% for 2 min, followed by a linear program to 94% until 15 min.

Mass spectrometry was performed using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems) and MS/MS was carried out in the multiple reaction mode (MRM) using APCI in positive mode. The values of the main parameters of the source are shown in Table 3.2.

Table 3.2. Source-dependent parameters for LC-APCI-MS-MS analysis.

Parameter	
Collision gas (psig)	6
Curtain gas (psig)	14
Ion source gas nebulizer (psig)	15
Nebulizer current (µA)	3
Temperature (ºC)	450
Entrance potential (V)	10

For each compound, two characteristic fragmentations were optimized, the most abundant one being used for quantitation, while the second one with lower intensity was used as a qualifier, as shown in Table 3.3.

Table 3.3. MRM conditions used for LC-APCI-MS-MS analysis.

Compound	MRM transition → product ion	
E1	271.1 → 133.2,	271.1 → 159.2
E2	255.0 → 159.1,	255.0 → 133.1
EE2	279.2 → 133.1,	279.2 → 159.2

3.2.11. Determination of estrogenic activity

The estrogenic activity in samples was measured by LYES by the procedure detailed in Chapter 2.

3.3. Results and discussion

3.3.1. Immobilization of laccase by encapsulation in sol-gel matrix

The sol-gel encapsulation method described by Veum et al. (2004) was used for the immobilization of *Myceliophthora thermophila* laccase. A scheme of the procedure is shown in Figure 3.4.

The first step of this well-established sol-gel immobilization protocol consisted on hydrolyzing the precursor compounds at acid pH and thus forming the sol. The most widely used precursors are alkoxides ($Si(OR_4)$, such as TMOS where R is a methyl group) and alkoxysilanes ($(R_1)_xSi(OR)_{4-x}$, such as MTMS where both R_1 and R are methyl groups), which were already used in the mid-1980s to prepare organically-modified silicates for the encapsulation of antibodies and enzymes (Glad et al. 1985). In this work, MTMS and TMOS precursors were selected and used at the proportions previously indicated once considering the best compromise solution between structural integrity of the material and laccase activity of the resulting biocatalyst. It has been reported that both precursors provided the highest efficiency for the encapsulation of bacterial cells and lipase enzymes, in comparison with other precursor compounds (Chen et al. 2004, Yang et al. 2010). Moreover, the hydrolysis reaction was performed at acid pH once it was proved that these precursors are almost 100% hydrolyzed under acid conditions (Veum et al. 2004) (see first reaction in the scheme in Figure 3.4). The methanol formed during this reaction was removed by evaporation aiming to avoid the inactivation of the enzyme during the gelation process, as occurred in previous works dealing with this technique (Buisson et al. 2001, Reetz et al. 2000), and its volume was replaced by distilled water.

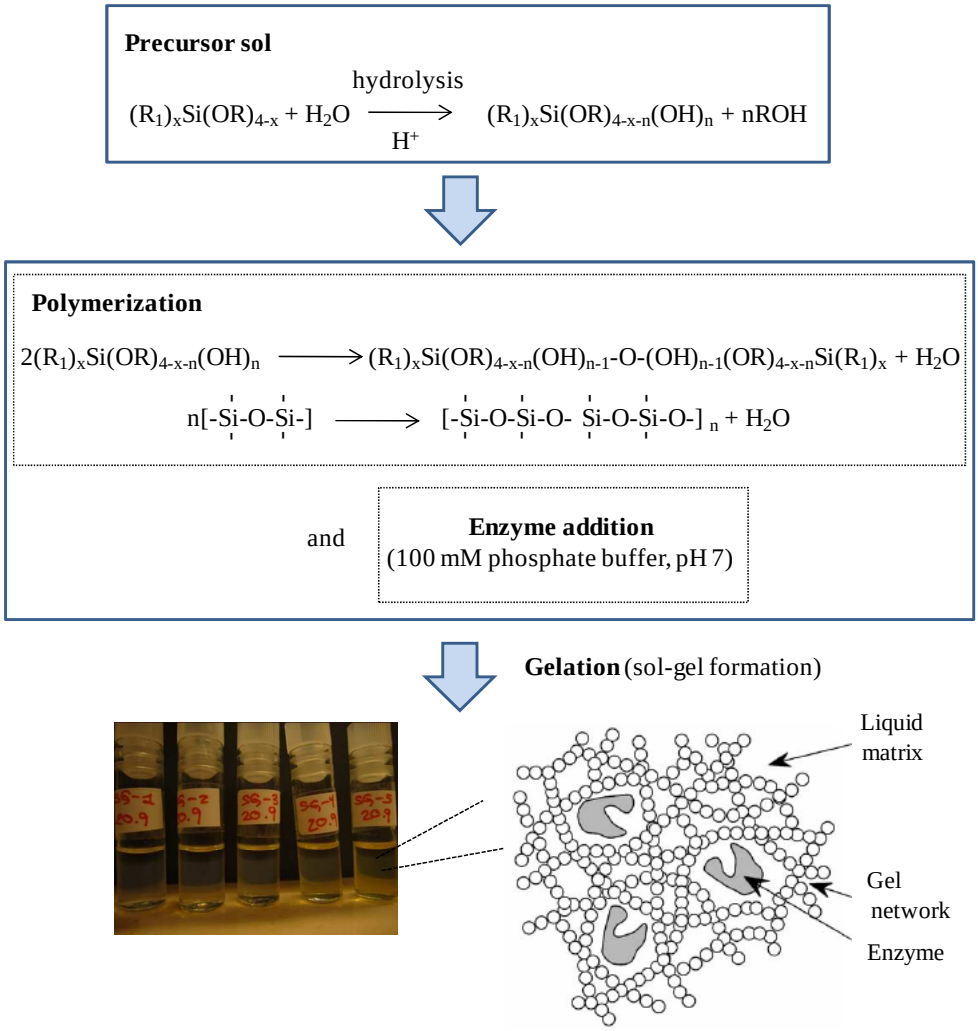

Figure 3.4. Scheme of the procedure applied for the encapsulation of laccase in a sol-gel matrix.

Following hydrolysis, the sol may be used immediately or stored to allow continued evolution of the sol particles. During that time, further hydrolysis and condensation reaction occur, resulting in a wide range of linear, branched and colloidal polysilicates (Jin and Brennan 2002). In order to avoid this and permit polymerization reaction only in the presence of laccase for its encapsulation, the solution was cooled as the sol was formed.

In the next step of the procedure, the precursor sol was mixed with the buffer solution containing laccase at pH 7. The change in the pH promotes a large extension of polymerization reaction (see reactions in Figure 3.4). Moreover, it

was reported that some enzymes may catalyze this polymerization reaction although the mechanism involved is quite controversial. Various studies have shown that protein backbones can act as templates to build the silica network, while other suggested that the enzyme accelerates the reaction by the fact that positively charged amino groups of the protein can attract the negatively charged silica oligomers and hence bring them closer to each other, favoring polymerization (Pierre 2004). Anyhow, this results in gelation of the solution and thus a hydrogel containing the encapsulated enzyme was formed.

Some authors reported the drying of the hydrogel for its optimum use, obtaining a xerogel when the drying is conducted by evaporation or an aerogel by supercritical drying (Pierre 2004). Nonetheless, these procedures cause shrinkage of the pores, and they are also likely to cause loss of activity. This could be due to the fact that capillary stress, which occurs by evaporation of the water phase of the hydrogel in open air, will cause partial collapse of the gel structure as the liquid-gas interface moves in through the gel. As suggested by Veum et al. (2004), as the gel shrinks, the enzyme could be also crushed.

This sol-gel technique allows the synthesis of chemically inert and hydrophobic gels with remarkable thermal and mechanical resistance, significant porosity (up to 80%) and high water content (50-80%) (Jin and Brennan 2002, Pierre 2004, Veum et al. 2004). Yang et al. (2010) obtained hydrogels by a similar method with the following characteristics: surface area about 60 m^2/g and pore volume of 0.043 cm^3/g. It has been also shown that several configurations are possible: the protein can be free inside the pores, adsorbed or associated with surface functional groups, in a variety of orientations or conformations, or even aggregated (Alstein et al. 1998). An uncertain fraction of the enzyme may also be in cages which are not accessible to the substrate (Wambolt and Saavedra 1996). Anyhow, most works suggested no interaction of the protein with the polymeric matrix (Lee and Hong 2000, Yamak et al. 2009), that the enzymes tend to retain its native conformation upon encapsulation (Jin and Brenan 2002) and moreover, that they are homogeneously dispersed in the gels (Ellerby et al. 1992, Wang 2000). Furthermore, previous investigations showed that although the protein motion was more restricted than in solution, the global motion of the encapsulated protein was not completely inhibited, which suggests that the protein became sequestered into pores that were both hydrated and sufficiently large to allow some degree of mobility (Bhatia et al. 2000).

In this work, the resulting hydrogel was cut in small particles with diameters smaller than 3-4 mm, which were then washed with 100 mM phosphate buffer

(pH 7) to remove unreacted silane and free protein, and stored at 4ºC. During the washing procedure, protein concentration was detected in the washing solution, initially attributed to non-encapsulated enzyme due to saturation of the sol-gel matrix or leaching through pores with diameter higher than the enzyme size.

The influence of the amount of laccase in the solution for hydrogel preparation was studied in the range of 2.2-22 mg of protein/mL sol. The results obtained are summarized in Table 3.4.

Table 3.4. Laccase activity and immobilization efficiency found for the different conditions evaluated during laccase encapsulation in a sol-gel matrix.

Added protein (mg/mL sol)	Immobilization efficiency (%)	Laccase activity (U/g biocatalyst)
2.2	82.9 ± 2.6	5.5 ± 0.2
4.4	77.7 ± 3.4	7.2 ± 0.3
6.6	69.2 ± 1.7	10.7 ± 0.2
11	63.7 ± 2.1	14.1 ± 0.3
22	59.2 ± 1.9	16.9 ± 0.3

In general, a high catalytic efficiency of the obtained biocatalysts was observed, with activities in the range 5.5-17.0 U/g and immobilization efficiency in terms of bound protein between 82.3 and 59.2%. However, it was observed that when the load of enzyme was increased, the immobilization efficiency decreased. In this way, an increase of the added laccase from 4.4 to 22 mg of protein/mL sol resulted in a decrease of immobilization efficiency from 77.7 to 59.2%. This phenomenon was also observed by Reetz et al. (1996) for sol-gel entrapment of lipases, and it was assumed to occur due to diffusional limitations of the substrate entering into the catalyst particles, which would lead to the decrease in the apparent immobilization efficiency. Another reason could be the aggregation of the enzyme that might occur at relatively high concentrations, resulting in lower degree of dispersion in the sol-gel matrix.

The ranges of encapsulation attained here match those reported in literature. For example, Sangeetha et al. (2008) achieved an immobilization efficiency of 78% after encapsulation of subtilisin using MTMS as precursor. Yang et al. (2010) investigated different silane compounds as precursors during sol-gel

preparation for the entrapment of lipase, obtaining variable immobilization efficiency between 42 and 99%. In addition, the results obtained by the encapsulation method for the immobilization of laccase are in the range of those attained by other methods such as the covalent bonding of the enzyme on cellulose-based carriers Granocel (Rekuc et al. 2009), Sepabeads EC-EP3 and Dilbeads NK supports (Kunamneni et al. 2008b), silica beads (Champagne and Ramsay et al. 2007) or even by the formation of cross-linked enzyme aggregates (Cabana et al. 2007a).

3.3.2. Immobilization of laccase on Eupergit supports

Commercial epoxy-activated acrylic polymers were used for the evaluation of covalent immobilization of laccase. Eupergit C and Eupergit C 250L are microporous beads with a diameter of 100-250 µm, made by copolymerization of N,N'-methylen-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide (Berrio et al. 2007, Boller et al. 2002, Katchalski-Katzir and Kraemer 2000). They differ in the content of oxirane groups and in their porosity: while Eupergit C has an average pore size of 20 nm and an oxirane density of 600 µmol/g dry beads, Eupergit C 250L has larger pores (200 nm) and lower oxirane density (300 µmol/g dry). These supports were selected once the immobilization of enzyme on their surfaces is rapid and easy at laboratory and it could be feasible even at industrial scale (Boller et al. 2002).

Mateo et al. (2000b) have proposed a two-step binding mechanism for this process. It is assumed that in the first step the enzyme is physically adsorbed on the carrier by hydrophobic interactions. This brings amino and thiol groups on the surface of the enzyme in close proximity to the oxirane groups of the carrier. Thus, Eupergit carriers bind via its oxirane groups which react with the amino groups of the enzyme, as indicated in Figure 3.5 (Katchalski-Katzir and Kraemer 2000). Immobilization of enzymes on Eupergit supports is usually carried out at high ionic strength (phosphate buffer 1 M was used here) and neutral or basic pH (pH 7 in this work) to favor hydrophobic interactions and reactivity of amino groups, respectively (Mateo et al. 2002). Eupergit beads can also bind with enzymes via their sulfhydril, hydroxyl and carboxyl groups in the acidic, neutral and alkaline pH range (Knezevic et al 2006, Turkova et al. 1978).

The high density of oxirane groups on the surface of the supports promotes multipoint attachment, which is assumed to increase conformational stability of the enzyme and hence, it improves long-term operational stability of the biocatalyst (Boller et al. 2002). Nevertheless, Mateo et al. (2000a) reported the

need of incubation of the enzyme for longer periods of time and even under stronger pH conditions to achieve the formation of significant multipoint attachment.

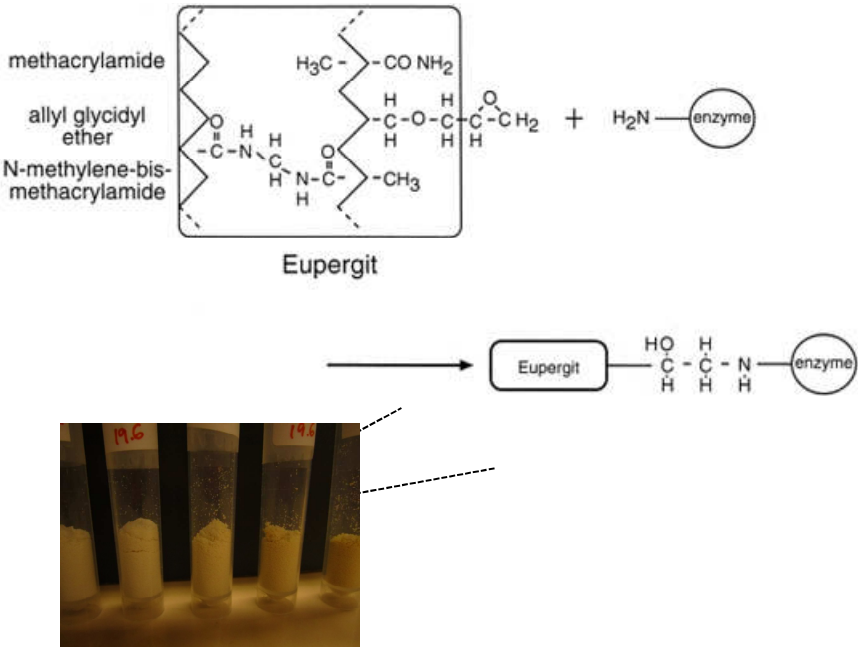

Figure 3.5. Eupergit supports structure and scheme of the reaction during enzyme immobilization on Eupergit supports.

Different amounts of laccase: 11, 22, 55, 110 and 220 mg (22-880 mg of preotein/g of support), were assayed to evaluate the impacts on laccase activity as well as on the bound protein and recovered activity in an attempt to optimize the ratio protein/carrier. The results obtained for both supports are shown in Table 3.5.

Table 3.5. Laccase activity and immobilization yields for the different conditions evaluated during laccase immobilization on Eupergit supports.

Added protein (mg/g support)	Bound protein[a] (%)		Activity[b] (U/g biocatalyst)		Activity recovery[c] (%)	
	Eupergit C	Eupergit C 250L	Eupergit C	Eupergit C 250L	Eupergit C	Eupergit C 250L
22	79.1 ± 2.3	99.3 ± 2.8	1.4 ± 0.1	9.7 ± 0.5	36.1 ± 1.8	88.4 ± 4.1
44	75.9 ± 5.1	89.1 ± 1.3	2.4 ± 0.1	15.9 ± 0.7	28.4 ± 1.1	86.9 ±3.9
110	74.2 ± 4.2	84.2 ± 0.5	7.4 ± 0.2	24.3 ± 0.3	26.9 ± 0.9	50.9 ± 2.5
220	61.3 ± 0.2	75.1 ± 2.4	10.1 ± 0.3	53.8 ± 1.1	23.9 ± 2.1	63.8 ± 3.0
440	45.1 ± 0.9	61.9 ± 4.6	16.0 ± 0.2	72.9 ± 1.1	21.1 ± 1.3	43.8 ± 2.1
880	43.8 ± 1.1	56.7 ± 2.5	17.1 ± 0.3	80.0 ± 0.9	19.2 ± 1.1	39.5 ± 0.9

[a]The amount of bound protein was calculated from the difference between the protein in the control and protein remaining in the supernatant of immobilization assay.

[b]Laccase activity was determined under standard conditions.

[c]Activity recovered was calculated as the ratio between the measured activity of the biocatalyst and the theoretical bound activity (difference between free laccase activity in the controls and the remaining in the supernatant of immobilization assay).

Specific activities of up to 17.1 U/g and 80.0 U/g were measured for Eupergit C and Eupergit C 250L, respectively. It is evident that increasing amounts of the enzyme implies that the percentage of bound protein decreases. For example, the increase in laccase load from 22 to 440 mg/g resulted in the decrease of efficiency from 79.1% to 45.1% for Eupergit C and more so, from 99.3% to 61.9%, in the case of Eupergit C 250L. It is generally acknowledged that the immobilization efficiency of enzymes on solid supports decreases when enzyme loading exceeds a certain value (Knezevic et al. 2006, Li et al. 2007). Furthermore, a saturation value at 440 mg protein/g support seems to be achieved once immobilization efficiency and laccase activity of the biocatalysts slightly changed for higher amounts of protein, as it was previously reported by Katchalski-Katzir and Kraemer (2000). Despite the high bound protein yields obtained, important loss of activity was observed, as commonly occurs during covalent immobilization of enzymes because of the diminished catalytic activity in comparison to their free form due to multipoint attachment to the carriers (López et al. 2010). Berrio et al. (2007) attained similar levels of bound protein between 10.3-66.5% and 10.2-34.2% for *Pycnoporus coccineus* laccase immobilized on Eupergit C and Eupergit C 250L, respectively.

Among both supports, Eupergit C 250L yielded the highest specific activity (80 U/g biocatalyst), which could be caused by its lower concentration of reactive groups but larger pores, as also suggested by Gomez de Segura et al. (2004) and Berrio et al. (2007). Due to its superior activity, only laccase immobilized on Eupergit C 250L was used for further experiments.

3.3.3. Characterization of the biocatalysts

In order to evaluate the effect of the immobilization, laccase immobilized by both methods was biochemically characterized in terms of effects of pH, temperature and stability over various operational conditions; also kinetic parameters were determined.

3.3.3.1. Optimum pH and temperature

In order to evaluate the effects of pH and temperature on the laccase activity of the immobilized enzyme, laccase activity was determined under various conditions (pH 2-7 and temperatures in the range 20-70ºC). The results found for encapsulated and immobilized laccase on Eupergit C 250L are shown in Figure 3.6; results found for free enzyme and presented in Chapter 2 are also included here to facilitate comparison.

The pH of maximum activity of free and immobilized laccases was found to be pH 3 (Figure 3.6.A). On the other hand, the relative activity of immobilized laccase by encapsulation as well as on Eupergit supports was approximately 10% higher than free laccase in the pH range of 3-7. Regarding the effect of the temperature, both free and immobilized enzymes presented it maximum activity at 60ºC, and once again, immobilized laccases presented a wider profile of optimum temperature. However, the encapsulated laccase provided a more remarkable broader profile since its relative activity was 10-30% higher than the free laccase over the whole range assayed (Figure 3.6.B).

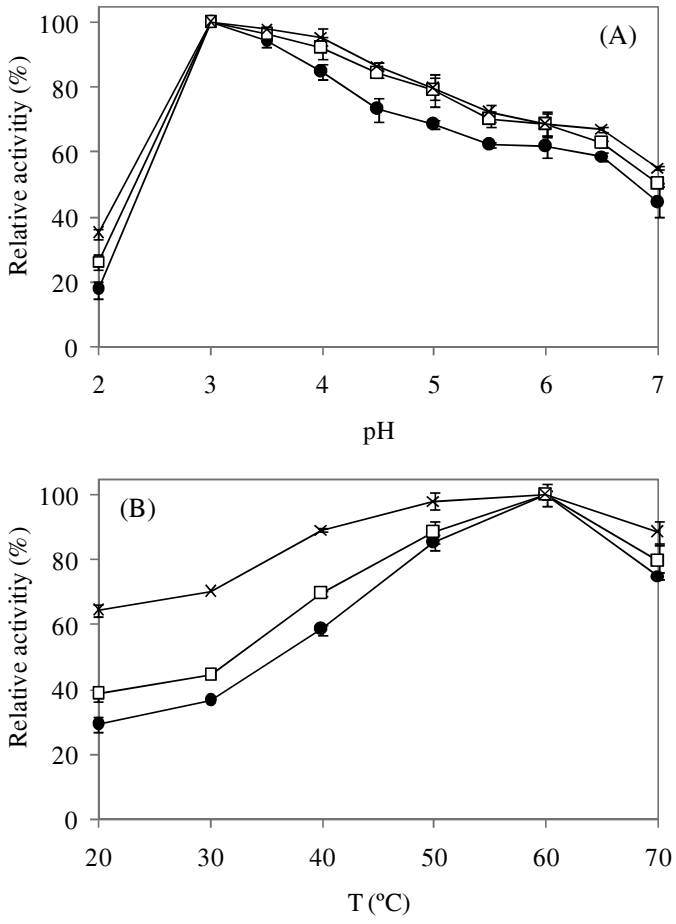

Figure 3.6. Influence of pH (A) and temperature (B) on the activity of free (●), encapsulated (×) and laccase immobilized on Eupergit supports (□).

3.3.3.2. pH and thermal stability

With the aim of studying the effect of pH and temperature on the stability, immobilized enzymes were incubated for 24 h at the corresponding conditions assessed (pH 2-8 and temperature 20-80ºC). The results of residual activities after 4 h of incubation are shown in Figure 3.7; the results for free enzyme, further detailed in Chapter 2, are also depicted for comparison.

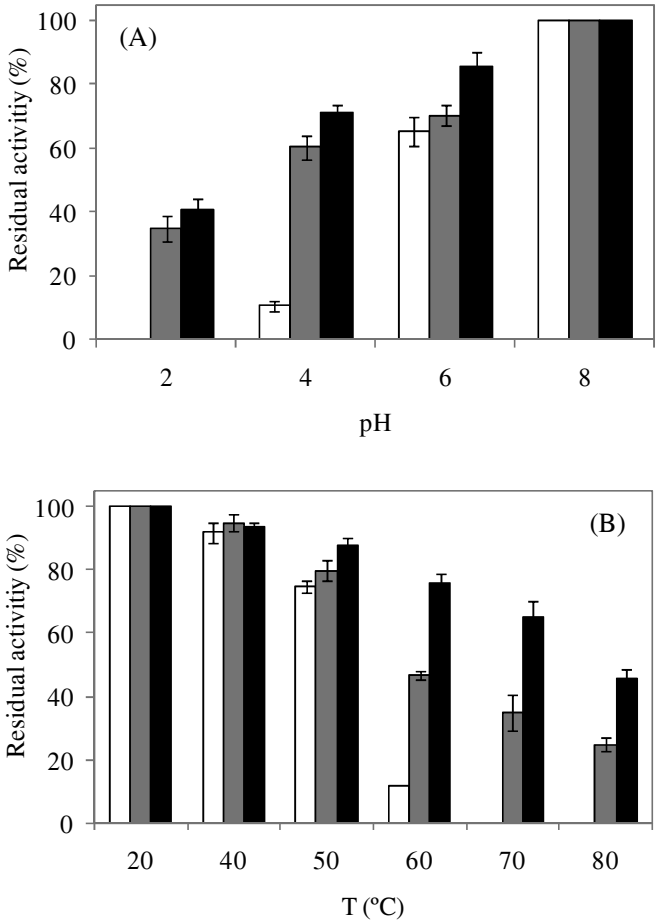

Figure 3.7. pH (A) and thermal (B) stability of free (□), encapsulated (■) and laccase immobilized on Eupergit supports (■).

While results were similar at pH values close to neutral pH, enzyme immobilization was demonstrated to significantly enhance enzyme stability at acid pH. For example, free enzyme was completely inactivated when incubated at pH 2 for 4 h, and it retained only 10% of its initial activity at pH 4. However, the

residual activity of immobilized laccase on Eupergit supports after incubation at the same conditions was 34 and 60% (Figure 3.7.A). Alptekin et al. (2010) reported that the covalent bonding of laccase to the resin resulted in more rigid enzyme less prone to pH-induced conformational changes, providing higher pH-stability.

Thermal stability of immobilized enzyme on Eupergit supports was also proved to be enhanced with respect to the free form; while free laccase was almost completely inactivated at 60, 70 and 80ºC, covalently immobilized enzyme retained 47, 35 and about 25% of its initial activity (Figure 3.7.B). Moreover, it was observed that the activity of free laccase dropped more rapidly than that of immobilized enzyme (data not shown). These results agree with the study carried out by Osma et al. (2010), who reported that immobilization affects the conformational flexibility of the enzyme since it causes an increase in enzyme rigidity and stability towards denaturation by high temperatures. The improvement on stability attained in the present study was similar to that achieved in previous investigations. Kunamneni et al. (2008b) and Forde et al. (2010), who carried out the immobilization of the *Myceliophthora thermophila* laccase on Sepabeads EC-EP3 and Dilbeads NK polymers and on mesoporous silicates particles, attained an enhancement of 10-15% after immobilization in a similar range of temperature.

In the case of encapsulated laccase, the improved stability towards pH was approximately of the same magnitude than that attained with covalent immobilization (Figure 3.7.A), whereas encapsulated laccase presented much higher thermal stability: residual activity of the biocatalyst after 4-h incubation at 60, 70 and 80ºC was up to 75, 65 and 45%, respectively (Figure 3.7.B). Even, free laccase was totally inactivated after 24 h of incubation at 60ºC, while encapsulated enzyme retained about 35% of activity (data not shown). Thermal stability depends on the microenvironment of the enzyme and its reorganization upon immobilization. In this sense, the improved stability of the encapsulated laccase could be due to the confinement of the enzyme in nanopores which in turn brings about an enhancement of the order and compactness of the structure, favouring intramolecular stabilizing forces (Sangeetha et al. 2008). Moreover, it is expected that the enzyme would preserve its original three-dimensional conformation inside the sol-gel matrix and the active sites would have no interaction with the polymeric matrices, which may imply that the optimum values of pH and temperature of the encapsulated laccase are similar to those of

the free enzyme (Lee and Hong 2000, Yamak et al. 2009), as it was verified in the previous section.

3.3.3.3. Stability against chemical inactivation

Stability of free laccase as well as immobilized enzymes in the presence of various inactivating agents was evaluated. Residual activity after 30 min of incubation in sodium acetate buffer containing different inhibitors: 30 µM NaN_3, 10 µM $ZnCl_2$, 10 µM $CoCl_2$, 10 µM $CaCl_2$, 25% (v/v) methanol and 25% acetone (v/v), are shown in Figure 3.8.

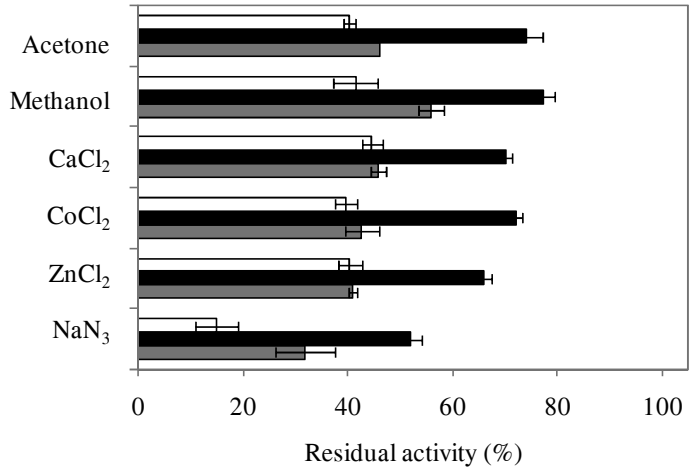

Figure 3.8. Stability of free (□), encapsulated (■) and laccase immobilized on Eupergit supports (■) in the presence of the different inactivating agents evaluated.

It was observed that the chemical inactivating agents evaluated affected the stability of immobilized laccase as the residual activity after 30 min of incubation in their presence (Figure 3.8) was lower than that for stability experiments at identical conditions but in the absence of any chemical: values between 70 and 90% of residual activity were found (data not shown).

However, immobilization of laccase by both methods provided significant enhancement of laccase stability against chemical inactivation when comparing with the results for free *Myceliophthora thermophila* laccase. For instance, free laccase presented only 15% of residual activity after incubation with NaN_3 while immobilized enzyme on Eupergit supports retained almost 32% and approximately 52% the encapsulated laccase. It seems that the immobilization diminished the structural alteration of the catalytic site of laccase induced by

azide binding (Cabana et al. 2007b). A slighter enhancement was observed in the case of $ZnCl_2$, $CaCl_2$ and $CoCl_2$ probably due to the small dimension of the ions that allowed them to easily migrate in the structure of the biocatalyst and inactivate the enzyme in a similar extent. On the other hand, a significant higher stability was observed for the encapsulated laccase in comparison not only to the free enzyme but also to the laccase immobilized on Eupergit C 250L.

3.3.3.4. Determination of kinetic parameters

Michaelis-Menten kinetic parameters of immobilized laccase were determined with ABTS as substrate (25-1500 µM) aiming to evaluate the effect of the both immobilization methods on the catalytic efficiency of laccase by comparing Michaelis-Menten constants (K_M). This will provide an idea about the affinitiy of the enzymes towards the substrate; that is, a higher constant value, a poorer affinity is expected.

A value of v_{max} for the covalently immobilized enzyme of 693 µM/min was found, and an approximately 3-fold higher K_M (150 µM) was determined in comparison to that of the free laccase (56 µM, see Chapter 2). Lower efficiencies for immobilized laccase and catalase from *Lentinus edodes* on Eupergit C were also reported by D'Annibale et al. (2000) and Alptekin et al. (2010), respectively. As expected, a more significant increase in K_M values for the immobilized laccase by encapsulation (645 µM) was observed, which agrees with the findings reported by Yamak et al. (2009) for the entrapment of laccase in sol-gel matrices based on semi-permeable polymers.

Several authors have suggested that poorer enzymatic properties of enzymes after immobilization could be related to limitations in the diffusion of the substrate, substrate partitioning, lower accessibility of the substrate to the active site, protein conformational changes and internal mass transport limitations (Davis and Burns 1992, Hernaiz and Crout 2000, Rekuc et al. 2010, Yamak et al. 2009). The poorer apparent catalytic efficiency for encapsulated laccase is likely to be caused by the fact that the enzyme in confined in the pores of the sol-gel matrix, whereas laccase immobilized on the Eupergit surface is significantly more accessible for the substrate.

3.3.3.5. Storage stability

Storage stability is one of the most important parameters to be considered in enzyme immobilization as it affects overall productivity. Free and immobilized laccases were stored at 4ºC and at room temperature, with periodical sampling

and monitoring. After 3 months at 4ºC, the residual activities of free and both immobilized laccases were around 98%. The residual activity of free laccase amounted to 95% after 3 months at room temperature, while immobilized laccases showed negligible loss of activity, about 1.5-2%. In a previous research work, covalently immobilized laccase from *Myceliophthora thermophila* on Sepabeads EC-EP3 carriers retained about 96% of its initial activity after storage at 4ºC for 4 months (Kunamneni et al. 2008b).

3.3.4. Decolorization of the dye AG27 in PBRs by encapsulated and immobilized laccase on Eupergit supports

In order to evaluate the feasibility of the above described PBRs with encapsulated and immobilized laccase on Eupergit supports, these bioreactors were applied for the continuous removal of AG27, which was as model compound due to its easy monitoring. Moreover, consecutive continuous cycles of 4-5 h were carried out aiming to check the potential reusability of the immobilized laccases. Decolorization percentages attained after each continuous cycle for both biocatalysts in the corresponding PBRs are presented in Figure 3.9, along with the elimination levels observed during the control experiments.

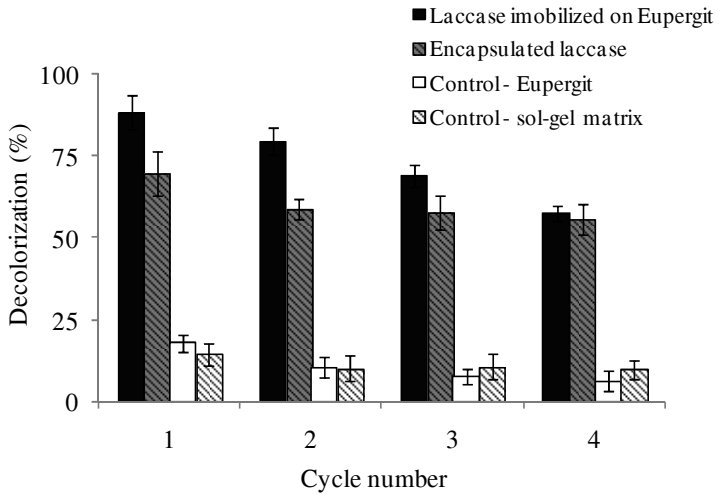

Figure 3.9. Decolorization percentages of AG27after each consecutive continuous cycle performed in the PBRs with both encapsulated and immobilized laccase on Eupergit supports.

As shown, a decolorization efficiency of 85% was found after the first cycle with laccase immobilized on Eupergit; however, the removal levels slightly

decreased after each consecutive operation. On the other hand, encapsulated enzyme provided lower decolorization yields but this efficiency was better conserved during the consecutive cycles: a decrease of approximately 10% was detected from the first to the second cycle but thereafter, the extent of decolorization was maintained.

The decrease on the decolorization efficiency by immobilized laccase on Eupergit supports during the different batches may be related to a slight enzyme inactivation produced by operational conditions and diffusion problems caused by dye or products adsorption onto the carrier surface (Rekuc et al. 2010). In order to verify this assumption, SEM pictures of the solid biocatalyst before and after 4 cycles of treatment of AG27 dye were analyzed (Figure 3.10). It was observed that the carriers retained its initial conformation, demonstrating their potential reusability. Nonetheless, small particles were observed on the support surface, which could be the dye or any degradation product adsorbed onto Eupergit beads.

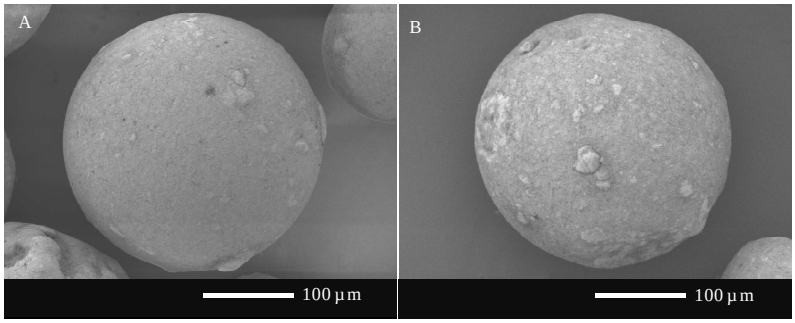

Figure 3.10. SEM pictures of Eupergit C 250L with immobilized laccase before (A) and after (B) four consecutive cycles performed in the PBR for the removal of AG27.

Anyhow, the enzymatic reaction was observed as the major contribution in the removal process, although this effect is likely to be dependent on the substrate: elimination percentages of AG27 of about 10-20% were found for the control experiments with both biocatalysts prepared with previously inactivated laccase (Figure 3.9). Moreover, negligible leaching of the immobilized enzyme was detected.

3.3.5. Continuous removal of E1, E2 and EE2 by encapsulated and immobilized laccase on Eupergit supports

3.3.5.1. Continuous removal in PBRs

The feasibility of both encapsulated and immobilized laccase on Eupergit C 250L supports on the continuous removal of E1, E2 and EE2 at initial concentration of 5 mg/L (each) in phosphate buffer (100 mM, pH 7) was evaluated in PBRs. For this purpose, the bioreactors previously applied for the decolorization of AG27 were used under identical conditions but now operating continuously for 8 h.

Adsorption of the substrates on the hydrogel and Eupergit supports might be an important role on the observed total removal of the target compounds, as reported by other authors dealing with the elimination of various compounds by immobilized laccases in similar carriers (Russo et al. 2008, Cabana et al. 2009).

The results found for each compound in the corresponding PBR with encapsulated or covalently immobilized laccase after 8 h of operation are shown in Table 3.6.

Table 3.6. Elimination percentages (%) of E1, E2 and EE2 attained by encapsulated and immobilized laccase on Eupergit supports in corresponding PBRs. Values in parenthesis correspond to elimination percentages during control experiments.

Target compound	Encapsulated laccase	Immobilized laccase on Eupergit supports
E1	55 (6)	65 (11)
E2	75 (14)	79 (22)
EE2	60 (11)	80 (20)

Despite the unfavorable kinetics found when using ABTS as substrate, still high removal yields of estrogens seem to be attained by both immobilized laccases: E1, E2 and EE2 were removed by 65-80% with covalently immobilized laccase, whereas 55-75% were obtained for encapsulated enzyme. The higher efficiency of laccase immobilized on Eupergit was presupposed since the enzyme is considerably more accessible when it is immobilized on a carrier rather than encapsulated in a matrix, once enzyme molecules are predominantly immobilized onto the external surface of the supports and consequently, exposed to the bulk solution. In addition, residual activity after 8-h operation was about 70 and 84% for encapsulated and immobilized laccase on Eupergit supports, respectively.

Nevertheless, considerable amount of removed estrogens was demonstrated to be caused by adsorption during control experiments: 6-14% of estrogens were eliminated when operating the PBR with hydrogel prepared with inactivated laccase; this effect was even higher when working with Eupergit carriers (11-22%). The adsorption levels appeared to be correlated with the hydrophobicity of each compound. The highest adsorption was seen for E2, which has the highest octanol-water partition coefficient ($logk_{ow}$ 3.9-4.0) (Suárez et al. 2008). This effect was probably favoured by the interaction between the hydroxyl groups of the estrogens and the epoxy residues of the support, once the block of unreacted epoxy residues (e.g. by methylamine) after immobilization procedure was not conducted (Hernaiz and Crout 2000).

Similar results were reported by Cabana et al. (2009) during the elimination of 5 mg/L of nonylphenol, bisphenol A and triclosan in a PBR by laccase covalently immobilized on Celite R-633 supports. The adsorption of these compounds on the support with inactivated laccase varied from 40 to 60% and their physical removal was also linked to the hydrophobicity of the different EDCs. Russo et al. (2008) observed adsorption of an anthraquinone dye on Eupergit supports with immobilized enzyme. These authors reported two different steps: during the first stage, dye adsorption on the carrier was extensive while enzymatic action was negligible; during the second step, the removal of the substrate was attributed to enzymatic conversion and an adsorption/desorption equilibrium was attained. A similar interaction between estrogens and the carrier could be expected here.

Although it cannot be asserted that similar degree of non enzyme-catalyzed elimination occurs during experiments with active enzyme (e.g. this elimination extent could be even higher because of the potential interaction between the substrates in the medium and adsorbed reaction products, or vice versa), E1, E2 and EE2 appeared to be eliminated mainly by the enzymatic action, which shows the immobilized laccase as a promising enzymatic system for the removal of estrogens. Moreover, the immobilization of laccase successfully allowed its application during continuous operation by its retention in the bioreactor, with negligible leaching over the whole continuous operation of the PBRs. Anyhow, the efficiency of the enzymatic treatment could be still enhanced by increasing the HRT of operation and even by evaluating other types of enzymatic reactor. These strategies were evaluated in the next phase of the research.

3.3.5.2. Continuous removal in a FBR

Previous experiments demonstrated the capability of immobilized enzyme to remove estrogens, obtaining the most favorable results as well as easier handling and application for laccase immobilized on Eupergit supports. Therefore, this biocatalyst was selected for the following work.

On the other hand, various disadvantages are associated to the use of PBRs. For instance: difficulties for the monitoring of laccase activity, passive or poor aeration, slow mass transfer, high pressure drop in the bed which can provoke its compactation and hence biocatalyst damage, possible formation of insoluble products would lead to clogging phenomena and formation of preferential channels (Gómez et al. 2007, Murry et al. 2002, O'Neill et al. 1971, Osma et al. 2010, Rekuc et al. 2009). With the goal of overcoming these challenges, a FBR was proposed for the application of immobilized laccase. Moreover, the continuous supply of air to maintain the fluidization of the bed was thought to improve the removal efficiency not only by favoring mass transfer and the maintenance of a homogenous reaction medium in the bioreactor, but also to enhance the reaction kinetics by increasing oxygen concentration, once it actively participates in the catalytic cycle of laccase (Wesenberg et al. 2003).

In a first experiment, the proposed FBR was operated for 10 h with a HRT of 50 min and was fed with a stock solution of estrogens of about 5 mg/L each (feed addition rate of 6 mg/L·h, except for E2 6.6 mg/L·h). The results demonstrated the efficient removal of these compounds in the steady-state operation of the FBR with removal percentages up to 92%, which corresponded to transformation rates higher than 5.4-5.9 mg/(L·h). Besides, residual activity after 10-h operation was up to 89%. Control experiments demonstrated that about 15-18% of estrogens elimination was due to adsorption on the support. However, even when considering that effect, the use of a FBR led to removal efficiencies higher than those found for PBRs. The improved aeration and dissolved oxygen concentration, which enhanced mass transfer together with more uniform flow distribution through the reactor, are believed to be key factors affecting the operation efficiency of the FBR. Similar beneficial effects were observed by González et al. (2001) after the comparative study of stirred tank and fluidized-bed bioreactors for the continuous biodegradation of phenol by immobilized *Pseudomonas putida*.

Aiming to study the feasibility of the enzymatic system to remove estrogens at lower initial concentration closer to environmental levels, as well as to evaluate

the stability of the biocatalyst during long-term operations, additional experiments were performed at concentrations of 100 and 10 µg/L for 15 and 16 days.

First, some conclusions can be drawn from the results of control experiments corresponding to these additional experiments. An increasing concentration of estrogens in the bioreactor effluent was observed during the initial operational period of experiments at 100 and 10 µg/L (about 5 hours and 2 days). It might be attributed to the saturation of the support by estrogens over that period of time. After that, a constant concentration value was observed, which represented about 5-9% of inlet concentration. This was related to an adsorption/desorption equilibrium to explain that the effluent concentration did not become equal to that determined in the influent over the rest of the operation (15-16 days). Furthermore, it can be concluded that after those periods of time the removal yields attained were mainly caused by the action of laccase.

Under this hypothesis, the amount of substrates per grams of support required to saturate the support was calculated; from this and considering the operational conditions in the PBR applied for the removal of estrogens at 5 mg/L, the 10 g of carrier used would be saturated within the first few minutes of operation. This proved that the levels of estrogens elimination observed after 8 h of control experiment in the PBR (Table 3.6) are related to the adsorption/desorption equilibrium, but not to a pending saturation of the carrier, although this hypothesis should be corroborated by a long-term operation of the PBR and a proper adsorption/desorption study. Anyhow, the situation of non-equilibrium would have a positive effect according to the findings reached by Rekuc et al. (2010): the authors reported a far more rapid dye removal by immobilized laccase than by free enzyme due to the fact that during the short period of non-equilibrium the dye transport to the carrier´s surface is boosted, which favors the rate of oxidation.

Results of removal rates and laccase activity profile for the experiment performed at 100 µg/L of estrogens (each) and a HRT of 50 min, which corresponded to a feed addition rate 120 µg/(L·h) are shown in Figure 3.11.

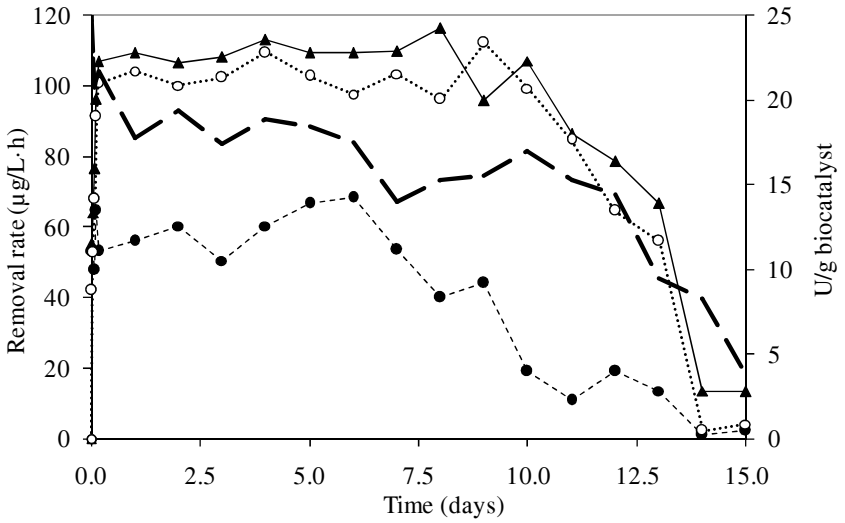

Figure 3.11. E1 (●), E2 (▲) and EE2 (○) removal rates (µg/L·h) attained during the operation of the FBR with immobilized laccase on Eupergit supports, with 100 µg/L of estrogens and 50 min of HRT. The biocatalyst activity profile during the operation (---) is also shown.

During the initial 10 days, the operation was steady and high removal rates of 110 µg/L·h were obtained for both E2 and EE2 (92% of removal), but E1 was eliminated with a lower rate of 50 µg/L·h. After this period, laccase activity decreased with the consequent drop in the removal efficiency. However, when the activity was 15 U/g, E2 and EE2 were still removed at high rates of 70-80 µg/L·h, which corresponded to removal percentages of 58-67%.

During the experiment carried out at 10 µg/L of the target compounds, the value of HRT was varied during the operation to study its effect and improve the removal efficiencies (see conditions in Table 3.1). Results of removal rates and laccase activity monitored during the assay are depicted in Figure 3.12.

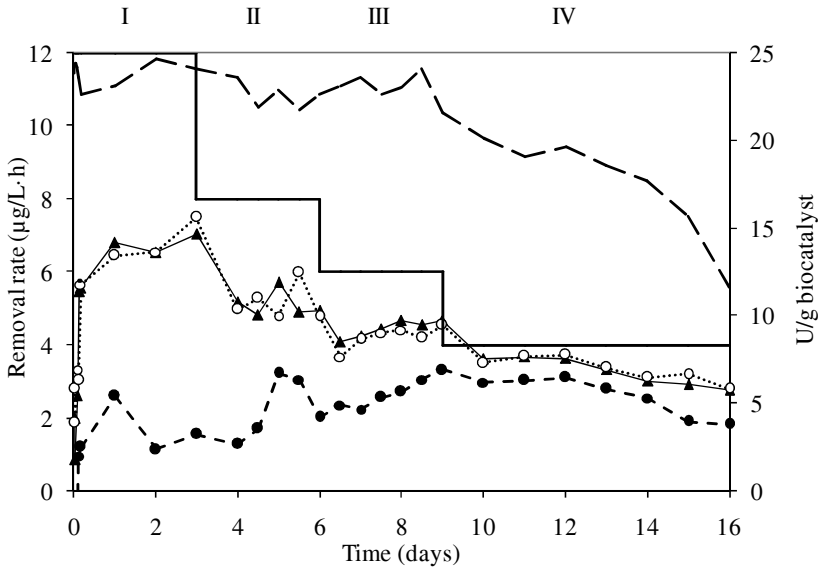

Figure 3.12. E1 (●), E2 (▲) and EE2 (○) removal rates (µg/L·h) attained during the operation of the FBR with immobilized laccase on Eupergit supports, with 10 µg/L of estrogens and variable HRT between 50 and 150 min (Table 3.1). Feed addition rates (—) and biocatalyst activity (---) profiles are also shown.

Between days 1 to 3 (stage I), when the HRT was maintained in 50 min, the percentages of removal were only 14, 54 and 55% for E1, E2 and EE2, respectively. With the goal of maximizing the removal capacity of estrogens in the reactor, HRT was progressively increased from 50 min to 150 min. In stage II with a HRT of 75 min and an addition rate of 8 µg/L·h, the removal levels increased to 2.2 µg/L·h (removal of 28%) for E1 and 5.2 µg/L·h (removal efficiency of 65%) for E2 and EE2. For a HRT of 100 min and addition rate of 6 µg/L·h (stage III), the concentration of the estrogens in the effluent slightly decreased; E1, E2 and EE2 were removed by 45, 74 and 70%, respectively (removal rates 2.7, 4.4 and 4.2 µg/L·h).

These percentages were significantly improved by the increase of HRT to 150 min and addition rate of 4 µg/L·h (stage IV). During the first three days of this stage, higher removal levels were obtained: 76% for E1 and more than 90% for E2 and EE2 (3.04 and 3.64 µg/L·h). However, after 13 days of operation a decrease in the capability of the system was observed in parallel to the biocatalyst inactivation, with removal values of 46% for E1 and 70% for E2 and EE2 (1.82 and 2.8 µg/L·h) at day 16.

The lower removal efficiencies found for E1 may be due to a possible lower laccase affinity for this substrate in comparison to E2 and EE2 or because of a poorer interaction with the carrier suggested by its lower hydrophobic character, especially during the first stage of operation when the support was still under saturation. Another explanation behind that may be the fact that E2 was reported to be transformed into E1 by activated sludge from sewage treatment plant or nitrifying activated sludge during treatment with *Sphingobacterium* sp. JCR5 (Skotnicka-Pitak et al. 2008) and even by enzymatic transformation with lignin peroxidase (Mao et al. 2009, 2010a, b), which would occur along with radical coupling reaction of the estrogens (Nicotra et al. 2004) and the formation of different degradation products (this aspect will be further investigated in Chapter 5). Probably, the transformation of E2 into E1 was the reaction pathway which predominated over the others when operating at low concentrations of estrogens with immobilized laccase, explaining the lower removal yields for E1 due to its concomitant removal and production.

With regard to the operational stability in these long-term experiments, the biocatalyst activity was maintained nearly constant for 10 days and after that, it decreased until 4 U/g at day 15 when the FBR was fed with a stock solution of 100 µg/L (Figure 3.11). However, in the following experiment (stock of 10 µg/L), laccase activity was maintained almost in its initial value for 12-13 days despite the changes made in the HRT. Thereafter, the enzyme activity decreased until 12 U/g at day 16 (Figure 3.12). The adsorption of substrates and/or products on the surface of the support with immobilized enzyme has been reported to cause diffusional problems which could lead to an apparent deactivation of the biocatalyst due to the poorer contact between the substrate and the immobilized enzyme (Rekuc et al. 2010).

Finally, the designed enzymatic system was also demonstrated to be effective not only on the removal of estrogens but also on the reduction of the associated estrogenic activity. A decrease of estrogenicity of approximately 90% was achieved under the best conditions evaluated during the last experiment (HRT 150 min, stage IV).

The continuous elimination of endocrine disrupting chemicals by immobilized laccase has been reported for bisphenol A by laccase on nylon membrane in non-isothermal bioreactors (Diano et al. 2007) and covalently bound onto alkylaminated controlled porosity glass column in combination with an electrolytic device (Iida et al. 2003). Cabana et al. (2007a, 2009) tested the elimination of bisphenol A, triclosan and nonylphenol by laccase immobilized

through the formation of cross-linked enzyme aggregates in a FBR and on Celite R-633 support in a PBR. The main advantage of the immobilization of laccase on Eupergit supports is the simplicity of the procedure as well as the reported chemical and mechanical stability of the beads and the fact that they are commercially available worldwide; besides, immobilization of enzymes on Eupergit is possible even without their purification (Katchalski-Katzir and Kraemer 2000, Knezevic et al. 2006).

Determination of the RTD

The hydrodynamic behavior of the FBR was characterized by determining the RTD. For this, the experiment using NaOH as tracer was performed at a HRT of 50 min. The results obtained by fitting the experimental data to the tank-in-series model (Levenspiel 1999) are shown in Figure 3.13.

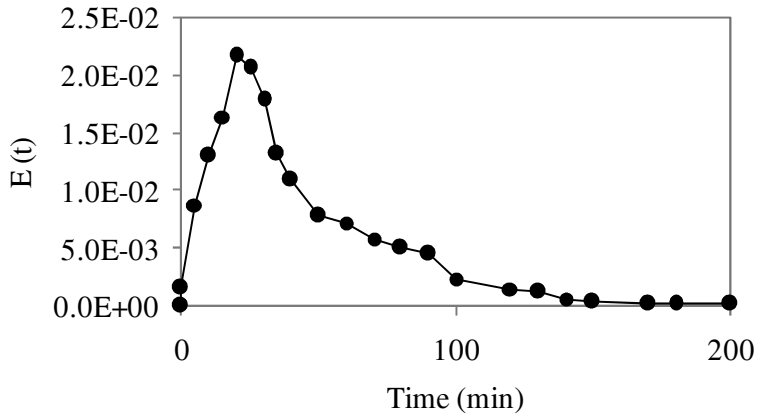

Figure 3.13. RTD obtained for the FBR.

From these experimental results, the number of tank reactors in series which would be analogous to the bioreactor was calculated: the hydraulic behavior of the bioreactor was found to be equivalent to a number of 1.7, which indicates a fairly similar behavior to a complete stirred tank reactor. The average residence time and the real reacting volume were calculated by means of appropriate correlations: values of 45 min and 200 mL were obtained, which are fairly similar to the theoretical values (50 min and 215 mL).

3.4. Conclusions

Immobilization of laccase from *Myceliophthora thermophila* was investigated aiming to facilitate its reuse and application in continuous operation. For this purpose, two different methods were evaluated: first, laccase was immobilized by its encapsulation in a sol-gel matrix based on silane compounds which hydrolyze and polymerize in the presence of the enzyme, resulting in a hydrogel with the laccase encapsulated inside; also, laccase immobilization was conducted by covalent bonding to commercial solid epoxy-activated acrylic supports, Eupergit C and Eupergit C 250L. Laccase was successfully immobilized by both procedures yielding bound protein percentages of up to 44-99 and 59-83% and activities 1-80 and 5-17 U/g, for covalently immobilized and encapsulated laccase, respectively. Eupergit C 250L provided higher immobilization efficiencies despite the lower oxirane groups content in comparison to that of Eupergit C, due to the presence of larger pores.

Enzymes immobilization often allows their stabilization and makes them less sensitive towards their environment. This assumption was corroborated by the biochemically characterization of the biocatalysts: the somewhat lower catalytic efficiency of laccase immobilized by both studied methods in comparison to that of free form (higher Michaelis-Menten constants were found for immobilized laccase) is balanced by its increased stability and broader operational window related to temperature, pH and chemical inhibitors. This effect was significantly higher in the case of encapsulated laccase probably due to the confinement of the enzyme in the hydrogel pores and the consequent enhancement of the order and compactness of the protein structure, whereas the enzyme is more accessible when it is immobilized on the surface of the Eupergit carriers.

Corresponding PBRs were designed for the application of encapsulated and immobilized laccase on Eupergit supports in continuous processes. First, these bioreactors were evaluated by their application on the removal of a synthetic dye used as model compound; moreover, the bioreactors were operated in consecutive continuous cycles aiming to verify the reusability of the biocatalysts. High decolorization yields were attained although in the case of laccase immobilized on Eupergit supports a slight decrease of the efficiency was observed over the consecutive cycles, potentially caused by the adsorption of the substrate and/or products. The proposed PBRs were applied for the continuous removal of E1, E2 and EE2, providing removal yields of 55-75 and 65-80% the encapsulated and the covalently immobilized laccase, respectively; 6-14 and 11-22% of elimination was attributed to adsorption of the substrates. Furthermore, the biocatalysts

retained about 70 and 84% of their initial activity after 8 h of continuous operation. These results present the immobilization procedures and the proposed PBRs as promising technology to favour the applicability of laccase in bioremediation processes.

Nevertheless, some limitations were identified related to the use of PBRs, such as poor or passive aeration, slow mass transfer and formation of preferential paths. These challenges were overcome by a FBR which was designed for the application of laccase immobilized on Eupergit supports on the removal of E1, E2 and EE2. With this system, higher removal yields were attained when operating under similar conditions than those in the PBRs: removal percentages up to 92% were found and a residual activity of 89% was detected after 10-h operation, probably because the fact that aeration enhances laccase action once it needs oxygen to initiate its catalytic cycle, and also due to the homogenous reaction mixture and improved mass transfer. The FBR was also proved to be effective on the removal of estrogens at concentrations as low as 100 and 10 µg/L, although HRTs of 150 min were needed to attain significant removal efficiencies. The system was fairly stable for more than 10 days of continuous operation. In addition, this technology was also demonstrated to be a promising system for the detoxification of influents containing estrogenic compounds once an estrogenicity reduction of 90% was achieved.

3.5. Acknowledgements

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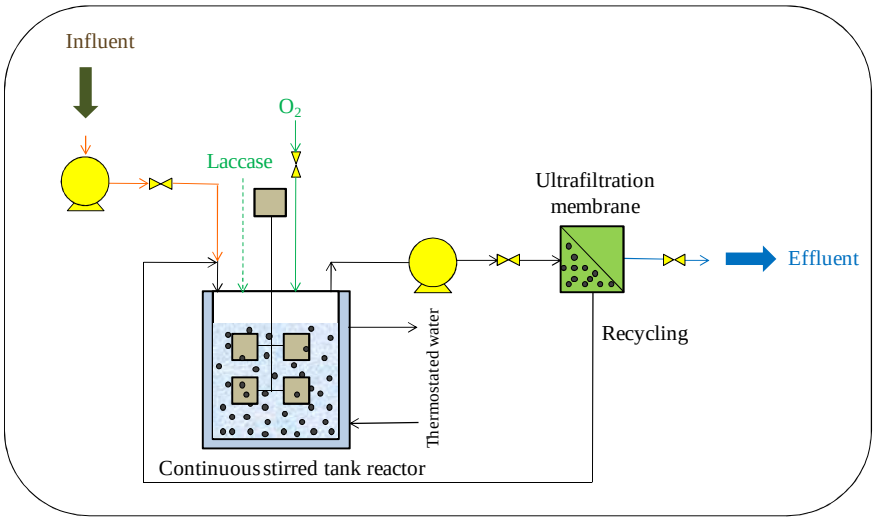
Chapter 4

Evaluating the application of an enzymatic membrane reactor for the continuous removal of estrogens by free laccase*



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L. Lloret, G. Eibes, G. Feijoo, M.T. Moreira and J.M. Lema, Application of response surface methodology to study the removal of estrogens in a laccase-mediated continuous membrane reactor, Biocatalysis and Biotransformation 31 (2013) 197-207.

L. Lloret, G. Eibes, M.T. Moreira, G. Feijoo and J.M. Lema, Removal of estrogenic compounds from filtered secondary wastewater effluent in a continuous enzymatic membrane reactor. Identification of transformation products, Environmental Science and Technology 47 (2013) 4536-4543.

L. Lloret, G. Eibes, G. Feijoo, M.T. Moreira and J.M. Lema, Degradation of estrogens by laccase from *Myceliophthora thermophila* in fed-batch and enzymatic membrane reactors, Journal of Hazardous Materials 213-214 (2012) 175-183.

Outline

4. Evaluating the application of an enzymatic membrane reactor for the continuous removal of estrogens by free laccase

4.1. Introduction

In Chapter 2 the ability of free laccase from *Myceliophthora thermophila* to remove estrogenic compounds: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), at neutral pH and in the absence of mediator was demonstrated batchwise. However, technology must be developed for the efficient application of the enzymatic treatment in continuous operation. In this sense, the enzyme was immobilized by different methods, as detailed in Chapter 3, once this is the most widely used strategy to facilitate the retention and thus the reuse of enzymes, which should be an imperative when a continuous prolonged operation is attempted (Cabana et al. 2007a, Fernández-Fernández et al. 2012, Kunamneni et al. 2008b, Osma et al. 2010). This allowed the application of the laccase-catalyzed transformation of estrogens in continuous bioreactors, as indicated in the same chapter. Nonetheless, although immobilized enzymes are more robust and resistant to environmental changes compared to free forms, a number of drawbacks related to diffusional limitatios or the difficulty of maintaining a constant activity level are evidenced when using immobilized enzymes (Diano et al 2007). Accordingly, these systems would require further study for their optimum operation.

In order to avoid these limitations, an alternative is developed and evaluated in the present chapter: the use of free laccase by means of an enzymatic membrane reactor (EMR) based on the use of a semipermeable membrane to ensure the retention of the biocatalyst. The main benefits of continuous operation with soluble enzymes are: i) the catalyst can be homogeneously distributed, thereby avoiding transport limitations, ii) simple determination and monitoring of enzymatic activity, and iii) no dependence on special immobilization know-how, besides reduction of costs and time associated to the immobilization procedure (Bódalo et al. 2001). Moreover, this bioreactor technology presents several advantages such as the possibility of easy addition of fresh enzyme in case of inactivation, high flow rates, reduced energy requirements, simple operation and control and straightforward scale-up (Eibes et al. 2007, Katchalski-Katzir et al. 1993, López et al. 2002, Rios et al. 2004).

With the goal of evaluating the possibility of applying an EMR for the continuous removal of estrogens, the first step of this work was focused on the development of fed-batch reactors to assess the influence of various variables:

aeration/oxygenation, laccase activity and contact time. This strategy was expected to permit not only the investigation of an interesting type of reactor configuration based on the feeding of substrates in pulses, but also to identify the main parameters affecting the process and to elucidate the potential best conditions for the following continuous experiments in the EMR. In fact, the obtained results were the basis for the subsequent EMR application for the preliminary evaluation of oxygen supply and hydraulic residence time (HRT) to optimize the continuous operation.

Nevertheless, the conventional method followed, "one factor at a time" approach, is not the most adequate for process optimization since it is very time-consuming, laborious and incomplete, being response surface methodology (RSM) an adequate alternative. This tool is extensively utilized in biotechnology once it is useful for designing experiments, building models, evaluating the significance of several variables besides determining optimum conditions; and also, it allows a reduction in the number of experiments to be undertaken (Claus et al. 2002, Ferreira et al. 2007, Roriz et al. 2009). For instance, RSM based on Box-Behnken design has been successfully applied to evaluate different multivariable systems, such as wastewater treatments (Fernández et al. 2011) or enzymatic removal of various pollutants (Bhattacharya and Banerjee 2008, Khouni et al. 2010, Tavares et al. 2009). Hence, this methodology was conducted in the present work with the aim of investigating the individual and interrelated effects of the studied parameters (enzyme activity, HRT and oxygenation rate) on the different responses evaluated: removal rate, removal rate per units of enzyme activity required and reduction of estrogenic activity.

With this, the feasibility of the bioreactor to remove estrogens at high concentration and from synthetic media (buffer solution containing the substrates) was proved. However, the design of a technology for the application of enzymatic remediation under more realistic conditions was still a challenge: the authors who attempted to test the laccase-catalyzed removal in real matrix only used spiked wastewaters and performed simple batch experiments (Auriol et al. 2007, 2008). In view of the promising elimination yields obtained before, the EMR was expected to be useful for the treatment of real wastewaters at a larger scale. Indeed, integrated systems including filtration are considered nowadays one of the most promising technologies used for the advanced treatment of secondary effluents, being both micro and ultrafiltration membranes techniques widely used as tertiary treatments (Acero et al. 2010, Muthukumaran et al 2011, Zhu et al. 2012). Therefore, a third and important objective was to investigate the possibility

of applying the EMR with free laccase to remove estrogens from polluted wastewaters at real environmental levels.

To sum up, the experimental work and results obtained during the process to accomplish the following main objectives are presented and discussed in this chapter: A) Operation of fed-batch reactors for preliminary evaluation of an EMR for the continuous removal of E1 and E2, B) Application of RSM to study the main parameters affecting the continuous removal of E1, E2 and EE2 in an EMR, and C) Evaluation of the potential applicability of an EMR for the continuous treatment of wastewaters containing E1, E2 and EE2.

4.2. Materials and methods

4.2.1. Chemicals and enzyme

E1, E2 and EE2 were purchased from Sigma-Aldrich. Deuterated 17β-estradiol (E2-d_4), with deuterium introduced in positions 2, 4 and 16, was purchased from Cambridge Isotope Laboratories. Stock solutions were prepared in methanol (J.T. Baker, HPLC grade, 99.8%). 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was purchased from Fluka. All other reagents used were of analytical grade.

The recombinant yeast *Saccharomyces cerevisae* was kindly provided by the Laboratory of Microbial Ecology and Technology (Labmet, Ghent University, Belgium). Laccase from *Myceliophthora thermophila* was supplied by Novozymes.

4.2.2. EMR configuration

Description of the bioreactor system

The EMR consisted of a stirred tank reactor (250 mL or 1.88 L) Biostat Q or MD (B. Braun-Biotech International) equipped with pH, temperature and oxygen sensors, which was coupled to an ultrafiltration polyethersulfone membrane (Prep/Scale-TFF Millipore) with a nominal molecular weight cutoff of 10 kDa, which permits the recycling of the enzyme to the tank. A scheme of the reactor is shown in Figure 4.1.

The additional volume held by the ultrafiltration unit and the interconnecting tubing was about 120 mL; therefore, the total volume of the systems was 370 mL or 2 L. PTFE tubing was used to prevent adsorption of the compounds to the inner surface of the tubing and the reaction mixture was continuously stirred (250 rpm)

using magnetic stirrers and Teflon-coated stir bars. The reactor was operated at 26ºC by circulating thermostated water through the tank chamber.

The influent containing a mixture of the estrogens was continuously fed into the tank by a peristaltic pump (Cole Parmer Instrument Co.), whereas laccase was only added in a single initial pulse. A second pump was used to circulate the reaction solution from the tank to the membrane, where the enzyme was retained and continuously returned to the reactor at a recycling/feed flow ratio 12:1. A valve located in the membrane module permitted the control of both effluent and recycling flow rates. An electrovalve located at the end of a flexible membrane tube controlled by a cyclic timer was used to inject oxygen with a pulsing flow of 1 bar for 30 s each pulse at the selected frequency.

At the start-up of the operation, the whole system (tank reactor, membrane and pipes) was filled with the reaction solutions and at time zero, the reaction was initiated by the addition of the enzyme into the reactor vessel. Corresponding controls lacking laccase were carried out and the results demonstrated that elimination of the substrates takes place only by laccase action.

Analysis of the membrane efficiency

The suitability of the selected membrane was demonstrated by circulating a solution of laccase through the membrane under certain conditions and measuring enzyme activity in permeate and retentate. No loss of activity in the permeate was observed, concluding that the membrane retained the enzyme efficiently.

Determination of the residence time distribution (RTD)

The determination of the RTD curve by stimulus-response experiments was carried out to characterize the hydraulic behavior of the 2-L EMR, as conducted for the fluidized bed reactor in Chapter 3. For this purpose, an aliquot of 1.5 mL of NaOH (20 M), which was used as tracer, was injected into the reaction tank during the operation of the bioreactor with distilled water. Afterwards, samples were periodically withdrawn from the bioreactor effluent to determine their conductivity and thus the NaOH concentration by corresponding calibration curves.

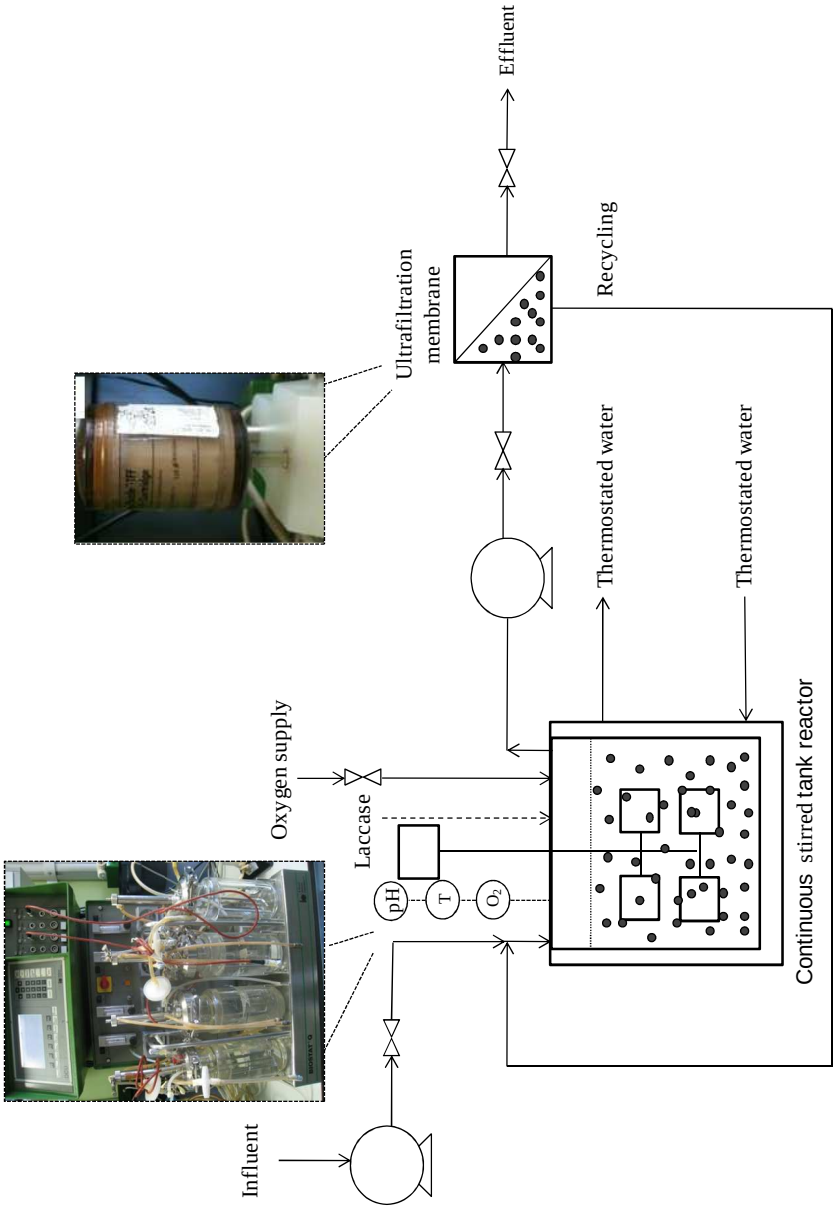


Figure 4.1. Scheme of the EMR used for the continuous laccase-catalyzed transformation of estrogens.

4.2.3. Experimental procedure

4.2.3.1. Objective A: Operation of fed-batch reactors for preliminary evaluation of an EMR for the continuous removal of E1 and E2

Fed-batch experiments

The oxidation of E1 and E2 in fed-batch mode was carried out in the 250-mL tank reactor used for the EMR design without the membrane coupling. The reaction medium consisted of a mixture of E1 and E2 (5 mg/L each) and a single initial pulse of laccase in 100 mM phosphate buffer (pH 7). Fed-batch addition of the estrogens (5 mg/L each pulse and compound) was carried during the course of the reaction. Temperature was controlled at 26ºC and continuous magnetic stirring at 250 rpm. Several experiments were conducted in order to evaluate the effect of different operational parameters: aeration and oxygenation, frequency of estrogens pulses and enzymatic activity.

An initial experiment was conducted with an initial laccase activity of 2,000 U/L and pulses of estrogens every hour. In a following step, the effect of aeration was analyzed by supplying 0.5 L air/min. Two additional strategies were investigated to improve removal efficiency: pulses of estrogens were performed every 2 h and pure oxygen was supplied periodically (1 bar for 30 s every hour); both strategies were assessed at two levels of laccase activity: 500 and 2,000 U/L. Finally, the combination of the conditions which provided the best results was evaluated.

Samples were withdrawn during the course of each operation to monitor laccase activity under standard conditions. Afterwards, reaction was stopped by the acidification of the samples to pH 2 by adding HCl to inactivate the enzyme, and samples were frozen until their analysis. Quantification of E1 and E2 was performed by high performance liquid chromatography (HPLC). Removal yields (%) of each compound were calculated at the end of each experiment by taking into account the total amount of each estrogen added during the experiment and the amount removed.

Preliminary continuous removal experiments

Continuous removal experiments of E1 and E2 were performed in the 370-mL EMR. The influent consisted of a mixture of estrogens at a concentration of 4 mg/L each. Considering the findings attained by the previous fed-batch experiments, experiments were performed at pH 7 (100 mM sodium phosphate buffer) with an initial laccase activity of 500 U/L. Moreover, two oxygen supply

strategies were assayed: i) a less regular addition of pulses every 1 h and ii) a more frequent addition every 30 min. The lowest oxygenation frequency was assayed at a HRT of 2 h (feed addition rate of 2 mg/L·h). In order to investigate the effect of the HRT and to improve the removal yields, the highest oxygenation frequency was evaluated at two different values of HRT: 2 and 4 h (feed addition rate 1 mg/L·h).

Samples were withdrawn at different periods from the reaction tank to measure the residual laccase activity. Also, samples were collected from the reactor influent and effluent to determine the associated estrogenic activity by yeast estrogen screen assay assisted by enzymatic digestion with lyticase (LYES), as well as the estrogens concentrations by HPLC. Both estrogens removal yields and reduction of estrogenic activity (%) were determined by comparing corresponding values for the influent and effluent. Removal rates of each target compound expressed in mg/(L·h) were also calculated.

4.2.3.2. Objective B: Application of response RSM to study the main parameters affecting the continuous removal of E1, E2 and EE2 in an EMR

Continuous removal experiments

While previous experiments were performed with E1 and E2, EE2 was now included in the study. Continuous removal experiments of the three compounds were performed in the 370-mL EMR. The influent consisted of a mixture of the estrogens at a concentration of 4 mg/L (each) in 100 mM phosphate buffer (pH 7); taking into account the results of preliminary experiments, the following variables were evaluated: enzyme activity (100-1,000 U/L), HRT (1-4 h) and oxygenation rate, conducting pulses of 1 bar for 30 s at selected frequencies between 30 min and 2 h, which ensure oxygenation rates in the range of 15-60 mg O_2/(L·h).

Samples were withdrawn from the reaction vessel in order to measure laccase activity, as well as from the reactor influent and effluent to determine both estrogenicity by LYES and estrogens concentrations by HPLC, in order to determine the following response variables under steady-state conditions: i) removal rate of the target compound mg/(L·h)), ii) removal rate per units used of enzyme (mg/(L·h·U)) and iii) reduction of estrogenic activity (%). The operation was monitored over 10-14 h of operation aiming to verify that steady-state conditions were achieved.

RSM and factorial design

A total of 15 experiments were carried out in order to study the influence of the evaluated factors (enzyme activity, HRT and oxygenation rate) on the continuous laccase-catalyzed removal of estrogens. The coded factors and levels and the experimental design are shown in Table 4.1.

Experimental results were analyzed with the software Statgraphics Centurion XVI (Statsoft Inc.) in order to link the response to the selected variables. The experimental Box-Behnken design and analysis of variance (ANOVA) were performed with the same program. The quality of the models was expressed by the coefficients of correlation (R^2 and $adjR^2$) and statistical significance was checked by the *F*-test. Finally, three-dimensional response surfaces were plotted by Matlab R2012a software (MathWorks Inc.).

Table 4.1. Box-Behnken factorial design, coded values and levels evaluated during the continuous removal of E1, E2 and EE2 in the 370-mL EMR.

Runs	Factors		
	x_1 Enzyme*	x_2 HRT*	x_3 Oxygenation*
1	-1	-1	-0.33
2	+1	-1	-0.33
3	-1	+1	-0.33
4	+1	+1	-0.33
5	-1	-0.33	-1
6	+1	-0.33	-1
7	-1	-0.33	+1
8	+1	-0.33	+1
9	-0.11	-1	-1
10	-0.11	+1	-1
11	-0.11	-1	+1
12	-0.11	+1	+1
13	-0.11	-0.33	-0.33
14	-0.11	-0.33	-0.33
15	-0.11	-0.33	-0.33

*Coded levels:
Enzyme activity (x_1): -1 (100 U/L); -0.11 (500 U/L); +1 (1,000 U/L)
HRT (x_2): -1 (1 h); -0.33 (2 h); +1 (4 h)
Oxygen (x_3): -1 (15 mg O_2/(L·h)); -0.33 (30 mg O_2/(L·h)); +1 (60 mg O_2/(L·h))

The response evaluated was related to the selected variables by a quadratic model as shown in Equation (4.1):

$$Y = \alpha_0 + \alpha_1 \cdot x_1 + \alpha_2 \cdot x_2 + \alpha_3 \cdot x_3 + \alpha_{12} \cdot x_1 \cdot x_2 + \alpha_{13} \cdot x_1 \cdot x_3 + \alpha_{23} \cdot x_2 \cdot x_3 + \alpha_{11} \cdot x_1^2 + \alpha_{22} \cdot x_2^2 + \alpha_{33} \cdot x_3^2 \quad (4.1)$$

where Y is the predicted response; x_1, x_2 and x_3 are the coded levels of the factors; α_0 is the intercept term; α_1, α_2 and α_3 the coefficients for linear effects; α_{12}, α_{13} and α_{23} are the cross-coefficients; and α_{11}, α_{22} and α_{33} are the quadratic coefficients. The choice of this response is justified by the fact that relatively few experimental

combinations of variables are required to estimate the potentially complex response function.

4.2.3.3. Objective C: Evaluation of the potential applicability of an EMR for the continuous treatment of wastewaters containing E1, E2 and EE2

Continuous removal experiments to evaluate the EMR real applicability

Finally, additional work was performed aiming to evaluate the potential applicability of the designed enzymatic bioreactor for the removal of estrogens at environmental concentrations and the treatment of real wastewaters. For this purpose, the 2-L EMR was operated at a HRT of 4 h and a frequency addition of oxygen pulses of 30 min, which accounted for an oxygenation rate of 60 mg O_2/(L·h); besides, an initial laccase activity of only 100 U/L was attempted in order to reduce enzyme requirements.

The following sequence of experiments was performed: the continuous removal of estrogens at high concentrations (to evaluate the effect of change of scale with respect to the previous experiments) and low levels (to test the feasibility of the enzymatic system to remove low concentrations of the pollutants) from buffered solutions, the treatment of spiked real wastewater (to study potential matrix effects), and finally, the treatment of wastewater at environmental concentrations. The sequence of experiments and corresponding operational conditions are detailed in Table 4.2.

Table 4.2. Experiments performed in the 2-L EMR for the continuous removal of E1, E2 and EE2 and corresponding operational conditions. The bioreactor was operated a HRT of 4 h, laccase activity 100 U/L and 60 mg O_2/(L·h)

Experiment	Matrix	Estrogens concentration	Feed addition rate
1	Phosphate buffer (100 mM, pH 7)	4 mg/L	1 mg/(L·h)
2	Phosphate buffer (100 mM, pH 7)	100 µg/L	25 µg/(L·h)
3	Spiked real wastewater	100 µg/L	25 µg/(L·h)
4	Real wastewater	Environmental concentrations: 0.29-1.52 ng/L	0.07-0.38 ng/(L·h)

The reactor was operated for 100 h in order to demonstrate the viability of the technology and the stability of both the membrane and the biocatalyst.

Samples were withdrawn at different periods from the reaction vessel in order to measure laccase activity, as well as from the influent and effluent to determine both the estrogenic activity by LYES and the estrogens concentrations. The performance of the technology was evaluated in terms of the removal percentage (%) of both target compounds and estrogenicity, as well as by the estrogens removal rates expressed in mg, µg or ng/(L·h).

Sampling

Samples preparation and quantification of estrogens by the appropriate analytical technique was conducted according to the range of estrogens concentration:
i) HPLC was used for the analysis of approximately 1-mL samples withdrawn during experiments performed at concentrations of mg/L;
ii) 20-mL samples were collected from the EMR influent and effluent during experiments at concentrations of µg/L and diluted in distilled water (pH 2 adjusted with HCl) in a final volume of 100 mL. Afterwards, solid phase extraction (SPE) was performed for subsequent derivatization and analysis by gas-chromatography mass spectrometry (GC-MS);
iii) For the quantification of estrogens in those samples taken during the experiment performed at environmental concentrations (ng/L), samples of 2-L (samples from EMR influent) or 5-L (EMR effluent) were withdrawn for subsequent SPE as well as further concentration for the analysis by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS-MS).

Characteristics of the real wastewater used

The real wastewater used was collected from the outlet of the secondary clarifier of the municipal wastewater treatment plant of Calo-Milladoiro (Ames, Spain). This water was filtered (0.45 µm) to remove particulate matter and suspended solids to avoid undefined biological transformation by bacteria and other microorganisms or even adsorption of the target compounds, and thus examining the sole role of the laccase. Thereafter, it was stored at 4ºC until its use. The wastewater was analyzed according to Standard Methods (1999) and quantification of estrogens was carried out by LC-APCI-MS-MS; the main characteristics are summarized in Table 4.3.

Table 4.3. Characteristics of filtered secondary effluent.

Estrogens	ng/L	Carbon and nitrogen content	Concentration (mg/L)
E1	1.52	inorganic carbon	5.6
E2	0.29	total organic carbon	5.6
EE2	0.74	total nitrogen	7.8
COD (mg O_2/L)	33.80	inorganic nitrogen	3.0
pH	6.85	total Kjendahl nitrogen	4.8
Anions	Concentration (mg/L)	Cations	Concentration (mg/L)
NO^{2-}	0.1	Na^{+}	38.2
Br^{-}	0.0	NH^{4+}	0.0
NO^{3-}	9.9	K^{+}	9.0
PO_4^{3-}	2.2	Mg^{2+}	2.9
SO_4^{2-}	21.3	Ca^{2+}	11.9

4.2.4. Determination of estrogens concentration

4.2.4.1. Quantification of estrogens at level of mg/L by HPLC

Determination of E1, E2 and EE2 concentration by HPLC was performed by applying the method described in Chapter 2.

4.2.4.2. Quantification of estrogens at level of µg/L

SPE of samples

Samples SPE was carried out with 60 mg OASIS HLB cartridges (Water closet) previously conditioned with 3 mL ethyl acetate, 3 mL methanol and 3 mL distilled water (pH 2). The cartridges were then dried with nitrogen for 45 min and eluted with 3 mL ethyl acetate.

GC-MS analysis

800 µL of the extract obtained by SPE was taken and 200 µL BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) was added for the derivatization of the

species. Afterwards, GC-MS analysis was performed by using a Saturn 2100T (Varian) system with a CP Sil column (CP Sil 8 CB-MS low bleed (30 m x 0.25 mm x 0.25 µm). The analytical conditions are detailed in Table 4.4.

Table 4.4. Equipment and conditions for the determination of estrogens by GC-MS.

Chromatographic parameters	
Split-splitless injector	
Splitless time	2 min
Injection temperature	280ºC
Gas flow (He)	1 mL/min
Injector (volume)	1 µL
Solvent	Ethyl acetate
Temperature program	
Initial temperature	70ºC
Initial time	2 min
1st ramp, Temperature 1	25ºC/min, 150ºC
2nd ramp, Temperature 2	3ºC/min, 200ºC
3rd ramp, Temperature 3	8ºC/min, 280ºC
Time 3	5 min
Mass Spectrometry	
Ionization mode	Electronic impact
Filament current	10 µA
Ion ramp temperature	220ºC
Transference line temperature	280ºC
Voltage	1700 – 1750 V
Scan velocity	1s/scan
Mass spectrum	50-550 m/z

Limits of quantification (LOQ) of the system were 10 µg/L for E1 and E2 and 40 µg/L for EE2. Anyhow, the concentration of the samples allowed the quantification of the compounds at concentrations of 1.5 and 6 µg/L, respectively.

4.2.4.3. Quantification of estrogens at level of ng/L

SPE and further concentration of samples

SPE of samples from experiments conducted at environmental concentrations was performed as described above. Then, additional concentration was carried out as follows: 2 mL of the 3-mL extract resulting from SPE were evaporated under nitrogen stream previously to resuspension in methanol; finally, further concentration by nitrogen was conducted to obtain 50 µL-samples.

LC-APCI-MS-MS analysis

Determination of E1, E2 and EE2 concentration by LC-APCI-MS-MS was performed by applying the method described in Chapter 3.

In this chapter, E2-d_4 was used as internal standard throughout the analytical procedure in order to corroborate the removal results of E1, E2 and EE2. The deuterated compound was added to the samples before the SPE (5 ng, which meant concentrations before SPE of 2.5 and 1 ng/L in the EMR influent and effluent samples, and 66.66 µg/L after concentration for subsequent LC/MS/MS analysis). The quantification of the estrogenic compounds was carried out using calibration curves obtained by plotting the ratio of analyte peak area/E2-d_4 peak area versus the analyte concentration; calibration standards contained increasing amounts of the analytes in the range 0.5-70 µg/L, and a fixed concentration of the surrogate of 66.66 µg/L. The surrogate presented the following transitions: 259.2 → 161.1 and 259.2 → 135.0 and retention time of 9.5 min.

LOQs were 0.5 µg/L; however, the concentration of the samples provided a reduction of the limits to 0.02 ng/L for the EMR influent samples and 0.01 ng/L for the samples withdrawn from the effluent.

4.2.5. Determination of laccase activity

Laccase activity was determined spectrophotometrically using ABTS under standard conditions as described in Chapter 2.

4.2.6. Determination of estrogenic activity

Estrogenic activity was determined by the LYES procedure detailed in Chapter 2.

4.3. Results and discussion

4.3.1. Objective A: Operation of fed-batch reactors for preliminary evaluation of an EMR for the continuous removal of E1 and E2

4.3.1.1. Fed-batch removal of E1 and E2

Fed-batch reactors were operated for the laccase-catalyzed removal of E1 and E2 and a number of operational parameters: aeration/oxygenation, substrates feeding rate and enzyme activity, were evaluated with the aim of elucidating the most suitable conditions for the subsequent development of the continuous reactor. The sequence of the experiments performed along with the removal percentages obtained is included in Table 4.5.

Table 4.5. Experiments performed in fed-batch reactors for the removal of E1 and E2, corresponding operational conditions and removal yields obtained.

Experiment	Aeration/ Oxygenation	Estrogens pulses frecuency (h)	Initial laccase activity (U/L)	E1 removal (%)	E2 removal (%)
1	--	1	2,000	70.9	91.5
2	Aeration	1	2,000	71.2	91.6
3	--	2	2,000	92.4	93.8
4	--	2	500	90.5	92.0
5	Oxygenation	1	2,000	90.1	93.5
6	Oxygenation	1	500	83.7	90.2
7	Oxygenation	2	500	94.1	95.5

Effect of frequency of estrogens addition

As expected in view of the results attained batchwise, E1 and E2 were removed by laccase within 1 h with significant yields, but residual estrogenic activity is still detected (see Chapter 2). Therefore, strategies must be applied aiming to improve the transformation efficiencies achieved in Experiment 1 (Table 4.5).

The first strategy proposed in order to enhance the removal percentages was the decreasing of estrogens addition frequency to 2 h (Experiment 3, Table 4.5) with the objective of increasing the contact time between the enzyme and substrates added in each pulse; concentration profiles during the experiment are shown in Figure 4.2. With this, the enzyme was capable to transform both compounds at percentages 92.4 and 93.8% for E1 and E2, respectively. These results were expected since only 2 pulses were performed during the operation, and thus the enzyme was exposed to lower loads of substrates.

These findings suggest that HRT values of 2 h or even higher would be necessary when operating a continuous bioreactor aiming to attain significant removal rates. Indeed, HRT effect was preliminarily evaluated in the 370-mL EMR and subsequently evaluated through response surface analysis, as it will be exposed below.

Effect of aeration/oxygenation

Oxygen actively participates in the catalytic cycle of laccase. It acts as the electron acceptor, which is in turn reduced to water whilst the oxidation of the laccase takes place for the subsequent oxidation of the substrate (Wesenberg et al. 2003). Thus, oxygen addition was conducted aiming to improve the kinetics of the reaction and thus, the removal yields; two strategies were evaluated aiming to increase the oxygen concentration inside the reactor, continuous aeration and addition of pure oxygen by pulses.

The supply of extra air in a continuous flow (Experiment 2, Table 4.5) exerted no significant effect in comparison with the non-aerated experiment (Experiment 1, Table 4.5), probably because agitation was sufficient to maintain the concentration of dissolved oxygen close to the saturation values and air addition did not increase these levels. However, when oxygen was supplied, the concentration of dissolved oxygen reached maximum values of 35-38 mg/L when pulses were applied. In fact, under oxygen pulses (Experiment 5, Table 4.5), the removal percentages increased, reaching an elimination of 90.1 and 93.5% for E1 and E2, respectively; concentration profiles for that experiment are shown in Figure 4.2. It can be observed than the enhancement was more noticeable for E1, and that similar removal yields were attained after only 1 h of treatment under oxygenated conditions than those found without oxygen supply after 2 h.

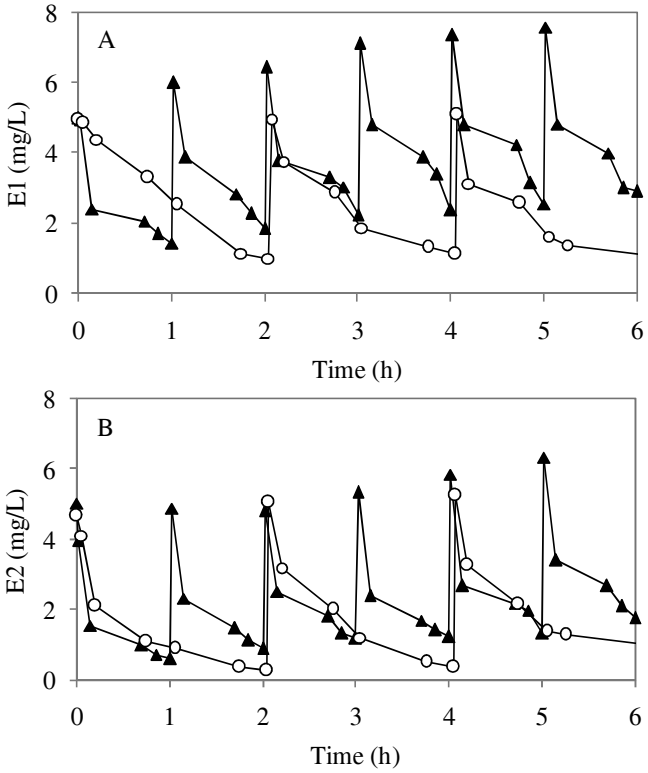


Figure 4.2. E1 (A) and E2 (B) concentration profiles during fed-batch experiments: Experiment 3 (○) and 5 (▲) from Table 4.5.

In view of these results, oxygenation rate was preliminarily evaluated in continuous experiments in the 370-mL EMR and further investigated and deeper discussed by response surface analysis.

Effect of enzyme activity

The primary objective of any engineering design should be to minimize the cost to make the process feasible. Consequently, a 4-fold reduction of the initial enzyme activity was considered once enzyme costs might be considered among the most representative of the technology and could limit its application (Ibrahim et al. 2001). A value of 500 U/L was tested for non aerated/oxygenated experiments with pulses of estrogens every 2 h as well as for oxygenated experiments with pulses every 1 h (Experiments 4 and 6, respectively, Table 4.5). Thus, the effect of initial laccase activity can be analyzed by comparing two pairs of experiments: Experiments 3-4 and 5-6. The results evidenced lower removal yields, but this reduction was not as significant as expected. For example, E1 and

E2 removal decreased from 92.4 to 90.5% and from 93.8 to 92.0%, respectively, for Experiments 3-4 (Figure 4.3).

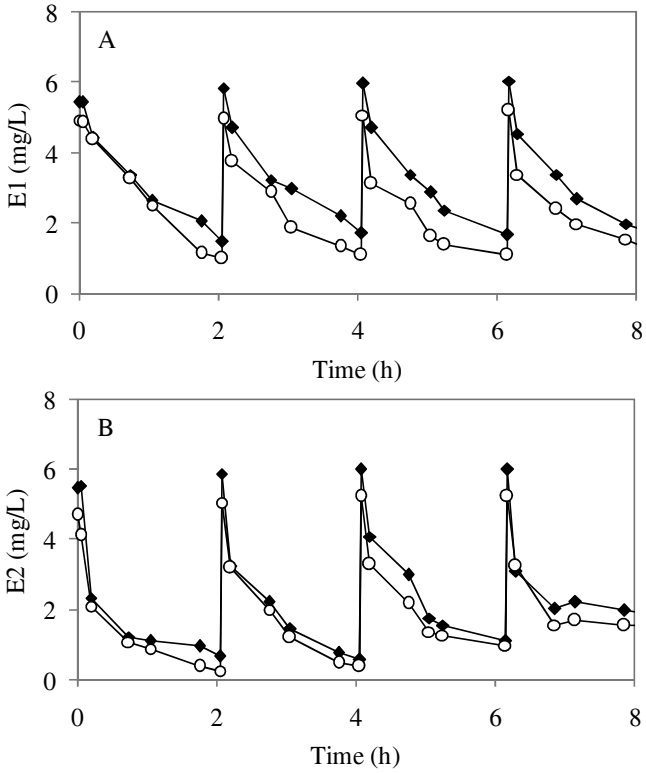


Figure 4.3. E1 (A) and E2 (B) concentration profiles during fed-batch experiments: Experiment 3 (○) and 4 (◆) from Table 4.5.

It was demonstrated that the enzyme activity can be down to 500 U/L without compromising the efficiency of the system; nonetheless, further optimization was done by response surface analysis.

Fed-batch operation under the best conditions

Finally, the strategies selected throughout this study were combined in order to obtain an efficient operation of the fed-batch reactor. The operational conditions were: compounds pulses every 2 h, oxygen supply by pulses every 1 h and 500 U/L of laccase activity (Experiment 7, Table 4.5); concentration profiles obtained are shown in Figure 4.4.

Under these selected variables, high degradation extent of E1 and E2 was observed during 8 h of operation, with transformation percentages of 94.1 and 95.5%, respectively.

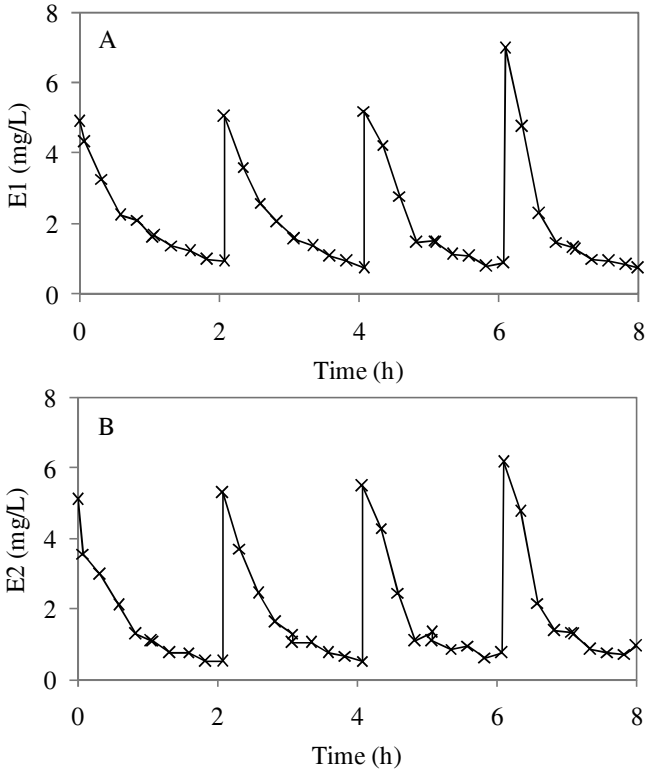


Figure 4.4. E1 (A) and E2 (B) concentration profiles during fed-batch experiment under the selected conditions (Experiment 7 from Table 4.5).

4.3.1.2. Preliminary experiments for the continuous removal of E1 and E2

Various experiments were performed in the 370-mL EMR for the continuous removal of E1 and E2; in this step, the operational parameters that are likely to affect the efficiency of the system were evaluated: laccase activity, oxygen supply and HRT. The experiments performed and operational conditions as well as the removal percentages results obtained are shown in Table 4.6. Besides, removal rates determined during each operation are depicted in Figure 4.5.

Table 4.6. Preliminary experiments performed in the 370-mL EMR for the continuous removal of E1 and E2, operational conditions and removal yields obtained.

Experiment	Feed addition rate (mg/L·h)	HRT (h)	Initial laccase activity (U/L)	O_2 pulses frequency	E1 removal (%)	E2 removal (%)
1	2	2	500	1 h	58.9	65.9
2	2	2	500	30 min	68.4	80.6
3	1	4	500	30 min	95.6	>99*

*Concentration of estrogens below detection limits.

Although previous experiments showed an important effect of the oxygen supply, the frequency of oxygenation was not evaluated. Hence, two different strategies for the oxygenation were tested: oxygen supply every 1 h, as assayed during fed-batch experiments, and oxygen supply every 30 min. As expected, higher oxygen supply led to a 10-15% of enhancement: values 68.4 and 80.6% were found for E1 and E2, respectively, which corresponded to removal rates 1.34-1.61 mg/(L·h) (Experiment 2, Table 4.6). It is interesting to highlight that enzymatic activity did not decrease throughout the experiment and thus, it was not affected by the oxygenation.

HRT is related to the desired conversion of pollutants, which is directly affected by the substrates concentration: a higher feeding rate (lower HRT) could lead to a faster but less efficient process. HRT values of 2 and 4 h were selected in order to study that effect and aiming to improve the enzymatic treatment efficiency. At the highest HRT assayed, E1 was removed up to 95.6 and E2 was not detected in the effluent; however, removal rates decreased to 0.96-0.98 mg/(L·h) (Experiment 3, Table 4.6).

There is no extensive information concerning *in vitro* degradation of estrogens in continuous bioreactors. For instance, Blánquez and Guieysse (2008) investigated the biodegradation of E2 by *Trametes versicolor* cultures and evidences of laccase involvement were found: removal rates (about 0.16 mg/(L·h)) in the same order of magnitude as those achieved by fungal enzymes, bacterial communities or bacterial isolates were reported. In this research,

estrogens transformation rates by laccase almost 10 times higher were achieved by using a continuous EMR.

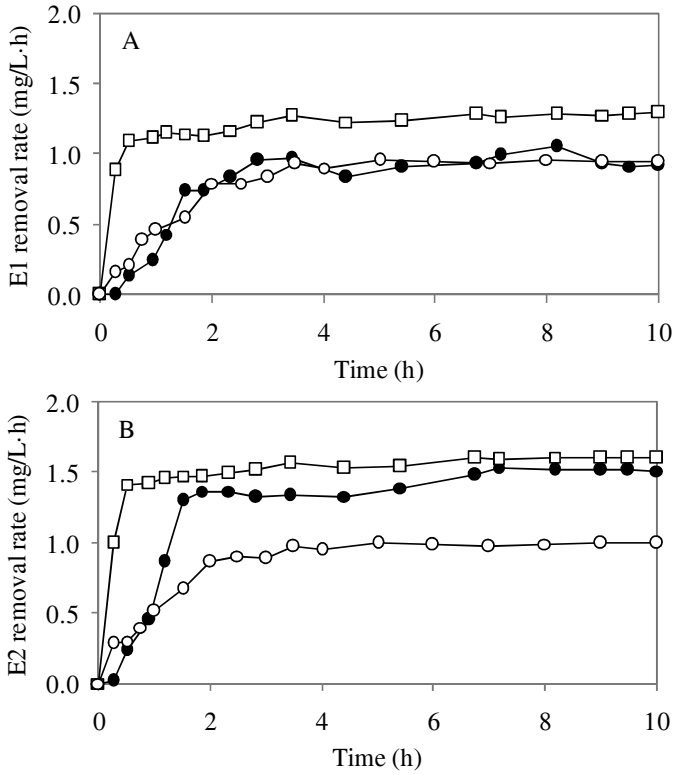


Figure 4.5. E1 (A) and E2 (B) removal rates attained during preliminary experiments in the 370-mL EMR: Experiments 1 (●), 2 (□) and 3 (○) from Table 4.6.

Furthermore, 97% of reduction of estrogenicity was measured for the best conditions evaluated. This indicates the potential of the developed technology not only for the removal of the estrogenic compounds but also for the detoxification of the effluent.

4.3.2. Objective B: Application of RSM to study the main parameters affecting the continuous removal of E1, E2 and EE2 in an EMR

Response surface analysis was performed aiming to adequately study the effects of the main parameters evaluated (laccase activity, HRT and oxygen supply) avoiding the "one factor at time" optimization.

Based on the Box-Behnken and RSM approach, the methodology involves three major steps: i) performance of statistically designed experiments, ii)

estimation of the coefficients of a mathematical model with the corresponding analysis of variance (ANOVA) and iii) prediction of the response and validation of the model (Box and Behnken 1960).

A complete application of RSM following those steps was carried out considering the removal rate (mg/(L·h)) as response variable (Y_1). Afterwards, the methodology was also applied for the following responses: Y_2 (mg/(L·h·U)), removal rates per units used of enzyme, and Y_3 (%) as reduction of estrogenic activity.

4.3.2.1. Analysis of removal rate

Fitting the model and analysis of variance

According to the Box-Behnken design, 15 different experiments corresponding to the continuous operation of the enzymatic reactor were performed. The levels of the factors along with the experimental results (actual values) are presented in Table 4.7.

In preliminary experiments, 500 U/L of laccase, HRT of 2 and 4 h and pulses of oxygen every 30 min and 1 h (which corresponded to oxygenation rates 60 and 30 mg O_2/(L·h), respectively) were assayed. The levels studied now were selected according to those previous results: 100 and 1,000 U/L were also assayed to minimize the consumption of enzyme or to improve the removal when operating at lower oxygenation and HRTs; a HRT of 1 h was also considered to maximize the removal rates; and oxygen supply of 15 mg O_2/(L·h) was included in the study to investigate its effect over a broader range. Moreover, EE2 was included in the study.

Table 4.7. Box-Behnken factorial design, comparison of experimentally obtained and theoretically predicted values for E1, E2 and EE2 continuous removal rates (Y_1, mg/(L·h)) and percentages of estrogenic activity reduction attained.

Runs	Factors			Removal rate (mg/(L·h))						Estrogenic activity reduction (%)
				E1		E2		EE2		
	x_1 Enzyme*	x_2 HRT*	x_3 Oxygenation*	Actual value	Predicted value	Actual value	Predicted value	Actual value	Predicted value	
1	-1	-1	-0.33	2.11	2.11	2.51	2.49	2.50	2.49	55
2	+1	-1	-0.33	2.71	2.69	3.11	3.09	3.11	3.15	71
3	-1	+1	-0.33	0.76	0.77	0.87	0.89	0.89	0.86	79
4	+1	+1	-0.33	0.95	0.95	1.00	1.02	0.99	0.99	96
5	-1	-0.33	-1	1.01	1.01	1.31	1.29	1.19	1.20	54
6	+1	-0.33	-1	1.46	1.47	1.77	1.75	1.76	1.72	78
7	-1	-0.33	+1	1.22	1.21	1.48	1.50	1.41	1.44	65
8	+1	-0.33	+1	1.62	1.62	1.90	1.91	1.85	1.85	84
9	-0.11	-1	-1	2.27	2.27	2.60	2.65	2.72	2.72	61
10	-0.11	+1	-1	0.79	0.77	0.90	0.89	0.89	0.92	84
11	-0.11	-1	+1	2.45	2.46	2.90	2.89	3.02	2.99	64
12	-0.11	+1	+1	0.91	0.91	0.99	0.97	0.98	0.98	93
13	-0.11	-0.33	-0.33	1.20	1.19	1.38	1.37	1.33	1.34	63
14	-0.11	-0.33	-0.33	1.21	1.19	1.36	1.37	1.31	1.34	64
15	-0.11	-0.33	-0.33	1.17	1.19	1.36	1.37	1.37	1.34	62

*Coded levels:
Enzyme activity (x_1): -1 (100 U/L); -0.11 (500 U/L); +1 (1,000 U/L)
HRT (x_2): -1 (1 h); -0.33 (2 h); +1 (4 h)
Oxygenation (x_3): -1 (15 mg O_2/(L·h)); -0.33 (30 mg O_2/(L·h)); +1 (60 mg O_2/(L·h))

Experimental data of removal rates of E1, E2 and EE2 were fitted to the second order polynomial model presented in Equation (4.1); the empirical models depicted in Equations (4.2) to (4.4) were obtained:

$$Y_1(E1) = 0.909 + 0.185 \cdot x_1 - 0.774 \cdot x_2 + 0.082 \cdot x_3 - 0.099 \cdot x_1 \cdot x_2 - 0.013 \cdot x_1 \cdot x_3 - 0.013 \cdot x_2 \cdot x_3 + 0.060 \cdot x_1^2 + 0.685 \cdot x_2^2 + 0.028 \cdot x_3^2 \quad (4.2)$$

$$Y_1(E2) = 1.024 + 0.179 \cdot x_1 - 0.932 \cdot x_2 + 0.079 \cdot x_3 - 0.117 \cdot x_1 \cdot x_2 - 0.012 \cdot x_1 \cdot x_3 - 0.041 \cdot x_2 \cdot x_3 + 0.109 x_1^2 + 0.755 \cdot x_2^2 + 0.089 \cdot x_3^2 \quad (4.3)$$

$$Y_1(EE2) = 0.975 + 0.189 \cdot x_1 - 0.966 \cdot x_2 + 0.077 \cdot x_3 - 0.132 \cdot x_1 \cdot x_2 - 0.028 \cdot x_1 \cdot x_3 - 0.052 \cdot x_2 \cdot x_3 + 0.066 \cdot x_1^2 + 0.846 \cdot x_2^2 + 0.100 \cdot x_3^2 \quad (4.4)$$

where Y_1 corresponds to the elimination rate (mg/(L·h)) of each estrogen at the steady-state, and x_1, x_2 and x_3 are the coded values of the studied variables: laccase activity, HRT and oxygenation rate. Positive values indicate synergism whereas negative values indicate antagonism. Predicted values provided by those models are shown in Table 4.7.

The statistical significance of the proposed models was investigated by ANOVA (Table 4.8). Moreover, pareto charts were plotted from the obtained results to analyze the standardized effect of each factor and their interdependence on the removal of the estrogens (Figure 4.6).

Table 4.8. Analysis of variance (ANOVA) of regression models of E1, E2 and EE2 removal rates (Y_1, mg/(L·h)).

Source	Degrees of freedom	Sum of squares			Mean square			F-value			p-value		
		E1	E2	EE2	E1	E2	EE2	E1	E2	EE2	E1	E2	EE2
x_1	1	0.249	0.233	0.257	0.249	0.233	0.257	569.90	202.46	131.57	0.000	0.000	0.000
x_2	1	4.544	6.603	7.072	4.544	6.603	7.072	10368.32	5742.97	3624.43	0.000	0.000	0000
x_3	1	0.049	0.047	0.045	0.049	0.047	0.045	112.26	41.00	2325	0.000	0.001	0.005
$x_1 \cdot x_2$	1	0.043	0.061	0.074	0.043	0.061	0.074	98.70	52.75	38.00	0.000	0.001	0.002
$x_1 \cdot x_3$	1	0.001	0.001	0.003	0.001	0.001	0.003	1.89	0.50	1.66	0.228	0.513	0.254
$x_2 \cdot x_3$	1	0.001	0.007	0.012	0.001	0.007	0.012	2.18	6.46	6.19	0.200	0.053	0.055
x_1^2	1	0.012	0.046	0.016	0.012	0.046	0.016	27.62	40.18	8.28	0.003	0.001	0.035
x_2^2	1	1.312	1.589	2.001	1.311	1.589	2.001	2993.60	1382.74	1029.32	0.000	0.000	0.000
x_3^2	1	0.002	0.023	0.029	0.002	0.23	0.029	4.57	20.7	15.13	0.086	0.007	0.012
Error	5	$2.0 \cdot 10^{-3}$	$5.7 \cdot 10^{-3}$	$9.8 \cdot 10^{-3}$	$0.4 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$	$1.9 \cdot 10^{-3}$						
Total	14	5.622	7.796	8.512									

E1: $R^2 = 0.999$, $adjR^2 = 0.999$; E2: $R^2 = 0.999$, $adjR^2 = 0.998$; EE2: $R^2 = 0.999$, $adjR^2 = 0.997$.

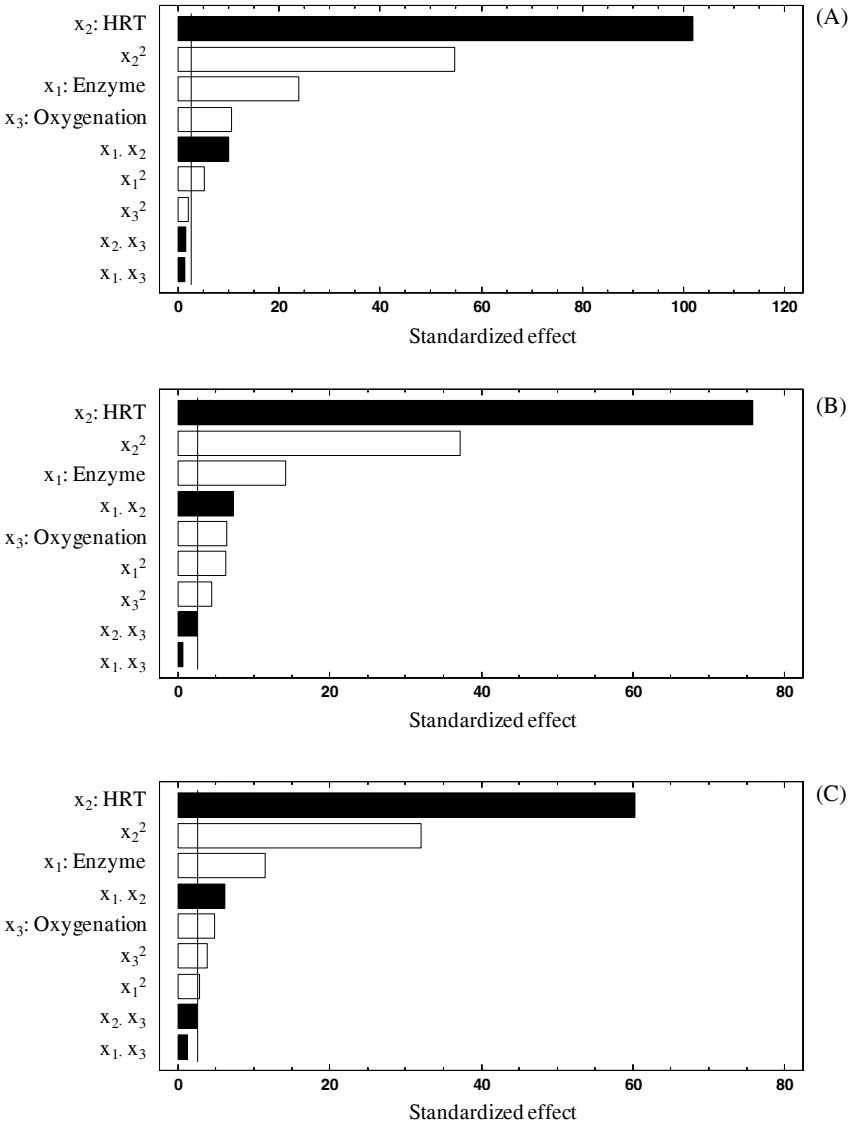


Figure 4.6. Pareto chart for E1 (A), E2 (B) and EE2 (C) removal rates (Y_1, mg/(L·h)). Black bars: antagonistic effect; White bars: synergistic effect.

It was assumed that the regression coefficients and the correlation between each factor can be considered statistically significant for p-values below 0.05. All the terms of the expression were found to be significant, except the cross-interactions $x_1 \cdot x_3$ and $x_2 \cdot x_3$ for the three compounds analyzed, as well as the quadratic effect x_3^2 in the case of E1. Hence, the polynomial models may be simplified to expressions with seven or six adjustable coefficients.

Besides, it was observed that the HRT of the compounds in the reactor is deemed to be the variable with the most significant effect, and as expected for a variable response defined per units of time (mg/(L·h)), that effect was negative. On the contrary, the HRT would have a positive effect if the response was the oxidation percentage. For instance, when increasing the HRT from 1 to 4 h, the removal rate of E1 decreased: 2.11 to 0.76 mg/(L·h) (runs 1 and 3, Table 4.7), while the removal percentages improved: 53 to 76% (data not shown). The quadratic effect of the HRT was the second most significant parameter followed by the linear effect of enzymatic activity. Oxygenation rate also presented an important effect on the removal rate of E1.

The removal rates of the target compounds predicted by the models are depicted in Table 4.7; their correlation with the experimental values was very close, indicating the goodness of the fitting. In fact, high correlation coefficients R^2 and adjR^2 between 0.997 and 0.999 were obtained, corroborating the adequacy of the models.

Response surface analysis

The effect of the variables tested and their interactions on the removal of the target compounds were analyzed by means of RSM; the obtained surface plots for the removal of E1 (slight differences were observed among the three compounds assayed) are shown in Figure 4.7.

Figure 4.7.A presents the removal rates of E1 as a function of laccase activity and the oxygen addition rate at a HRT of 2 h. The efficiency of the treatment was enhanced by increasing both laccase activity and oxygenation. Such improvement was quite linear in the case of E1 and E2, while EE2 presented a lower slope at higher oxygenation values (data not shown). According to the results obtained, the most interesting strategy to optimize the removal of estrogens by commercial laccase would be either the use of high laccase activity or, alternatively, low enzyme activity combined with high frequency of oxygen supply. Indeed, the results obtained with 1,000 U/L of enzyme and oxygenation rate of 30 mg O_2/(L·h) (run 2, Table 4.7) were similar to those obtained with 500 U/L but with 60 mg O_2/(L·h) of oxygen supply (run 11, Table 4.7).

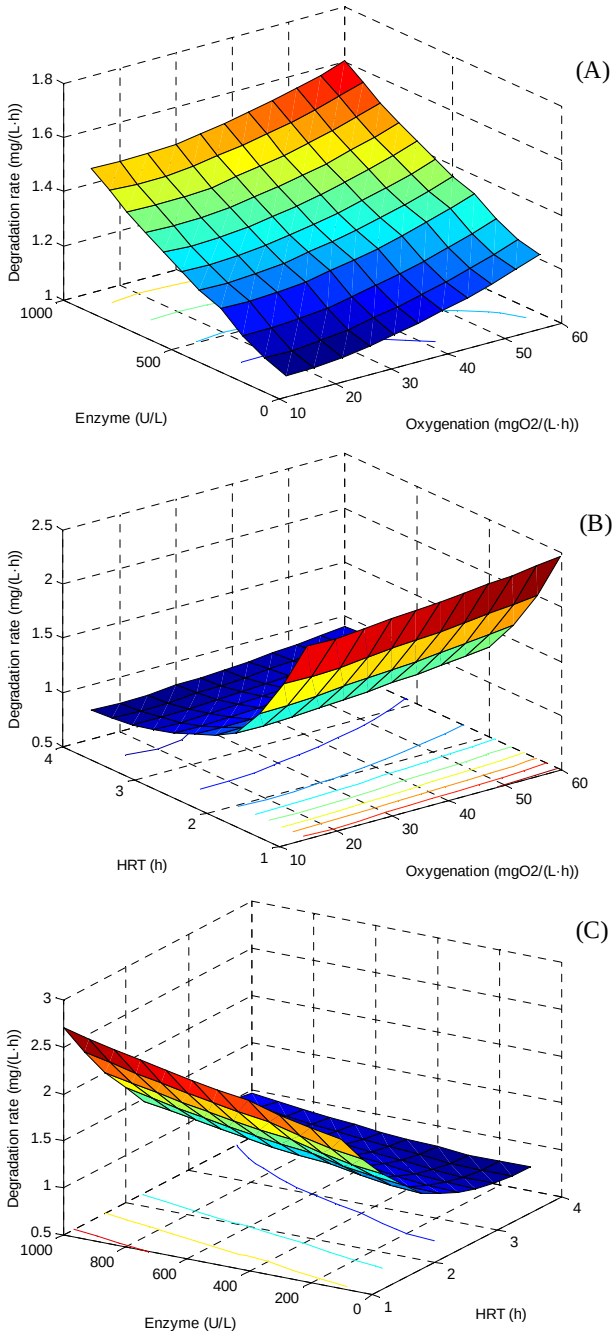


Figure 4.7. Response surface plots for E1 removal rate (Y_1, mg/(L·h)) as a function of: (A) oxygenation and enzyme activity at HRT 2 h; (B) oxygenation and HRT at 500 U/L; (C) enzyme activity and HRT at oxygenation rate of 30 mg O_2/(L·h).

The relevance of oxygen on the laccase catalytic cycle is undeniable as laccase requires oxygen to oxidize the substrates with the reduction of one molecule of oxygen to give one molecule of water plus theoretical four oxidized substrate molecules (Yaropolov et al. 1994). Hence, high oxygen concentrations in the reactor may facilitate oxidation and lead to improved efficiency. Previous investigations reported the enhancement on the laccase-catalytic action during biobleaching (Fillat and Roncero 2009) and phenol removal (Ghosh et al. 2008) caused by oxygen supply. However, Dasgupta et al. (2007) reported no effect of dissolved oxygen concentration on the removal of phenolic compounds by laccase. Nevertheless, oxygen supply is not a common variable studied by RSM to optimize the laccase action on bioremediation processes.

In the current research, oxygenation rates in the range 15-60 mg O_2/(L·h) were examined by injecting oxygen in pulsing flow by means of an electrovalve. For this purpose, pulses of 30 mg/L of oxygen (1 bar for 30 s each pulse) were performed with a frequency between 2 h and 30 min. Under these studied conditions, the dissolved oxygen concentration of the bioreactor was maintained at the saturation level (8 mg/L) during the operation, but increased instantly to 35-38 mg/L when the pulses were made and decreased due to the super-saturation within few minutes to recover the base value and re-establish the equilibrium. On the other hand, it was observed during experiments conducted without oxygen supply that oxygen dissolved was constantly maintained at 8 mg/L, which could prove that oxygen depletion during the reaction was immediately restored by the air from the headspace of the bioreactor, which was favored by the agitation. Besides, oxygen concentration in the reactor should not be a limiting factor in terms of stoichiometry even when assuming a theoretical stoichiometry ratio of 4 (Kurniawati and Nicell 2007a, Riva 2006). Therefore, it can be concluded that the oxygen supplied by pulses was not consumed by the reaction, but led to an important enhancement of the kinetics once the reaction was speeded up during the short periods of high oxygen concentration. It means that although the oxygen is not a limiting reactant, the reaction rates between the laccase and the oxygen increased due to the high concentration of one of the reagents, yielding in better removal results.

The results obtained by RSM for variable HRT and different oxygenation rates at a constant enzyme activity of 500 U/L are plotted in Figure 4.7.B. As expected, the surface plot shows a decrease in the estrogen elimination rates at increasing HRT values. The selected oxygenation rate slightly influenced the removal yields of the target compounds in comparison to the HRT effect in the

range considered and at that value of enzyme activity. Surprisingly, E1 removal rate was not so sensitive to alterations of laccase activity as assumed over the range studied and at an oxygenation rates of 30 mg O_2/(L·h) (Figure 4.7.C). Regarding the effect of HRT, values from 3 to 1 h led to an important increase of removal rate, which may be related to the significance of the quadratic effect of HRT as observed in the corresponding pareto chart (Figure 4.6.A), while values above 3 h showed slight differences on the response.

Optimum conditions and model validation

According to the models, shown in Equations (4.2)-(4.4), the optimum conditions would be: 1,000 U/L, HRT of 1 h and oxygen supply of 60 mg O_2/(L·h), in order to attain the maximum response: 2.82, 3.24 and 3.22 mg oxidized/(L·h) of E1, E2 and EE2, respectively. In order to evaluate the adequacy of the models proposed, three additional replicate runs were carried out under those optimum conditions to verify the optimized response. Average oxidation rates of 2.80, 3.21 and 3.20 mg/(L·h) were found, which are very close to the theoretical predicted values. Furthermore, an estrogenic activity reduction of 75% was found.

Thus, it was confirmed that the RSM was effective and reliable for predicting and optimizing the removal process of the tested estrogens by commercial laccase. Nonetheless, it is also important to take into account that RSM is considered a black box methodology (Cox and Baybutt 1981) and thus, the capacity of prediction when some variation occurs (e.g. variables values outside the ranges evaluated) may be limited.

4.3.2.2. Analysis of removal rate per units of activity and estrogenicity reduction

RSM was applied again to investigate the optimal conditions for the maximization of the following responses: Y_2, removal rates per unit of enzyme used (mg/(L·h·U)), to maximize removal rate and minimize laccase consumption; and Y_3, reduction of estrogenic activity (%), which should be one of the main concerns when removing this type of compounds. The experimental data used for Y_2 optimization were calculated as the ratio between those presented in Table 4.7 and the units of laccase used in each run. Experimental results of estrogenic activity reduction presented in the same table were used for Y_3 evaluation; similar conclusions would be drawn when applying RSM to maximize the removal percentages of estrogens.

Pareto charts obtained for both responses are shown in Figures 4.8. Interestingly, oxygenation rate was not an important effect when considering not only the removal rate but also the units of laccase required (Figure 4.8.A). Moreover, only three terms were found to be significant: laccase activity, followed by its quadratic effect and the HRT influence. On the contrary, oxygenation was the third parameter affecting the elimination of estrogenic activity, after HRT and laccase activity (Figure 4.8.B). Indeed, the percentage of estrogenicity reduction was the variable response most influenced by the addition rate of oxygen, indicating the need of taking this factor into account aiming to achieve high removal rates as well as effective detoxification of the effluent. Response surface for Y_3 response (Figure 4.9) corroborated the important effect that both laccase activity and oxygenation rate have on the reduction of estrogenic activity.

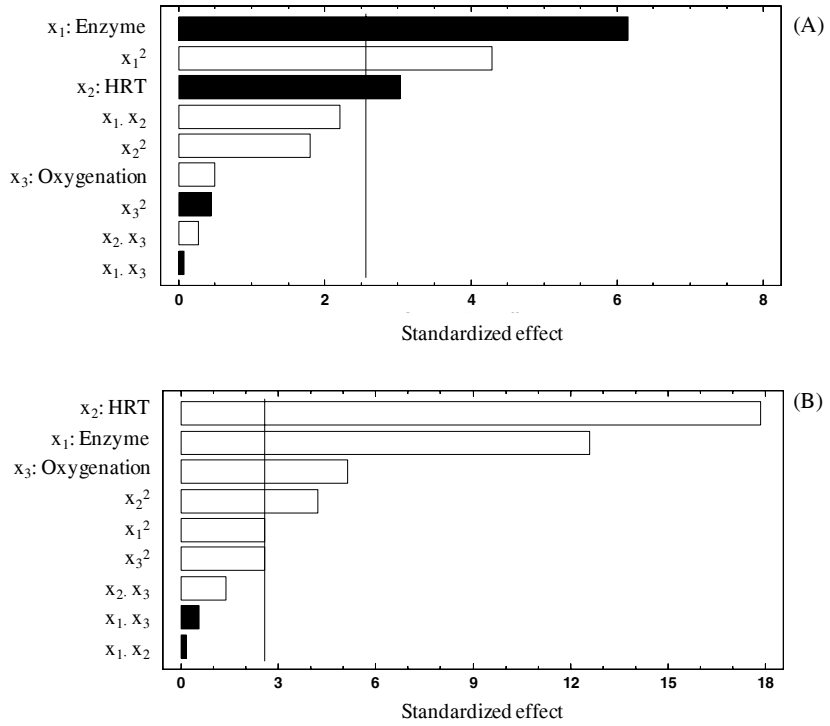


Figure 4.8. Pareto charts for E1 removal rate per units of enzyme used (Y_2, mg/(L·h·U)) (A) and for estrogenic activity reduction (Y_3, %) (B). Black bars: antagonistic effect; White bars: synergistic effect.

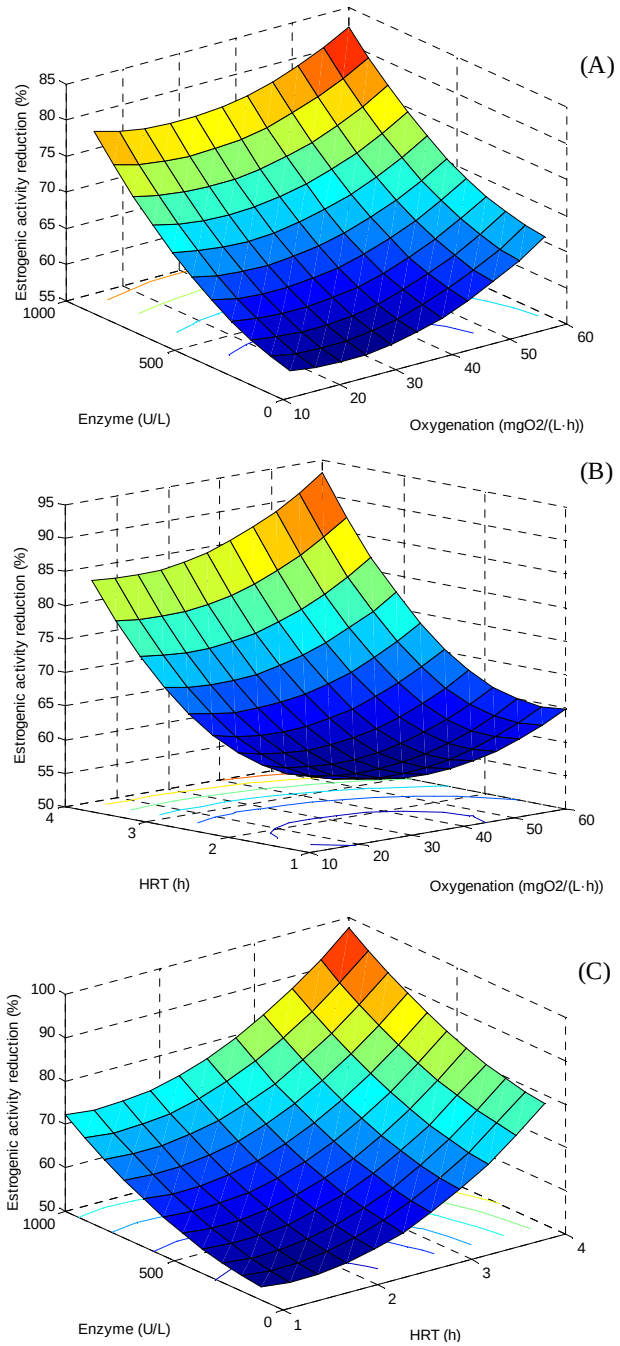


Figure 4.9. Response surface plots for estrogenic activity reduction (Y_3, %) as a function of: (A) oxygenation and enzyme activity at HRT 2 h; (B) oxygenation and HRT at 500 U/L; (C) enzyme activity and HRT at oxygenation rate of 30 mg O_2/(L·h).

4.3.2.3. Comparison of optimal conditions

Optimal conditions and corresponding optimized results obtained for all the responses studied using E1 as model compound are shown in Table 4.9.

Table 4.9. Optimal conditions and corresponding predicted responses for the different response variables analyzed by RSM.

Response	Optimal conditions			Responses		
	Enzyme (U/L)	HRT (h)	Oxygenation rate (mg O_2/(L·h))	mg/(L·h)	mg/(L·h·U) ·10^3	% of estrogenic activity reduction
Y_1 (mg/(L·h))	1,000	1	60	2.82	7.6	75
Y_2 (mg/(L·h·U))	100	1	60	2.28	60.4	60
Y_3 (%)	1,000	4	60	0.99	2.7	99
	1,000	4	30	0.95	2.6	96
	500	4	60	0.91	4.9	93

It was observed that while 1,000 U/L should be used to achieve the highest oxidation rate (2.82 mg/(L·h)) without taking into account the amount of required enzyme, only 100 U/L of laccase are needed to attain both the maximum amount of estrogen oxidized and minimum enzyme consumption (0.06 mg/(L·h·U)) at the same oxygenation rate and HRT. Nevertheless, the removal of estrogenic activity would decrease from 75 to 60%.

On the other hand, the highest values assayed: 1,000 U/L, 4 h of HRT and oxygenation rate of 60 mg O_2/(L·h), provided the best results in terms of estrogenicity reduction, up to 99% and almost complete elimination of the target compounds, which meant removal rates of 1.00 mg/(L·h). However, it was experimentally demonstrated that the decrease of oxygenation to 30 mg O_2/(L·h) or the enzyme activity to 500 U/L led to a decrease of estrogenic activity to 96 and 93%, respectively (runs 4 and 12, Table 4.7), which implies an efficiency descent of only 3-6%, and moreover, estrogens removal of 95-100 and 91-99%, respectively (data not shown). Thereby, the extreme values examined may not be

the best conditions in case of considering the costs of enzyme and oxygen since high detoxification levels are still attained at minimal conditions of enzyme activity and oxygenation. A compromise solution should be reached aiming to maximize not only the removal rate of estrogens but also to minimize the consumption of reagents and maximize the estrogenicity elimination.

The high removal rates and estrogenicity reduction obtained present the system as a promising tool for the detoxification of wastewater polluted with estrogenic compounds, which was indeed attempted in the next step of the research.

4.3.3. Objective C: Evaluation of the potential applicability of an EMR for the continuous treatment of wastewaters containing E1, E2 and EE2

Continuous removal experiments to evaluate the EMR real applicability

A 2-L EMR was proposed for the evaluation of the technology for the continuous removal of estrogens at low concentrations once the feasibility of a similar 370-mL bioreactor for the removal of E1, E2 and EE2 was proved before. In this step of the work, the bioreactor was operated with an initial laccase activity of only 100 U/L aiming to reduce the enzyme requirements and thus the associated costs, and an oxygenation of 60 mg O_2/(L·h) (pulses every 30 min) and a HRT of 4 h were used to favour both estrogens and estrogenicity elimination. A sequence of experiments (see Table 4.2) was designed and performed with the goal of evaluating the potential of the designed system to remove estrogens at environmental concentrations and to treat real wastewater containing these pollutants.

First, the EMR was fed with a buffer solution (phosphate buffer solution, pH 7) containing high estrogens concentration (4 mg/L each) to study the effect of the change of scale and the reduction of laccase activity. Removal percentages of 80, 87 and 85% of E1, E2 and EE2, respectively, were attained under steady-state conditions, which implied degradation rates in the range of 0.80-0.87 mg/(L·h), as shown in Figure 4.10. The use of a much lower laccase activity slightly decreased the removal yields by 11-13%: removal percentages between 91 and 99% were previously found using 500 U/L (run 12, Table 4.7), although this experiment was conducted in the 370-L EMR. Anyhow, it was demonstrated that the change of scale did not strongly affect the efficiency of the treatment: equation models obtained by RSM for the 370-mL EMR predicted values between 83 and 90%, which are quite in agreement with those found here for the 2-L EMR (80-87%).

Moreover, no biocatalyst inactivation was detected over the 100 h of operation and the estrogenicity was reduced by 84%.

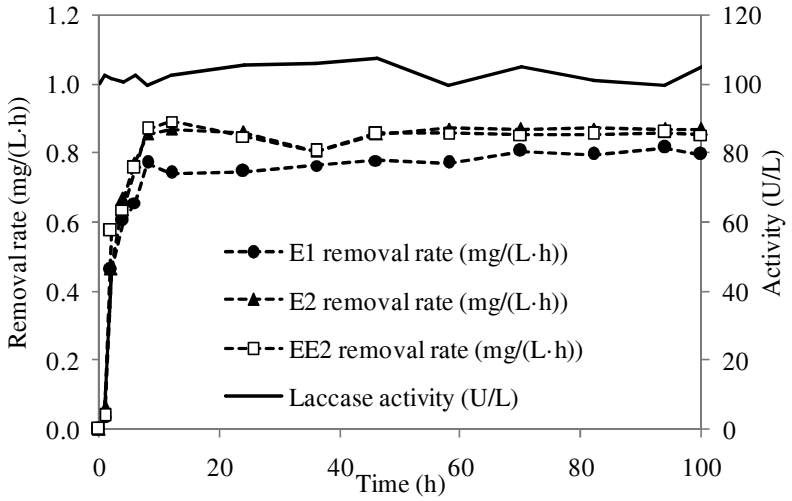


Figure 4.10. E1, E2 and EE2 removal rates and laccase activity during the operation of the 2 L-EMR for the treatment of synthetic water containing 4 mg/L of each estrogen (feed addition rate 1 mg/(L·h)) by 100 U/L of initial laccase activity.

The next experiment was conducted with 100 µg/L (each) of the estrogens in buffer solution as an attempt to work at lower concentrations and closer to environmental levels. The results evidenced the capability of the enzymatic technology to remove these pollutants even at such low concentration, obtaining significant efficiencies: E1 was eliminated by 88% (removal rate 22 µg/(L·h)) and E2 and EE2 were not detected (below detection limits), which meant removal percentages up to 99 and 94%, respectively (Figure 4.11). Moreover, a reduction of 95% of estrogenic activity was found and the biocatalyst retained its total initial activity after 100 h.

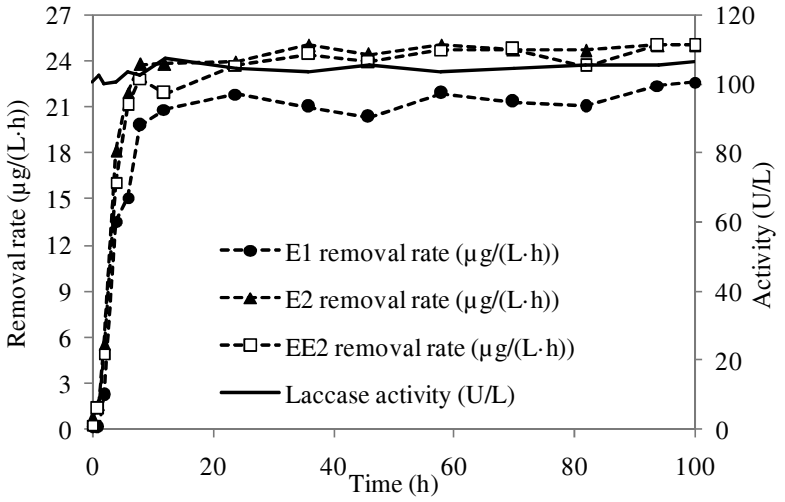


Figure 4.11. E1, E2 and EE2 removal rates and laccase activity during the operation of the 2 L-EMR for the treatment of synthetic waster containing 100 µg/L of each estrogen (feed addition rate 25 µg/(L·h)) by 100 U/L of initial laccase activity.

With the goal of studying the matrix effect to investigate the possibility of the real implementation of this technology, the bioreactor was operating with real wastewater (filtered secondary effluent) previously spiked with 100 µg/L of the estrogens; the results are shown in Figure 4.12.

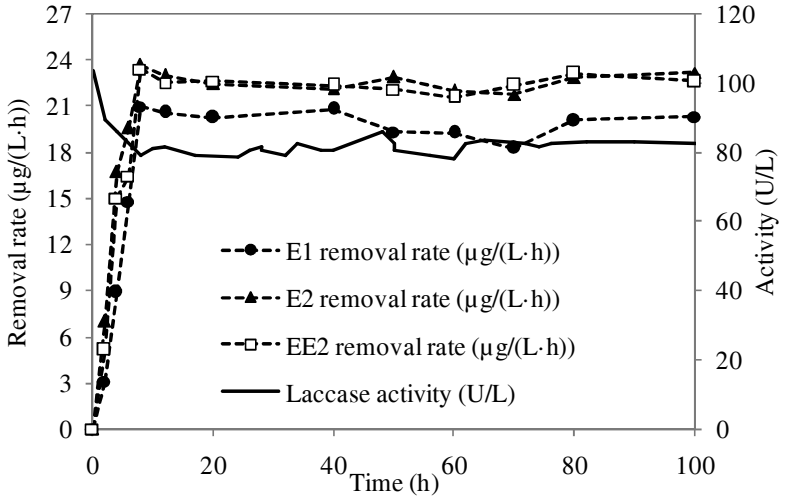


Figure 4.12. E1, E2 and EE2 removal rates and laccase activity during the operation of the 2 L-EMR for the treatment of real wastewater containing 100 µg/L of each estrogen (feed addition rate 25 µg/(L·h)) by 100 U/L of initial laccase activity.

Despite a partial inactivation of laccase (20%) during the first hours, probably due to the constituents of the wastewater, an activity close to 80 U/L was constantly maintained after 100 h of operation until the bioreactor was stopped. Furthermore, high removal percentages of the estrogens were achieved: 80-92%, which implies degradation rates of 20-23 µg/(L·h), under steady-state conditions. This loss of removal efficiencies observed when comparing both experiments with buffer solution and real wastewater may be caused by the slight biocatalyst inactivation and/or by the presence of other compounds which could compete with the target compounds for the enzyme. As expected, the lower oxidation yields led to diminished estrogenicity reduction, although still a significant decrease of 90% was detected. Consequently, the potential detrimental constituents of the real matrix (colloidal particles, organic matter, nitrogen-based compounds, etc.) exerted low impact on the removal yields and on the enzyme activity. Promising performance, stability and catalytic efficiency of this technology were demonstrated, evidencing its noticeable applicability on the treatment of real wastewaters. Indeed, although composite fouling (i.e., a combination of biofouling and inorganic fouling) often occurs in membrane systems because of the nature of wastewaters (Liao et al. 2004), neither fouling nor changes in the membrane pressure (1-1.5 bar) was detected here.

Finally, the capability of the system to remove the estrogens from wastewaters at real environmental concentrations was assessed. In this case, the bioreactor was fed with real non-spiked municipal wastewater containing the estrogens at real environmental concentrations of 0.29-1.52 ng/L. Under these conditions, E1 was removed by 98% (degradation rate 0.37 ng/(L·h)), while E2 and EE2 were not detected in the effluent, which corresponds to oxidation percentages up to 97 and 99%, respectively (removal rates of 0.07 and 0.18 ng/(L·h)). Thus, excellent results were found in spite of the possible presence of compounds which also may act as laccase substrates such as phenols. Few authors reported the oxidation of E2 under abiotic conditions by various mechanisms, such as by manganese oxides or through nitration in the presence of high nitrate concentrations (Gaulke et al. 2008, Sheng et al. 2009) as well as in the presence of model vegetable matter and the influence of the molecular oxygen during that mechanism (Marfil-Vega et al. 2012). Anyhow, control experiments corroborated the only implication of the laccase on the estrogenic compounds removal even when working with real wastewaters at environmental concentrations and under oxygenated conditions.

Determination of the RTD

The study of the hydrodynamic behavior of the 2-L EMR operated under the conditions previously applied for the removal of estrogens (HRT of 4 h and 60 mg O_2/L·h, which means pulses every 30 min) was carried out by the determination of the RTD. A stimulus-response technique was performed by using NaOH as tracer. The results obtained by fitting the experimental data to the tank-in-series model (Levenspiel 1999) are shown in Figure 4.13.

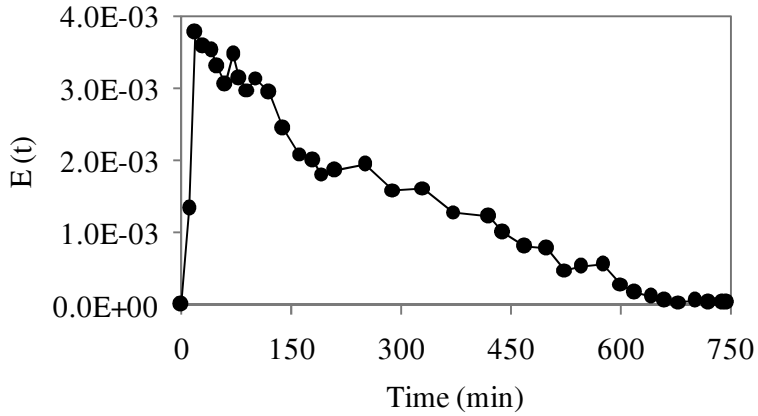


Figure 4.13. RTD obtained for the 2-L EMR.

The shape of the curve, with consecutive slight peaks while the concentration of the tracer is decreasing after attaining its maximum represents the typical tendency of a reactor system with partial recycling of the effluent. From these experimental results obtained, the number of tank reactors in series which would be analogous to the bioreactor was calculated: the hydraulic behavior of the bioreactor was found to be equivalent to a number of 0.7 complete stirred tank reactors, which suggest a fairly similar behavior to a complete stirred tank reactor. Furthermore, the average residence time and the real reacting volume were calculated using appropriate correlations, obtaining values of 225 min and 1.9 L. Although the difference was not deeply significant, the fact that those calculated values were lower than the theoretical ones (240 min of HRT and 2 L of volume) could be attributed to the existence of dead or stagnant zones. For example, the volume corresponding to the membrane module and tubing (approximately 120 mL), which was considered as part of the total volume of the reactor system, may not be effective volume.

4.4. Conclusions

Fed-batch reactors were found to be an interesting strategy for the removal of estrogens by laccase as well as useful systems to investigate the influence of various operational parameters affecting E1 and E2 transformation: laccase activity, substrate frequency addition and aeration/oxygenation, aiming to elucidate the most suitable conditions for the following continuous operation. The influence of aeration was negligible, whilst oxygen pulses provided enhanced removal efficiencies. Moreover, the enzyme activity was decreased to 500 U/L without significantly compromising the efficiency of the system, especially when oxygenation was applied in comparison with non-oxygenated experiments.

An EMR was designed based on a continuous stirred tank reactor coupled to an ultrafiltration membrane which allowed the recovery of enzyme while the estrogens could pass through it. The feasibility of a 370-mL bioreactor was preliminary evaluated for E1 and E2 removal: E1 was eliminated up to 95% and E2 was not detected in the effluent under steady state conditions. Additionally, the residual estrogenic activity was significantly reduced by more than 95%.

Although the effects of oxygenation and HRT were evaluated in previous experiments, a three-level Box-Behken factorial design combined with the RSM was successfully applied to study the continuous transformation of E1, E2 and EE2, avoiding the inadequate "one factor at a time" optimization. Three main factors affecting the treatment efficiency were considered: enzyme activity (100-1,000 U/L), HRT (1-4 h) and oxygenation rate (15-60 mg O_2/(L·h)), obtaining estrogenic activity reductions in the range between 54 and 96% and removal rates between 0.76 and 3.11 mg/(L·h). The statistical approach applied was presented as a feasible tool to study the effects and interactions of the tested parameters and predict the response for selected values.

As expected, laccase activity and HRT showed great effects and, interestingly, the relevance of oxygen in the improvement of oxidation reaction kinetics through the rise of the oxygen dissolved above saturation levels was demonstrated. Significant differences on the optimal conditions were found for the different responses evaluated. When considering removal rates as response, optimal conditions were: 1,000 U/L of laccase, 1 h HRT and 60 mg O_2/(L·h) of oxygenation rate, predicting 2.82-3.24 mg eliminated/(L·h), which meant 71-81% of oxidation. These optimum conditions were successfully validated, and 75% of estrogenicity reduction was achieved. On the other hand, only 100 U/L were found as optimal to maximize the efficacy of the enzyme: E1 was oxidized by 0.06 mg/(L·h·U), although the removal of estrogenicity decreased to 60%. The

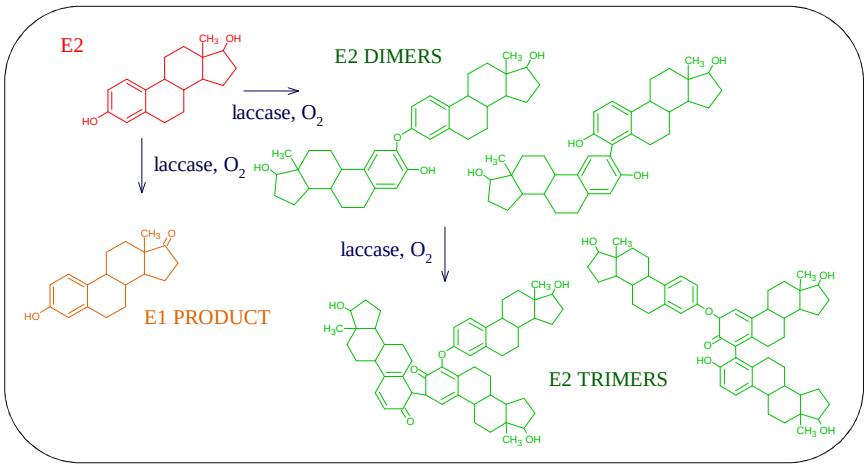
methodology was also applied to maximize the reduction of estrogenic activity: the highest values assayed (1,000 U/L, HRT 4 h and 60 mg O_2/(L·h)) provided nearly complete detoxification.

Finally, a 2-L EMR was successfully operated for 100 h with minimal requirements of laccase (only 100 U/L) for the transformation of E1, E2 and EE2 from both buffer solution and real wastewater (filtered secondary effluent). When the experiments were performed at high and low concentrations of the target compounds, 4 mg/L and 100 µg/L, not only high removal yields (80-100%) but also remarkable reduction of estrogenicity (about 84-95%) were attained. When the EMR was applied for the treatment of municipal wastewaters with real environmental concentrations of the different compounds (0.29-1.52 ng/L), excellent results were also achieved despite partial inactivation of the enzyme (about 20% within the first hours). It was concluded from these findings that the potential detrimental constituents of the real matrix exerted low impact on both estrogens removal yields and enzyme stability. Moreover, neither fouling nor changes in the membrane pressure were detected.

Overall, the results reported are encouraging as they present an innovative technology to remove natural and synthetic estrogens found in sewage effluents and thus demonstrated the potential implementation of this novel technology as an alternative advanced oxidation process in conventional treatment plants.

Chapter 5

Assessment of transformation products and reaction mechanisms*



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Outline

5. Assessment of transformation products and reaction mechanisms

5.1. Introduction

In the previous chapters the feasibility of using laccases for the removal of various estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), and anti-inflammatory compounds was proved; moreover, the reduction of estrogenic activity was demonstrated for the estrogens and the increase of the aerobically biodegradability after laccase transformation of diclofenac (DCF) was also demonstrated. However, the identification of the main biotransformation products as well as the reaction pathways was a pending objective.

In fact, there is an evident lack of knowledge with regard to the identification of laccase-catalyzed transformation products of estrogens, despite of the extensive emerging research on the application of ligninolytic enzymes for the oxidation of endocrine disrupting chemicals and other emerging pollutants. The elimination of E1, E2 and EE2 has been commonly assumed to occur by means of polymerization (Cabana et al. 2007b). Indeed, the enzymatic-catalyzed oxidative coupling of phenolic substrates has been studied by several authors, who suggested that laccase oxidizes organic substrates to free radicals, which undergo oxidative coupling reactions, producing dimers, oligomers and polymers (Garcia et al. 2011, Kunamneni et al. 2008a, Hollmann and Arends 2012). In the specific case of estrogens, only few works supported this hypothesis by reporting the formation of dimers of E2 and EE2 (Nicotra et al. 2004, Tanaka et al. 2009) whilst no other degradation products were identified. Furthermore, E1 byproducts resulting from laccase-catalyzed oxidation have not been characterized up to the date.

Advanced oxidation processes (AOPs) provided different outcomes with regard to the products formed. For instance, Bila et al. (2007) reported the generation of hydroxylated E2 (OH-E2) by ozonation, whilst Mazellier et al. (2008) demonstrated the formation of quinine methide and quinone derivates. Other authors reported the transformation of E2 to E1 by biological treatments such as activated sludge from sewage treatment plants or nitrifying activated sludge (Skotnicka-Pitak et al. 2008). Nevertheless, none of the products resulted from those reactions were found after laccase-catalyzed oxidation of estrogens.

Regarding anti-inflammatory compounds and according to Ziylan and Ince (2011), the removal of these drugs takes place mainly due to photodegradation and biodegradation processes while hydrolysis does not contribute to their

removal. Specifically, DCF was proved to be degraded by hydroxylation and subsequent C-N cleavage by various AOPs (Hofmann et al 2007, Vogna et al. 2004); nonetheless, scarce information can be found in literature dealing with the enzymatic removal of that compound. Marco-Urrea et al. (2010b) described the formation of various DCF transformation products by *Trametes versicolor* fungal cultures (Figure 5.1), including hydroxylated compounds as also demonstrated by other investigations using different fungi (Rodarte-Morales 2012a, Hata et al. 2010).

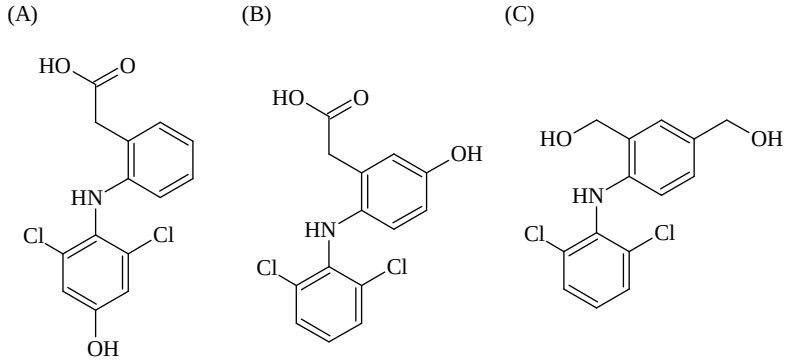


Figure 5.1. Structures of DCF transformation products proposed by Marco-Urrea et al. (2010b).

The goal of this chapter was to perform the identification of the reaction products of the estrogens: E1, E2 and EE2, as well as those of DCF, formed by laccase-catalyzed transformation, as a first step to elucidate the main reaction pathways. Estrogenic compounds were assayed by using *Myceliophthora thermophila* laccase, as done through the whole research, while DCF experiments were conducted by *Trametes versicolor* laccase because it provided higher removal extent of this substrate as discussed in Chapter 2. For this purpose, different techniques were utilized: gas chromatography mass spectrometry (GC-MS) was applied in an attempt to characterize transformation products of all the substrates by analyzing the obtained spectra and the possible fragmentation patterns, whereas liquid chromatography atmospheric pressure chemical ionization (LC-APCI) and liquid chromatography electrospray time-of-flight mass spectrometry (LC-ESI-TOF) was also used to identify the estrogens biotransformation compounds.

5.2. Materials and methods

5.2.1. Chemicals and enzymes

E1, E2, EE2 and DCF were purchased from Sigma-Aldrich. Stock solutions of the target compounds were prepared in methanol (J.T. Baker, HPLC grade, 99.8%). 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was purchased from Fluka. All other reagents used were of analytical grade.

Laccase from *Trametes versicolor* was also purchased from Sigma-Aldrich and laccase from *Myceliophthora thermophila* was supplied by Novozymes. All the other chemicals were of analytical grade.

5.2.2. Determination of laccase activity

Activity of both *Trametes versicolor* and *Myceliophthora thermophila* laccases was determined spectrophotometrically as described in Chapter 2.

5.2.3. Identification of laccase-catalyzed transformation products of estrogens

5.2.3.1. Estrogens laccase-catalyzed transformation experiments

With the objective of identifying the oxidation products of E1, E2 and EE2 and establishing the transformation pathways, experiments were conducted in batch reactors of 250 mL (Erlenmeyer flasks). Each reactor contained 100 mL of 5 mg/L of each estrogen in phosphate buffer (100 mM, pH 7) or distilled water. An initial activity of *Myceliophthora thermophila* laccase of 2,000 U/L was used aiming to ensure the transformation of the compounds and the subsequent formation of reaction products at significant concentrations. Each estrogen was assessed separately in order to identify the corresponding transformation products. Moreover, different flasks with the same initial conditions were used of each monitored reaction time. Corresponding controls without laccase and without the substrate were carried out. The reaction was stopped after 8 or 24 h by adding HCl to reach pH 2, and then samples were frozen until subsequent analysis.

5.2.3.2. GC-MS analysis

For the identification of the transformation products of the estrogens by GC-MS, 20 mL of the acidified samples of experiments performed with phosphate buffer were withdrawn after 8 h and diluted in 100 mL of distilled water (pH 2) for their

subsequent solid phase extraction (SPE). Thereafter, samples were analyzed after derivatization of the species. Both SPE and GC-MS methods applied were detailed in Chapter 4.

5.2.3.3. LC-APCI analysis

Acidified samples taken after 24 h of the experiments performed in distilled water were analyzed by LC-APCI following the method described in Chapter 3. However, for this study the mass spectrometer was used in the full scan mode rather than in the tandem MS-MS mode, and the range selected for the mass spectrometer full scan was between m/z ratios of 50 and 900.

5.2.3.4. LC-ESI-TOF analysis

Samples analyzed by LC-APCI were also assayed by LC-ESI-TOF to obtain information concerning accurate masses. For this purpose, a LC system (Alginet 1100) equipped with a Zorbax Eclipse XDB (C18 3 mm x 250 mm, 5 µm) (Alginet) analytical column was used. A binary solvent comprised acetonitrile (ACN) was used as the mobile phase. Flow rate was set at 0.6 mL/min and the gradient was programmed as following: 0 min, 40% ACN; 10 min, 60% ACN; 40 min, 80% ACN; 43 min, 100% ACN; 44 min, 100% ACN; and the injection volume was 15 µL. This LC system was connected to a Microtof Bruker Daltonics mass spectrometer with an ESI source operated at negative mode and under the following conditions: capillary, 4.5 kV; drying gas, 8 L/min; gas temperature, 200ºC.

5.2.4. Identification of laccase-catalyzed transformation products of DCF

5.2.4.1. DCF laccase-catalyzed transformation experiments

In an attempt to detect and identify the laccase-catalyzed transformation products of DCF, which was the anti-inflammatory compound removed to the largest extent (see Chapter 2), the monitoring of transformation products was performed during batch experiments at pH 4 (100 mM acetate buffer) with an initial concentration of DCF of 5 mg/L and 2,000 U/L of *Trametes versicolor* laccase (the biocatalyst which provided the highest DCF removal yields) in a final volume of 50 mL, under continuous stirring and at room temperature. Different flasks with identical initial conditions were used for each monitored reaction time and corresponding controls without laccase and without DCF were performed.

After the corresponding intervals, the reaction was stopped by the acidification of the mixture, which was then frozen until the subsequent procedure.

5.2.4.2. GC-MS analysis

For their analysis, 10 or 50 mL of the acidified samples were diluted in 100 mL of distilled water (pH 2) for their subsequent SPE following the method described in Chapter 4 for estrogens quantification. Then, derivatization of the species and GC-MS analysis were performed by applying the procedure detailed in the same chapter; only temperature profile was changed with respect to the method applied there. The GC oven was programmed as follows: 1 min at 50ºC, first ramp at 10ºC/min to 180ºC, which was held for 7 min, and second ramp at 10ºC/min to 230ºC, held for 25 min. The transference line and the ion trap temperature were set at 280 and 230ºC, respectively.

5.3. Results and discussion

5.3.1. Identification of laccase-catalyzed transformation products of estrogens

As indicated, the identification of biotransformation products of estrogens by *Myceliophthora thermophila* laccase was attempted by different analytical methods. First, GC-MS was applied for the preliminary assessment of the identification of E1 and E2 byproducts generated by reaction in buffered medium; afterwards, LC-APCI and LC-ESI-TOF techniques were performed aiming to identify the potential products with high MW produced from E1 and E2 but also EE2 by catalyzed reaction in distilled water (phosphate buffer was also assayed but it implied unfavorable signal when analyzing by LC-APCI).

5.3.1.1. Identification by GC-MS

A preliminary identification of the transformation products of estrogens was performed by GC-MS for E1 and E2. In case of E1, a single byproduct (E1-P1), with a major ion at m/z 340, was found. The chromatograms for the samples after 8 h of laccase-catalyzed transformation and those for the corresponding controls lacking enzyme are shown in Figure 5.2.A. The chromatograms show a new peak detected at a retention time of 31.6 min, which was initially attributed to E1; however, mass spectra corresponding to the derivatized compounds were different (Figures 5.2.B and C, respectively). With regard to E2, two different byproducts (E2-P1 and E2-P2) were observed in the chromatogram corresponding to the 8-h

sample (Figure 5.3.A); none of these peaks were detected in samples either at time 0 or in controls. The mass spectra of these metabolites are presented in Figures 5.3.B and C. In spite of the effort done, the identification of the detected products was not possible.

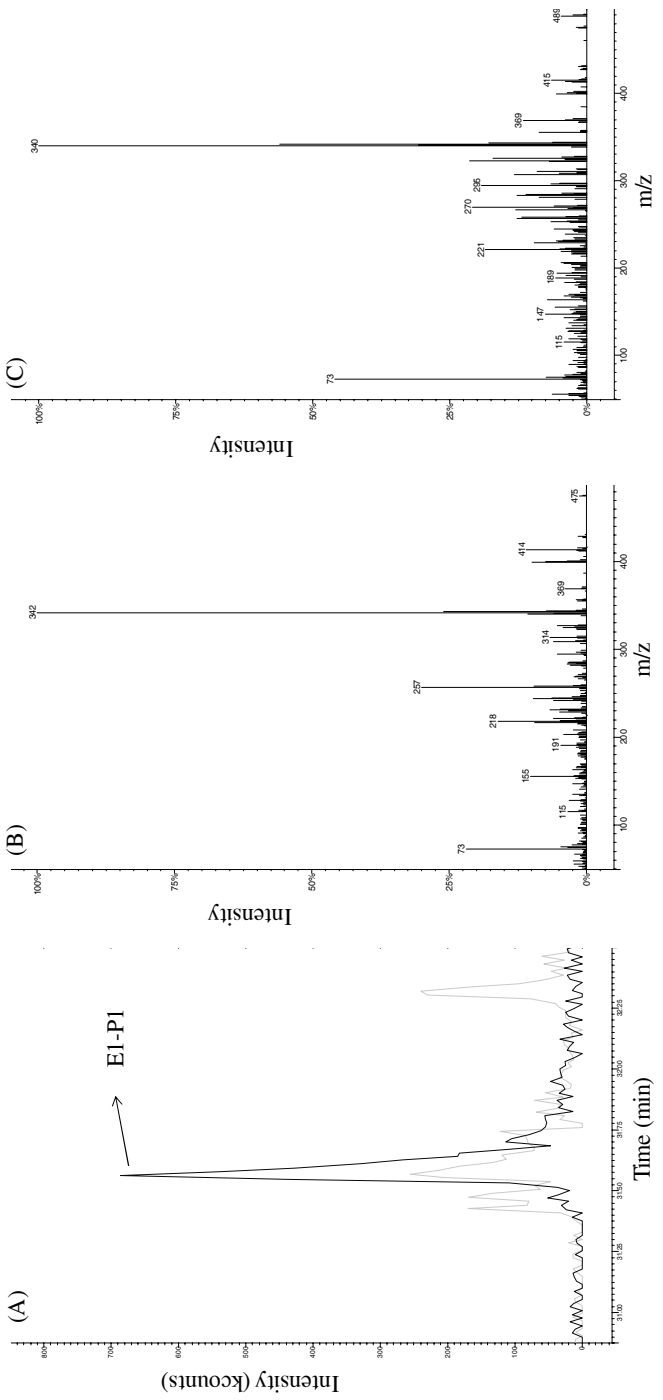


Figure 5.2. Selected ion monitoring chromatogram (m/z 340) for the sample after 8 h of laccase-catalyzed transformation of E1 (black line) and for the corresponding control (grey line) (A) and mass spectra corresponding to E1 (B) and E1-P1 (C) obtained by GC-MS.

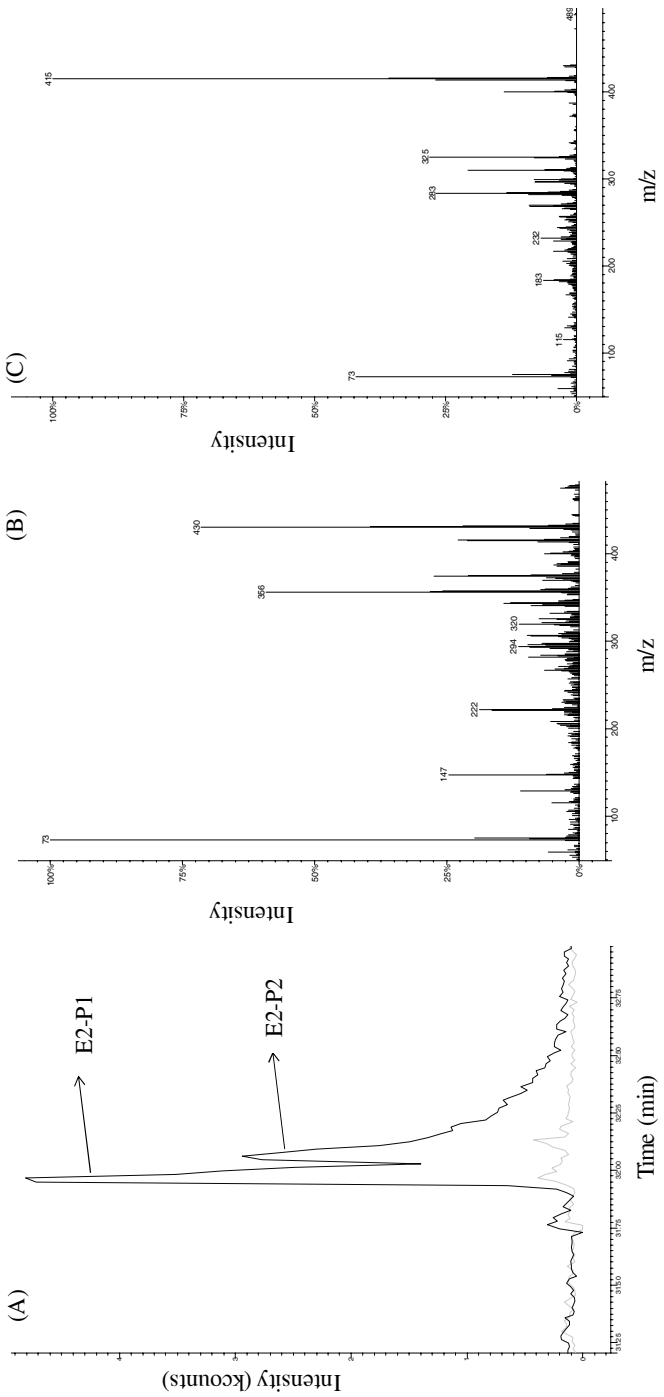


Figure 5.3 Selected ion monitoring chromatogram (m/z 430+356) for the sample after 8 h of laccase-catalyzed transformation of E2 (black line) and for the corresponding control (grey line) (A) and mass spectra corresponding to E2-P1 (B) and E2-P2 (C) obtained by GC-MS.

5.3.1.2. Identification by LC-APCI and LC-ESI-TOF

Some authors suggested that the removal of estrogens could occur by polymerization since these compounds possess a para-substituted phenol structure and the enzyme may catalyze the oxidative coupling of phenolic compounds (Cabana et al. 2007b, Nicotra et al. 2004, Tanaka et al. 2009). This assumption has been stated in several investigations dealing with laccase-catalyzed treatment of estrogens to explain the disappearance of the target compounds and the difficulties in characterizing the reaction products. Nonetheless, only few works demonstrated this hypothesis by experimental assays and the appropriate analytical methods. For instance, the formation of dimers of E2 by laccase has been previously reported by Nicotra et al. (2004) while Tanaka et al. (2009) reported the formation of a single dimer of EE2 by laccase-mediated treatment.

With the goal of verifying the radical coupling of estrogens and characterizing the laccase-catalyzed reaction products of E1, E2 and EE2, a further study was conducted by using a LC-APCI system coupled to a tandem mass spectrometer operated in the full scan mode, which allowed the detection of products with high m/z ratio. All precursor ions in APCI positive were the results of a simple protonation. Moreover, in the case of E2 and EE2 the analytes underwent a loss of water in the source, as reported by Vanderford et al. (2003). Thus, the compounds based on E2 and EE2 were detected as $[M+H-H_2O]$ in the first quadrupole of the mass spectrometer (Q_1), and [M+H] in the case of E1. The use of the Q_1 under full scan monitoring in the range m/z 50-900 revealed new peaks in total ion chromatograms (TIC) of the samples treated with laccase for 24 h. Although the identification of some of the products was not possible, most of them were characterized by their molecular weight (MW) by analyzing the TIC and compounds spectra (Figures 5.4 to 5.6) and as summarized in Table 5.1.

In the case of E1, some new peaks were detected in the TIC corresponding to 24 h of laccase-catalyzed transformation (Figure 5.4.A). The first peaks displayed in the TIC (those no indicated by their corresponding retention times) were also observed in controls and time 0 samples and thus, they were not considered as reaction products. Therefore, only the peaks marked in the figures are supposed to be reaction products and were identified as shown in Table 5.1. A new product was observed at a retention time of 13.7 min and showed a m/z of 539. This compound could be an E1 dimer with MW 538: mass of E1 270 x2 -2H = 538, and after protonation the molecular ion would be 539, as shown in the corresponding spectrum (Figure 5.4.B). Another compound with a molecular ion

at m/z 269, which seems to be that found by GC-MS, was detected at a retention time of 8.1 min but its identification was not possible.

Table 5.1. Characterization of the products detected by LC-APCI formed after 24 h of E1, E2 and EE2 laccase-catalyzed transformation observed in the corresponding TIC after subtracting the signals of controls and time 0 samples.

Parent compound	Retention time (min)	Molecular ion	Molecular weight	Suggested product
E1	8.1	269	268	not identified
	13.7	539	538	E1 dimer
E2	7.9	271	270	E1
	9.7	525	542	E2 dimer I
	10.9	525	542	E2 dimer II
	12.2	525	542	E2 dimer III
	13.0	795	812	E2 trimer I
	13.6	795	812	E2 trimer II
EE2	7.7	295	312	not identified
	8.9	573	590	EE2 dimer I
	9.6	573	590	EE2 dimer II
	10.9	867	884	EE2 trimer I
	11.8	867	884	EE2 trimer II

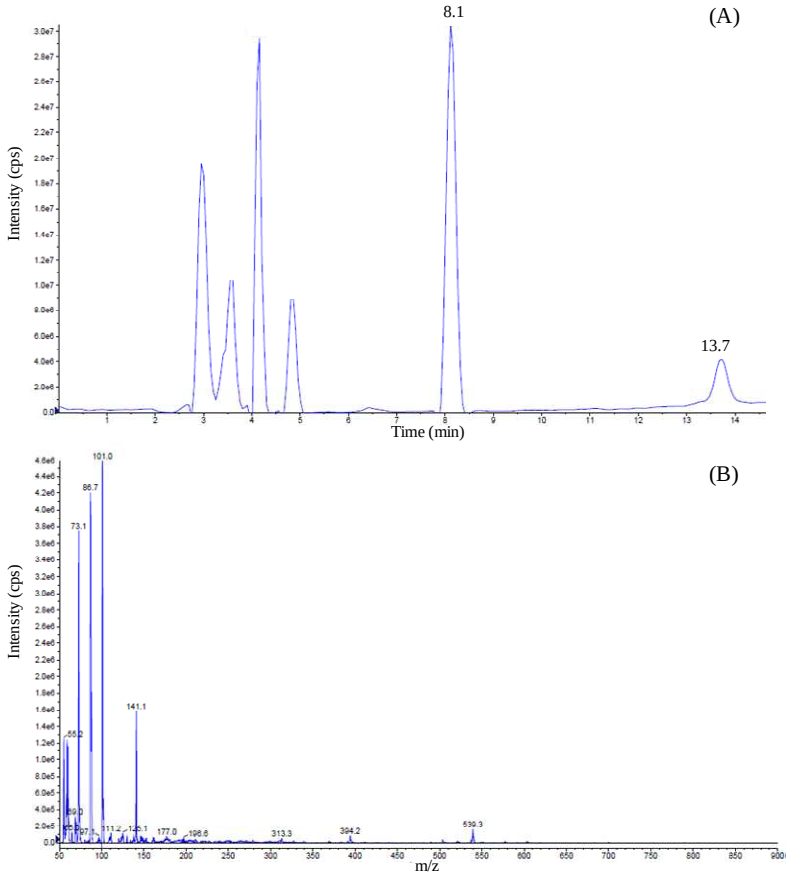


Figure 5.4. TIC of the sample after 24 h of laccase-catalyzed transformation of E1 (A) and mass spectrum of E1 dimer (B) obtained by LC-APCI.

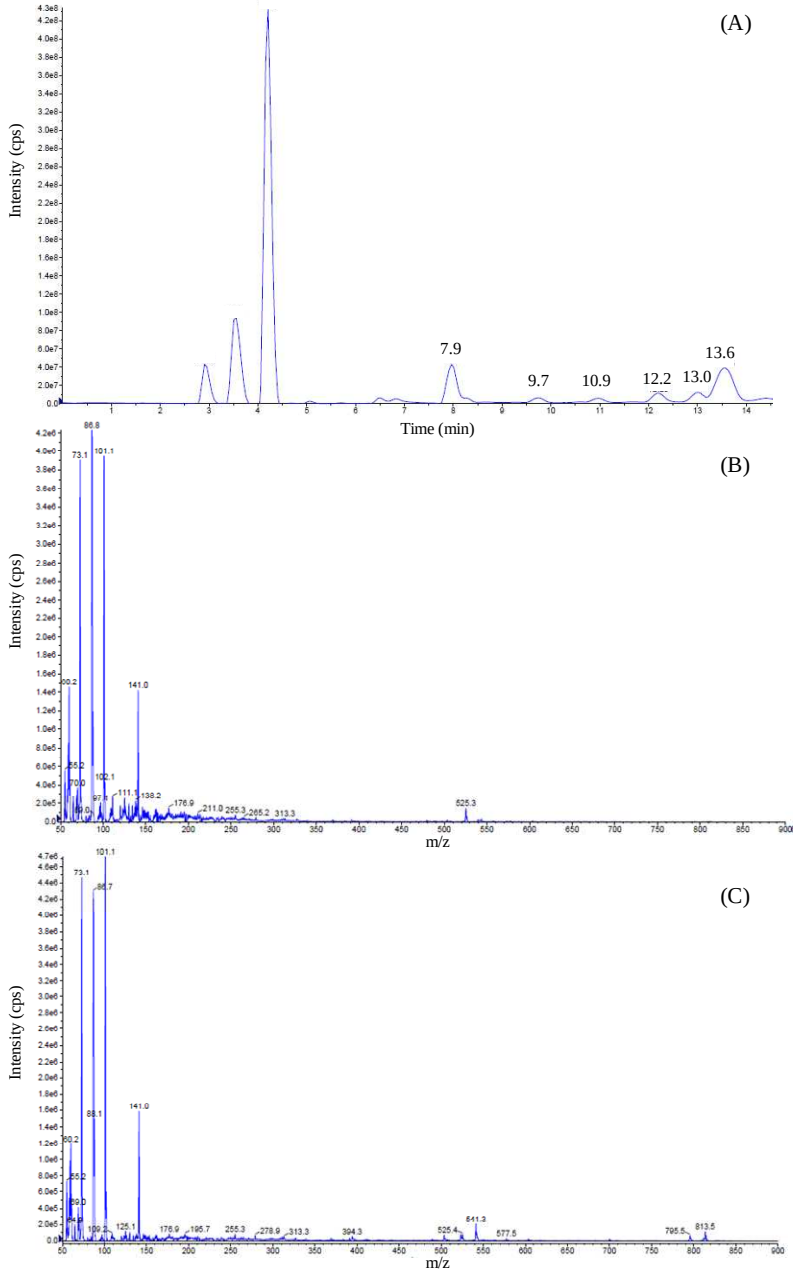


Figure 5.5. TIC of the sample after 24 h of laccase-catalyzed transformation of E2 (A) and mass spectra of E2 dimer I (B) and trimer I (C) obtained by LC-APCI.

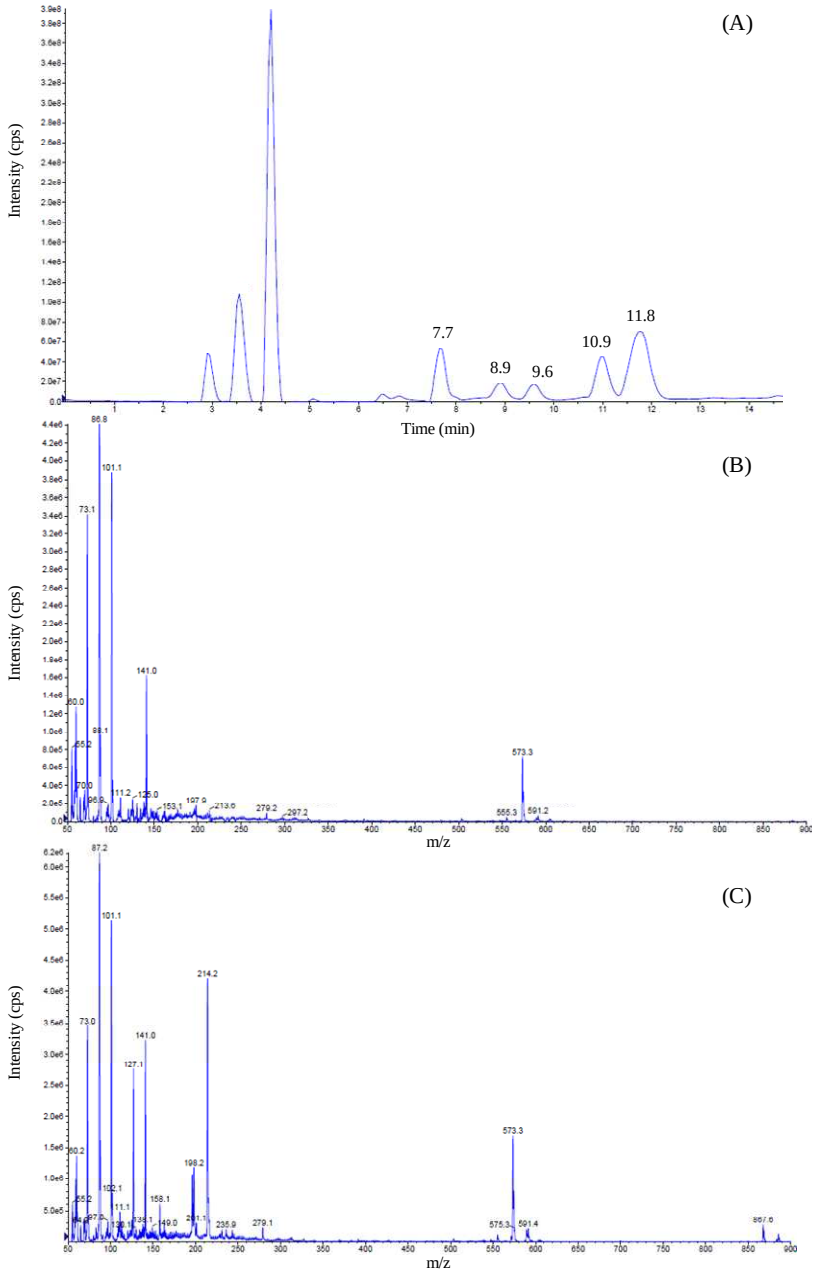


Figure 5.6. TIC of the sample after 24 h of laccase-catalyzed transformation of EE2 (A) and mass spectra of EE2 dimer I (B) and trimer I (C) obtained by LC-APCI.

Regarding E2, different new peaks were observed in the TIC after subtracting the signal of controls and time 0 samples (Figure 5.5.A) and were identified as indicated in Table 5.1. First, the results revealed a new compound with m/z 271 at a retention time of 7.9 min. This compound was presumed to be E1 formed upon oxidation of E2, since its spectrum corresponded to that of E1 standard. It could explain the apparent lower removal yield of E1 in comparison to E2 and EE2 when a mixture of the three compounds was treated with laccase. Other authors also reported the transformation of E2 to E1 by biological processes based on activated sludge or nitrifying activated sludge (Skotnicka-Pitak et al. 2008). Nevertheless, this is the first time that E1 is characterized as E2 transformation product by laccase. Furthermore, it is known that transformation of E2 into E1 is rather unspecific and it can be carried out by many bacteria (Ke et al. 2007). However, although these experiments were not run under sterile conditions, the corresponding controls lacking laccase discarded bacterial transformation of the target compounds.

Moreover, three new peaks were observed at retention times of 9.7, 10.9 and 12.2 min. These products were identified as dimers of E2 due to their molecular ions: dimer of E2 would have a MW of 542 (mass of E2 272 x2 -2H = 542); however, the compounds are seen as [M+H-H_2O] in the Q_1 and therefore, the molecular ion would be 525, as appeared in the spectrum of the E2 dimer I (Figure 5.5.B). Also, two trimers of E2 (MW 812) appeared at retention times of 13.0 and 13.6 min and presented a molecular ion at a m/z of 795 (Figure 5.5.C). These results are in agreement with those reported by Mao et al. (2009, 2010a, b), who demonstrated the formation of dimers and trimers, as well as E1, after the enzyme-mediated transformation of E2 using lignin peroxidase.

The formation of dimers and trimers was also demonstrated by analyzing the new peaks in the TIC corresponding to EE2 (Figure 5.6.A). Two dimers were observed at retention times of 8.9 and 9.6 min, which have MW of 590 (mass of EE2 296 x2 -2H = 590) although presented a molecular ion of 573 (590+H-H_2O) as observed in the spectrum of the EE2 dimer I (Figure 5.6.B). Besides, two trimers of EE2 (MW 884) were found at 10.9 and 11.8 min of retention time and presented a molecular ion at m/z of 867 (Figure 5.6.C). A new peak was also detected at a retention time of 7.7 min with a molecular ion of 295 (Table 5.1). Previous authors reported hydroxylation reactions from EE2 by fungi and algae, and in some cases a subsequent methoxylation of the hydroxyl derivate (Cajthaml et al. 2009). In this way, this compound might also correspond to a hydroxylated product of EE2: its MW would be 312 (mass of EE2 296 –H +OH = 312), and it

would be seen as 295 (312+H-H_2O). However, a further study should be conducted to ensure this premise.

As detailed above, the assessment of identification was based on the MW of the products, and thus on the molecular ions, since the structures and MW were known once presupposed compounds were attempted to be identified. Besides, mass spectra of the compounds may corroborate the hypothesis. As example, spectra of EE2, EE2 dimer and EE2 trimer including the corresponding structures and possible fragmentation patterns of the products are shown in Figures 5.7 to 5.9. Mass spectrum of EE2 (Figure 5.7) demonstrated the exhibition of [M+H-H_2O] as molecular ion of the compounds: a m/z 279 is shown, as well as m/z 297 which corresponds to the protonated molecule. Mass spectrum shown in Figure 5.8 may correspond to a EE2 dimer as it presents not only m/z values at 591 (corresponding to the protonated dimer) and 573 (corresponding to the molecular ion), but interestingly, the spectrum also presents a m/z at 279, which may correspond to the molecular ion of EE2 and thus indicating that indeed, the spectrum should be the one of a compound based on EE2 monomers. Similarly, the mass spectrum of EE2 trimer (Figure 5.9): m/z 885 and 867, corresponded to the protonated and molecular ion of EE2 trimer, but the spectrum also contains the molecular ions corresponding to the dimer and the monomer (m/z 573 and 279, respectively).

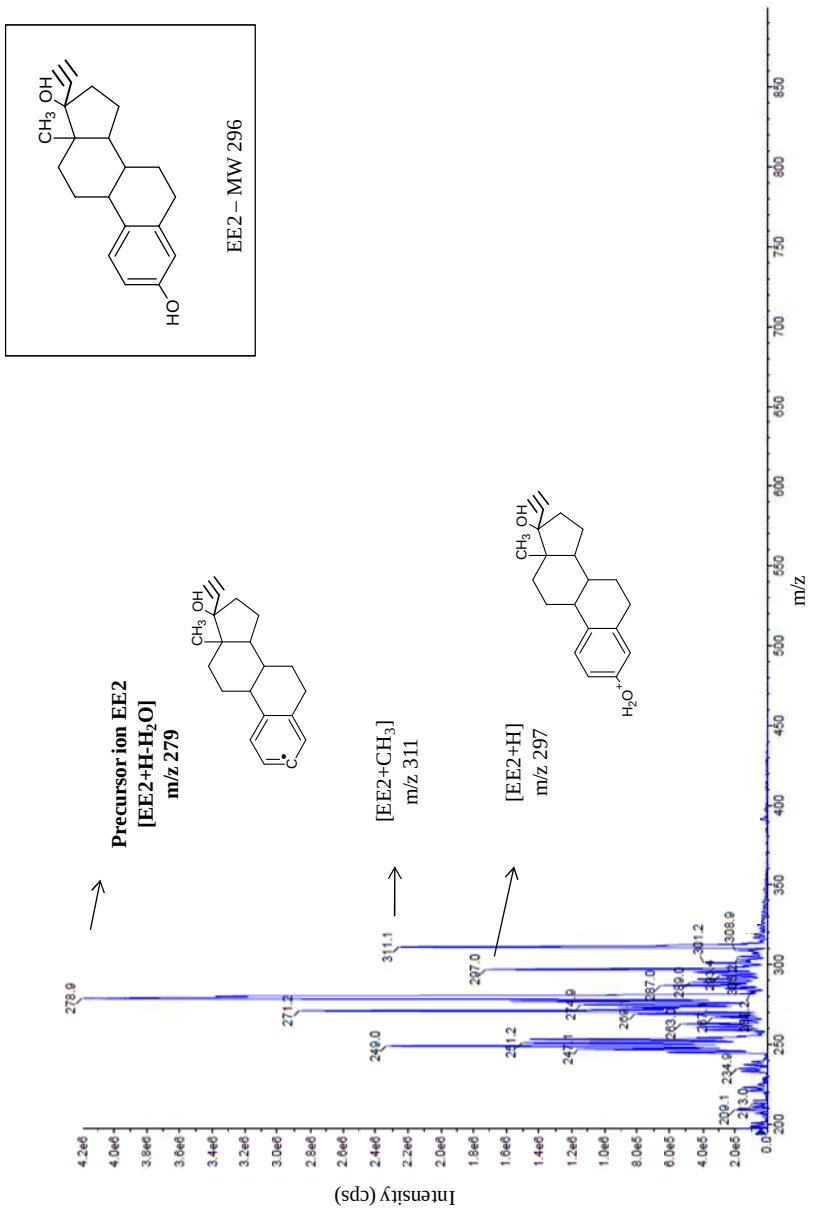


Figure 5.7. Mass spectrum of EE2 detected by LC-APCI including structures.

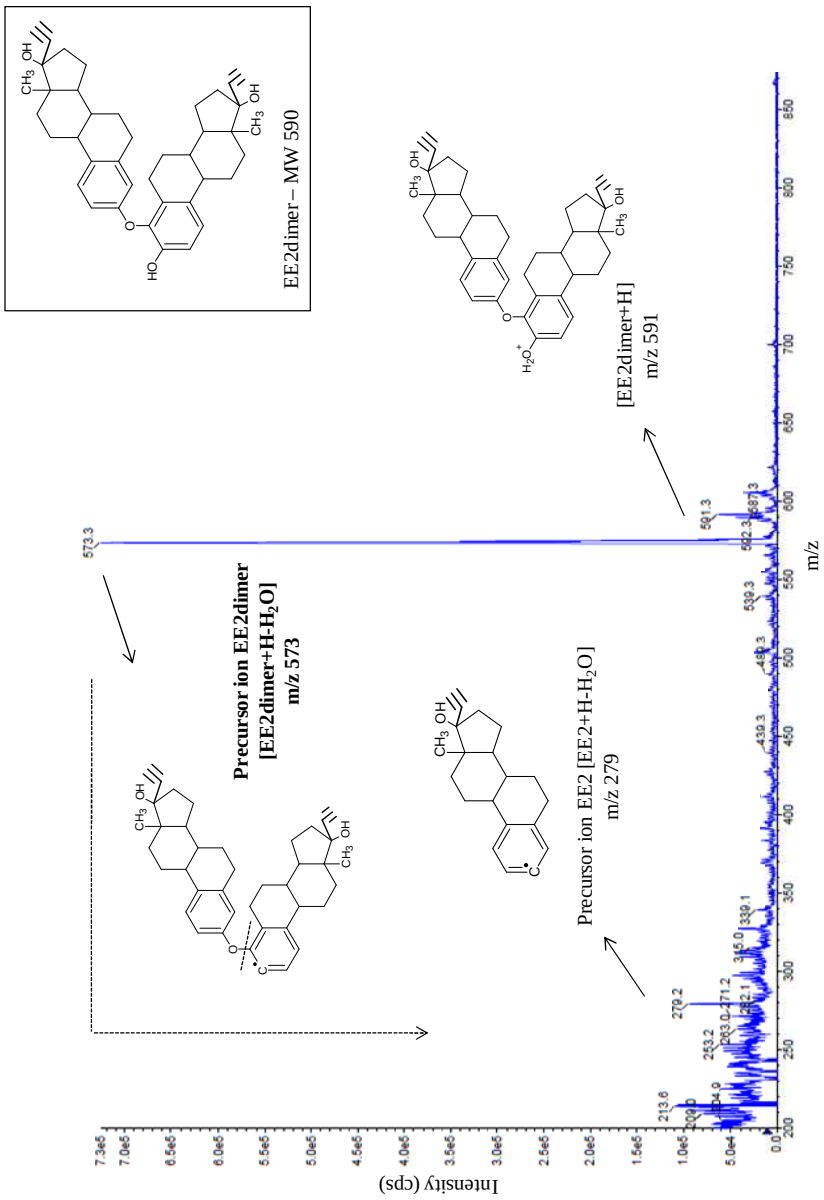


Figure 5.8. Mass spectrum of EE2 dimeric product detected by LC-APCI including structures and possible fragmentation patterns.

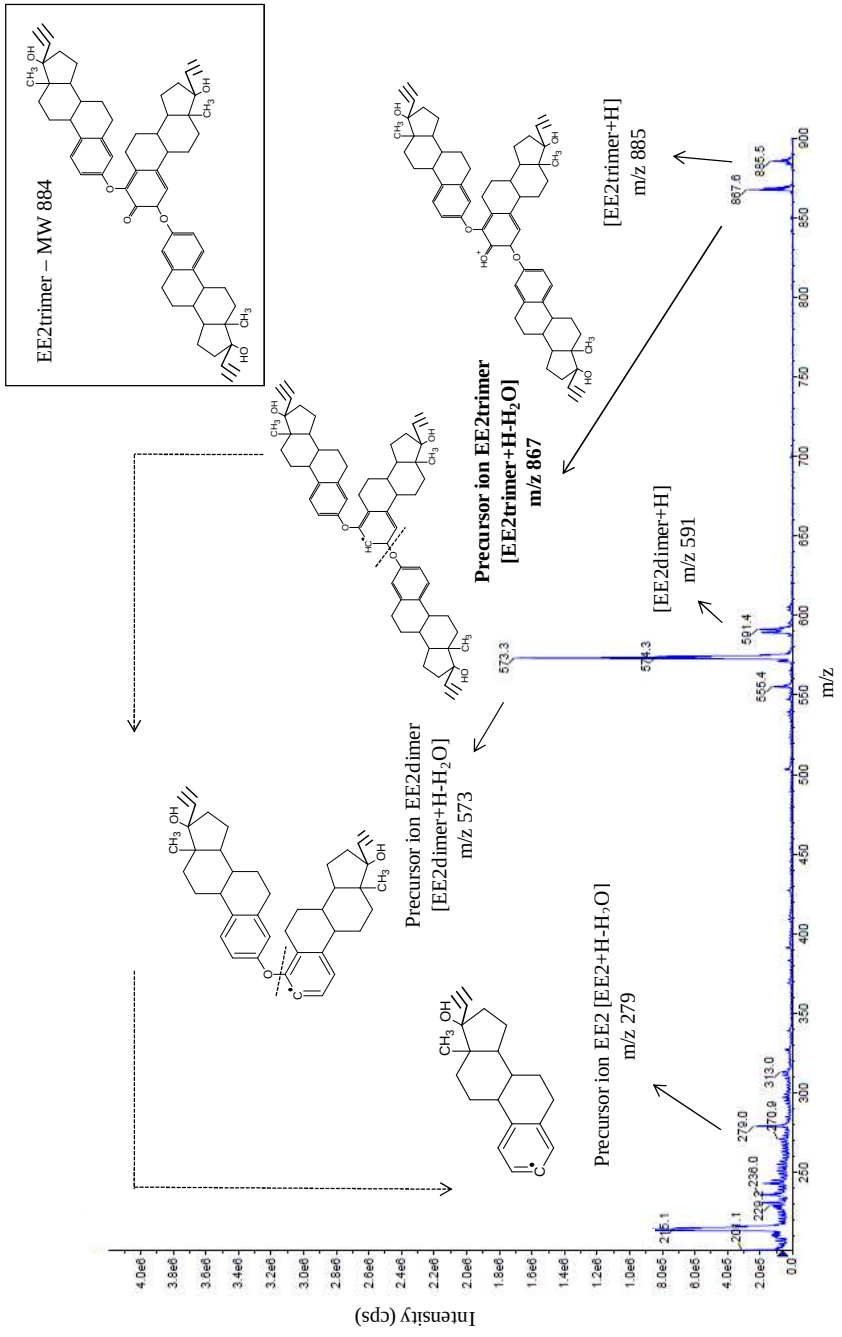


Figure 5.9. Mass spectrum of EE2 trimeric product detected by LC-APCI including structures and possible fragmentation patterns.

These findings increased the possibility that the biotransformation products are dimers and trimers. Anyhow, samples were analyzed by LC-ESI-TOF in order to obtain accurate masses information of the biotransformation products. Considering the chemical formula of E1, E2 and EE2: $C_{18}H_{22}O_2$, $C_{18}H_{24}O_2$ and $C_{20}H_{24}O_2$, respectively, the dimers and trimers of these target compounds would be: $C_{36}H_{42}O_4$ and $C_{54}H_{62}O_6$, $C_{36}H_{46}O_4$ and $C_{54}H_{68}O_6$, and $C_{40}H_{46}O_4$ and $C_{60}H_{68}O_6$, respectively. Once ESI was used as ionization source in negative mode, parent compounds were deprotonated and seen as M-H. As observed in Table 5.2, chemical formula of the detected biotransformation products matched with the deprotonated dimers and trimers of the estrogens, and also E1 was confirmed to be an E2 product.

Table 5.2. Accurate masses of the transformation products (deprotonated compounds) detected by LC-ESI-TOF.

Parent compound	Suggested product	Elemental composition	Theoretical mass	Experimental mass	mDa	error (ppm)
E1	Dimer	$C_{36}H_{41}O_4$	537.3010	537.3037	-2.6	-5.0
				537.3038	-2.7	-5.2
E2	Dimer	$C_{36}H_{45}O_4$	541.3323	541.3320	0.3	0.6
				541.3315	0.8	1.5
				541.3334	-1.1	-2.0
				541.3325	0.9	-0.4
	Trimer	$C_{54}H_{67}O_6$	811.4943	811.4954	-1.1	-1.4
				811.4942	0.1	0.1
				811.4943	0.0	0.0
	E1	$C_{18}H_{21}O_2$	269.1547	269.1536	-1.6	4.1
EE2	Dimer	$C_{40}H_{45}O_4$	589.3323	589.3312	1.2	1.9
				589.3328	-0.4	-0.8
				589.3330	-0.6	-1.2
				589.3323	0.0	0.0
	Trimer	$C_{60}H_{67}O_6$	883.4943	883.4949	-0.5	-0.7
				883.4963	-2.2	-2.3
				883.4959	-1.6	-1.8
				883.4954	-1.1	-1.3

Furthermore, experimental accurate masses were in agreement with the calculated ones with errors varying from -5.2 to 4.1 ppm and also, the values fitted those found in literature (Chen et al. 2007, Pezella et al. 2004). A larger number of dimeric and trimeric compounds were detected by LC-ESI-TOF in comparison to LC-APCI analysis, probably due to the higher sensitivity of the method.

The formation of dimers would be also expected after the treatment of environmental concentrations considering the findings of previous investigations (Mao et al. 2009, Pezella et al. 2004). Although the identification of coupling products by LC-APCI in the samples collected after 4 h of laccase-catalyzed transformation of estrogens from secondary effluent was not possible, dimers of E2 and EE2 were successfully identified by LC-ESI-TOF: products with accurate masses of 541.3343 and 541.3342 ($C_{36}H_{45}O_4$) and 589.3330 ($C_{40}H_{45}O_4$), respectively, were found with relative errors from -3.7 to -1.2 ppm. Although the possibility of other reaction mechanisms could not be discarded, these results indicated that laccase-catalyzed radical coupling reactions occur even at such low concentrations.

5.3.1.3. Proposed reaction pathways for laccase-catalyzed transformation of estrogens

As mentioned, the formation of dimers and trimers suggests the elimination via radical coupling reactions by laccase-catalyzed oxidation of the substrates to generate free radicals, which may couple covalently to each other subsequently. In fact, the products followed the pattern of nMW-2(n-1), where n is the number of monomers and MW the mass of the parent compound. However, it is interesting to highlight that in the case of E1, other species having smaller MW than the initial compounds were found, which may indicate that radical coupling is not the only transformation via but also different degradation mechanisms are involved. These mechanisms may include cleavage of the aromatic rings as suggested by Suzuki et al. (2003) and confirmed by Nicotra et al. (2004) using nuclear magnetic resonance when applying laccases for the removal of estrogens. Besides, hydroxylation reaction may occur as observed for EE2 transformation.

Anyhow, reaction pathways were proposed for E2 including radical coupling and transformation into E1: the suggested products structures and possible reactions are schematized in Figure 5.10.

Regarding the oxidative radical-radical coupling, the reaction is initiated by the laccase-catalyzed formation of the primary oxidation product by abstracting

one electron from the -OH group of the original molecule. Thus, the free radical is formed and the unpaired electron may delocalize through resonance to the respective conjugated positions (E2 radical intermediates: compounds 1-3). Thereafter, the subsequent covalent bonding between radical intermediates could occur through C-C or C-O bond formation. Mao et al. (2010a) reported that oxygen atoms have higher charges and lower spin density than carbon atoms, making bond formation at these sites kinetically less favorable. Anyhow, both possibilities were considered and the possible structures of the dimers are indicated as C-O and C-C dimeric products in Figure 5.10 (compounds 4-7).

Due to the remaining laccase activity and that the coupling products are still substrates of the enzyme due to their phenolic groups, radical coupling reaction can be further performed. Thus, the abstraction of other electron from one of the -OH groups of the dimeric products would occur. Once different dimeric products may have been formed and they present various -OH groups, there exist several possibilities of forming dimer radical intermediates. For instance, it is indicated in Figure 5.10 the radical intermediates formed from the C-O (compound 4) and C-C (compound 6) dimeric products resulted from the abstraction of one electron of the -OH groups indicated (marked with asterisks) to form the corresponding oxygen radicals than can delocalize to carbon-located radicals (intermediates 8 and 9 for the C-O dimer and intermediates 10 and 11 for the C-C dimer). Afterwards, the presence of radical intermediates of E2 in the reaction medium could lead to the formation of E2 trimers via radical-radical coupling mechanism. As example of possible E2 trimers formed, the products resulted from the covalent bonding between the second radical form of each pair of dimer radical intermediates (compounds 9 and 11) and both radical forms 1 and 2 of E2 are shown (compounds 12-15).

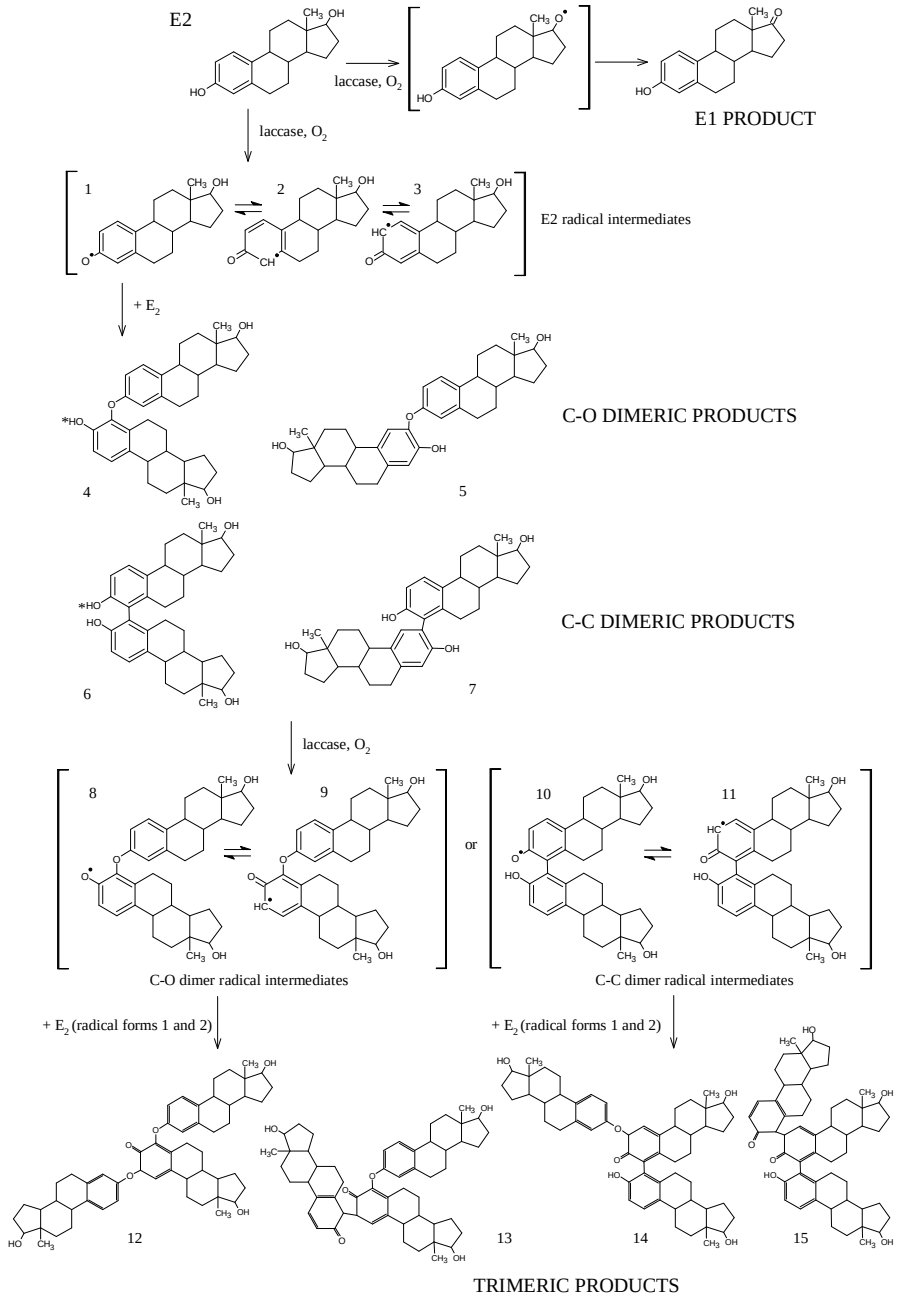


Figure 5.10. Proposed reaction pathways of E2 laccase-catalyzed transformation.

5.3.2. Identification of laccase-catalyzed transformation products of DCF and proposed reaction pathways

Prior to the analysis of the samples by GC-MS for the monitoring and detection of laccase-catalyzed transformation products of DCF, BSTFA was used for the derivatization of the species by silylation: the hydrogen in the carboxylic acids of the compounds was replaced by a trimethylsilyl group (-$SiMe_3$) with MW of 73. Accordingly, DCF with a MW of 296 increases to 368 after derivatization (296-1+73). Nevertheless, deprotonation occurred during the GC-MS analysis and thus, DCF is seen as a compound with MW of 367.

The chromatograms of the samples at different reaction times proved the degradation of DCF (retention time 26.90 min) into two products: P1 (23.05 min) and P2 (24.57 min), as shown in the chromatograms (Figure 5.11), once both compounds were not detected either in the samples at time 0 or in the controls. It was demonstrated, as observed during the analysis by high-performance liquid chromatography of samples from batch experiments in Chapter 2 that nearly complete removal of DCF occurred within 4 h of laccase-catalyzed reaction (Figure 5.11.A). Product P1 was found to be formed in parallel with the disappearance of DCF (Figure 5.11.B) whereas product P2 appeared after 15 min, then its concentration increased at 30 min and it was maintained practically constant after 2 h (Figure 5.11.C). Furthermore, analysis of samples taken at time 8 and 24 h evidenced P1 and P2 degradation after complete elimination of DCF: P1 and P2 were removed by 48 and 75% after 8 h, respectively, and nearly complete transformation was observed after 24 h (percentages calculated in peak area basis with respect to P1 and P2 peak areas found at 4 h). These results suggest that the generation of P1 and P2 by DCF removal occurs concomitantly to their transformation and thereby, they are probably laccase substrates; this may explain the low P1 and P2 peak signals in contrast to those of DCF, although comparison of concentrations cannot be made as no standards of the products were available. Unfortunately, the detection of other products which would result from the P1 and P2 transformation was not possible. Mass spectra corresponding to DCF and the biotransformation products detected by GC-MS, P1 and P2 are shown in Figure 5.12.

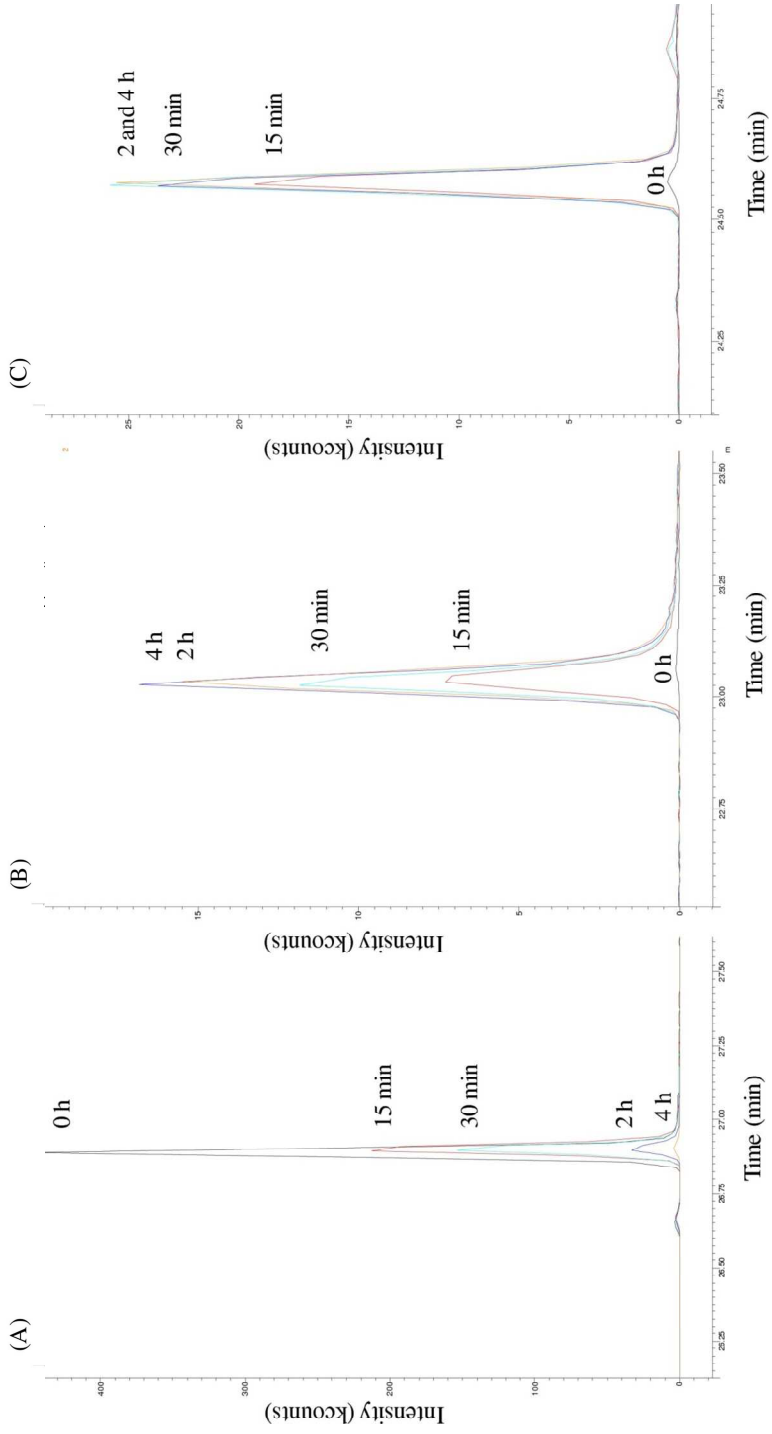


Figure 5.11. Selected ion monitoring chromatograms for DCF (m/z 367+277) (A), P1 (m/z 265+167) (B) and P2 (m/z 339+214) (C) of the samples withdrawn at initial time and after 15 and 30 min and 2 and 4 h of laccase-catalyzed transformation of DCF.

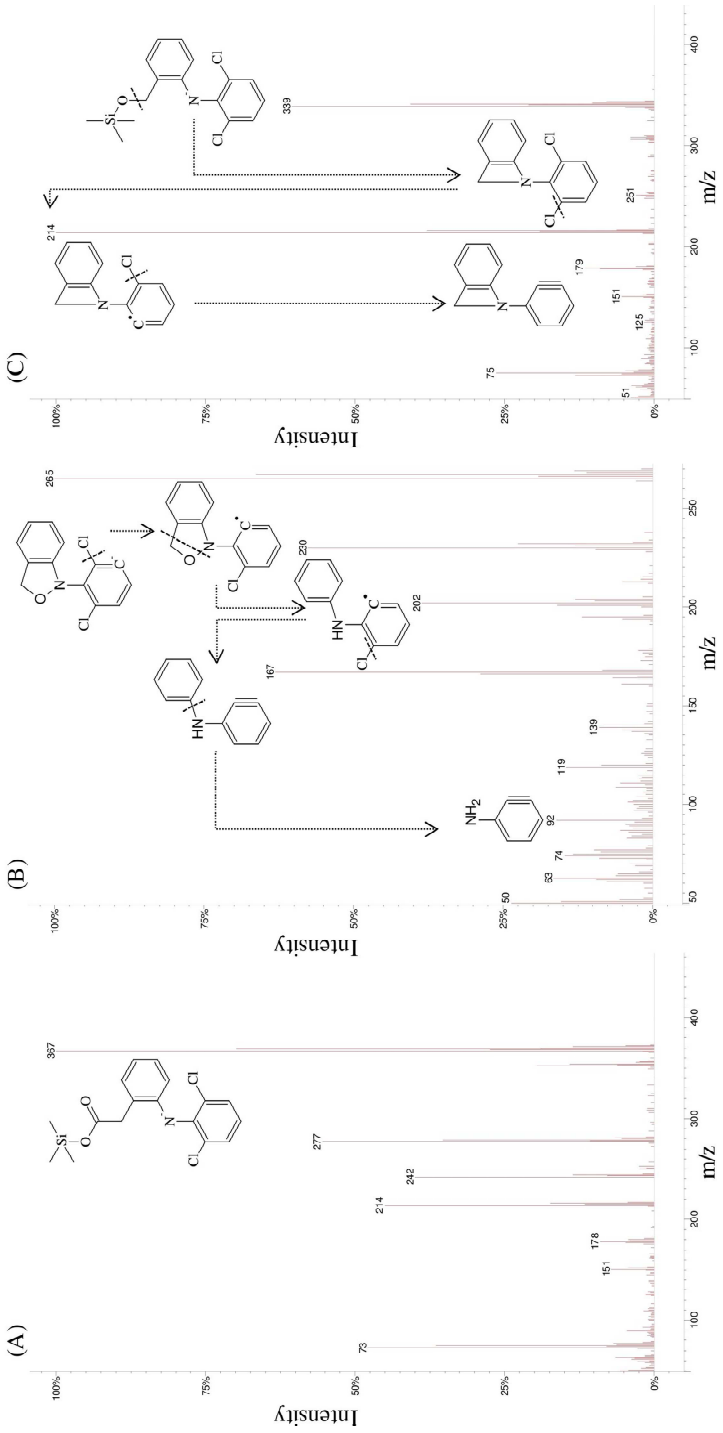


Figure 5.12. Mass spectra corresponding to derivatized and deprotonated DCF (A), deprotonated P1 (B) and derivatized and deprotonated P2 (B) including the proposed fragmentation patterns for the products.

Previous authors described different outcomes regarding the identification of anti-inflammatories transformation products and moreover, scarce reports can be found on the enzymatic removal of DCF. Marco-Urrea et al. (2010b) detected 4-(2,6-dichlorophenylamino)-1,3.benzenedimethanol as biotransformation product of DCF by laccase when dealing with the identification of reaction products resulted from the *in vivo* treatment assays using *Trametes versicolor* pellets (Figure 5.1.C); but that compound was not detected here. The same authors also demonstrated the formation of hydroxylated products from DCF: 4'-OH-DCF and 5-OH-DCF (Figures 5.1.A and B). These compounds were widely found when using different fungi. For instance, Rodarte-Morales et al. (2012a) identified 4'-OH-DCF as reaction product product of DCF by free and immobilized *Phanerochaete chrysosporium* and Hata et al. (2010) detected not only 4'-OH-DCF and 5-OH-DCF but also 4',5-OH-DCF after fungal transformation with *Phanerochaete sordida* YK-624. Nevertheless, only products with MW lower than that of DCF were detected in this work.

On the other hand, AOPs such as heterogeneous catalytic oxidation with H_2O_2, UV/H_2O_2 or ozonation led to hydroxylation reactions and subsequent C-N cleavage by the attack to the N-H bond followed by the opening of the ring (Hofmann et al. 2007, Vogna et al. 2004). In addition, laccase-catalyzed ring-cleavage of aromatic compounds has been reported (Claus 2004). However, none of the compounds resulted from these reactions were identified in this research once their mass spectra would not fit with those of P1 and P2.

Regarding the identification of P2, the difference of 28 units between its molecular ions and that of DCF suggests a primary alcohol structure in P2 instead of the carboxylic group of DCF (Figure 5.12.C). In addition, the isotopic distribution, i.e. presence of an ion at m/z 339 with an abundance about 2/3 of molecular ion, is characteristic of the presence of two chlorine atoms in the chemical structure, indicating that the product molecule still contains these atoms. Hence, a decarboxylated derivative of DCF with MW 268 was proposed as P2 compound, which would be seen as 339 due to derivatization and deprotonation. Besides the ion at m/z 339, other ion at m/z at 251 is depicted in the derivatized and deprotonated P2 spectrum, which could correspond to dehydration and loss of the trimethylsilyl group bound. The subsequent loss of a chlorine atom would produce the ion m/z 214 and the loss of the second chlorine atom would lead to the m/z 179 ion. This possible fragmentation mechanism corroborated the identification of P2 as the decarboxylated compound namely [2-[(2,6-dichlorophenyl)amino]phenyl]methanol. According to these results, the laccase-

catalyzed transformation of DCF to P2 implies a loss of CO_2 to form 2,6-dichloro-N-o-tolylbenzenamine (not detected), and afterwards this reaction may undergo $^{\bullet}OH$ bonding to yield the product P2 (Figure 5.13). Indeed, decarboxylation has been previously proposed as an example of laccase-catalyzed oxidative reactions (Majeau et al. 2010). The formation of the same transformation product was previously reported by Soufan et al. (2012) when investigating the chlorination of DCF, as well as by Martinez et al. (2011) after DCF degradation through heterogeneous photocatalysis.

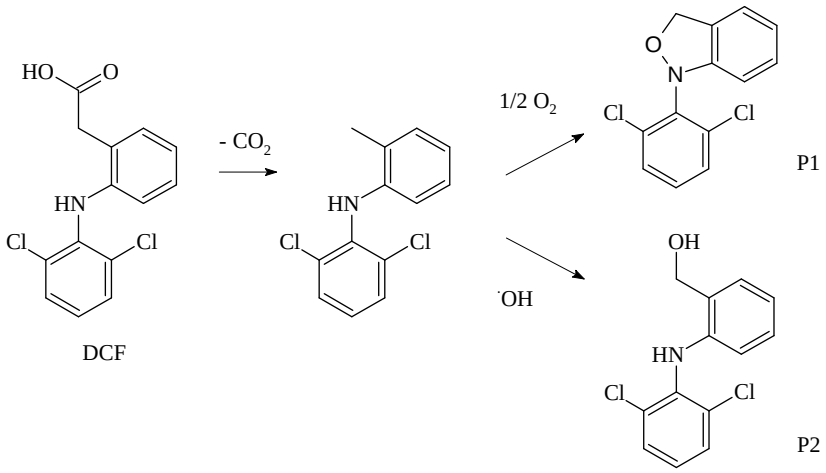


Figure 5.13. Proposed laccase-catalyzed biotransformation pathways of DCF into P1 and P2.

Following the same hypothesis, P1 was proposed as biotransformation product resulted from decarboxylation reaction of DCF and subsequent incorporation of oxygen (Figure 5.13). This compound would have a MW of 266 and would be seen as its deprotonated but no derivatized form with a MW of 265. Indeed, the proposed fragmentation mechanism of that product would adequately fit the m/z values of P1 spectrum (Figure 5.12.B).

The potential identification of P1 and P2 as decarboxylated products from DCF may explain the fact that these products are removed along with their generation by DCF transformation, as suggested above. It is known that carboxylic acid group is a strong electron withdrawing group (EDG) and thus, it diminishes the availability of the molecule towards oxidative attack. Therefore, P1 and P2 might be potential laccase substrates once they do not contain such EDG. In fact, their peak area markedly decreases after 4 h when DCF, which is

present in the medium at much higher concentration and thus being more rapidly transformed, was completely removed.

As laccases are known to oxidize certain organic substrates to free radicals which undergo oxidative coupling reactions resulting in the formation of dimers, oligomers and polymers (Hollmann and Arends 2012), as found for laccase-catalyzed transformation of estrogens, further work could be performed aiming to elucidate the complete laccase-catalyzed degradation mechanism of DCF by attempting the identification of dimers and trimers of this substrate by means of analytical techniques which permit to evaluate m/z rations higher than those examined by GC-MS, such as LC-APCI.

5.4. Conclusions

As commercial standards of the compounds were not available, an accurate identification was not possible; anyhow a tentative characterization of the transformation products of E1, E2, EE2 and DCF was conducted and the corresponding reaction pathways were proposed in this chapter.

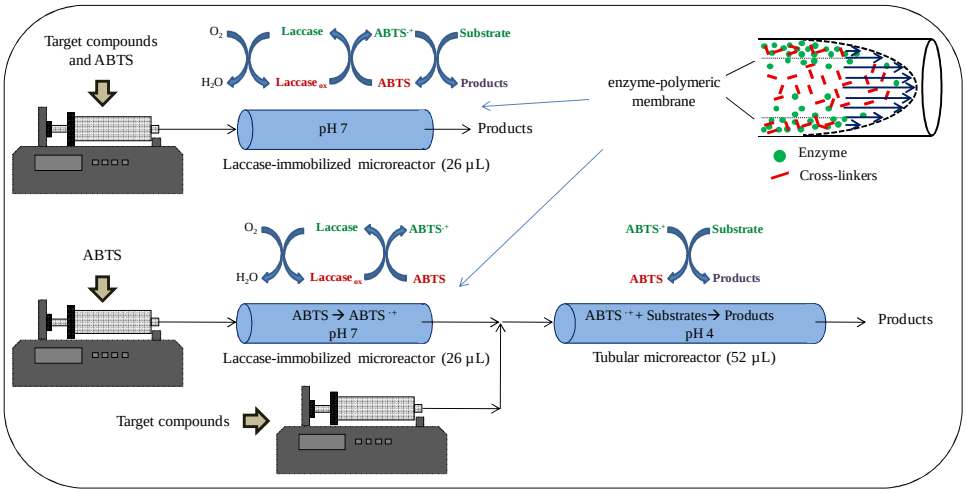
GC-MS analysis of samples taken after transformation of E1 and E2 by *Myceliophthora thermophila* laccase demonstrated the formation of byproducts although their identification was not possible. Anyhow, LC-APCI in full scan mode allowed the successful characterization of different dimers not only of E2 and EE2, but also of E1. Additionally, the formation of E2 and EE2 trimers as well as the transformation of E2 into E1 by laccase-catalyzed treatment was demonstrated. In addition, these findings were corroborated by the application of LC-ESI-TOF to obtain accurate masses of the products, which adequately matched the theoretical values and were also in agreement with those found in literature. In view of these valuable outcomes, reaction pathways were proposed, using E2 as model, mainly based on radical coupling reactions. However, the detection of products with MW lower than that of the parent compounds suggested that different degradation mechanisms might be also involved.

Samples withdrawn during the removal of DCF by *Trametes versicolor* laccase were analyzed by GC-MS aiming to monitor the presence in the reaction medium of both DCF and biotransformation products. The results evidenced the transformation of DCF into two different compounds which appeared to be progressively formed along with the DCF elimination until 4 h of reaction. Interestingly, both products were removed by laccase once DCF was completely transformed. Thereby, it was concluded that the products might be laccase substrates, which may explain the low peak areas found of these compounds.

However, the results showed that after 24 h of experiment no presence of DCF or the transformation products were detected, suggesting either DCF mineralization or DCF and products transformation to non-detectable metabolites. Regarding their spectra and considering the presence of chlorine atoms suggested by the isotopic distribution, both biotransformation products were identified as the compounds resulted from DCF decarboxylation and subsequent incorporation of oxygen or hydroxide.

Chapter 6

Development and application of laccase-immobilized microreactors: exploring an emerging technology*



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Outline

6. Development and application of laccase-immobilized microreactors: exploring an emerging technology

6.1. Introduction

Microreactor systems are a novel and promising technology in the fields of chemistry, chemical engineering and biotechnology thanks to a number of advantages. These reactors can be assembled by microfabrication techniques or by the modification of microcapillaries and use reaction apparatus with dimensions in the range of micrometers (μm) with handling volumetric capacities in the range of microliters (μL) (Kiwi-Minsker and Renken 2005).

These systems take the advantages of micro- or nano-fluidics to enable the use of drastically reduced volumes of reactant solutions and offer high efficiency and repeatability, better selectivity and flexible production (Asanomi et al. 2011, Chovan and Guttman 2002, Miyazaki et al. 2006). Furthermore, they present important benefits in the performance of chemical reactions in comparison with traditional methods: increased heat exchange and mass transfer, process intensification, relatively large surface and interfacial areas, and moreover the streams in microfluidics mainly form a laminar flow which allows a strict control of the reaction conditions (Honda et al. 2005, Miyazaki et al. 2008). Rapid screening and low material requirements are also potential advantages of miniaturized systems (Alam et al. 2011, Bolivar et al. 2011). Moreover, the use of microreactors favors the scale-out of the system by the parallel operation of several reaction devices, which permits the extension of reaction conditions optimized in a single reactor; thus, eliminating scale-up problems arising from the conventional process (Bolivar et al. 2011, Miyazaki et al. 2008).

These features make the microreactor technology suitable for its application on catalytic reactions such as the biotransformation of a wide range of compounds, biosynthesis and bioanalysis. Hickey et al. (2009) carried out the conversion of benzamide to benzoic acid by γ-lactamase using capillary tubes packed with cross-linked enzyme and Pohar et al. (2012) considered packed-bed microreactors for the synthesis of butyl butyrate by lipase. Another interesting example is the work reported by Matsuura et al. (2011), who developed a microreactor containing lipase-nanoporous material composites for the hydrolysis of a triglyceride. Regarding the use of microreactors for analytical techniques, Heijnis et al. (2012) performed the in-line quantification of peroxidase-catalyzed cross-linking of α-lactalbumin in a Y-shaped microreactor. Interestingly, Yamaguchi et al. (2009) applied protease-immobilized microreactors for the

analysis of the protein sequence with improved results in terms of promptness and reliability, probably due to large area-to-volume ratio and reduced diffusional constraints in the microsystems.

The microstructured flow reactor also constitutes a potent scale-down system in which a range of process conditions can be investigated in a relatively short time. Thus, microreactors are also useful in screening of substrates, enzymes, reaction conditions as well as for the determination of kinetic parameters. For example, Matosevic et al. (2011) prepared microreactors based on the attachment of His_6-tagged enzymes via Ni-NTA linkage to the surface of capillaries for the screening of multi-step conversions and the determination of kinetic parameters in the synthesis of chiral amino alcohols.

Despite all of these benefits, there are only few studies that consider microreaction systems for the application of laccases. For instance, Roman-Gusetu et al. (2009) prepared a capillary-size microreactor packed with encapsulated laccase by interfacial cross-linking for its coupling off-line to capillary electrophoresis for the monitoring of oxidation reactions. Lin et al. (2010) prepared magnetic microreactors with laccase immobilized on magnetite nanoparticles which were adhered on the inner wall of the microreactor due to external magnetic field forces. These methods require complicated multi-step procedures which imply undesirable costs and time of performance. Additionally, the use of a support in a packed-bed microreactor may lead to significant pressure drop, not suitable for long operational periods. Thus, the reduction of costs, effort and time in the manufacture of laccase-immobilized microreactors as well as the high performance and stability of the microreactors are the main challenges towards their implementation.

In this chapter, a straightforward method to immobilize laccases on microchannels is developed with the objective of improving the efficiency of laccase-catalyzed microreactions as well as broadening the range of application of these enzymes. The proposed procedure is based on the formation of an enzyme-immobilized membrane on the inner wall of microtubes as a result of the cross-linking polymerization reaction between the enzyme and bifunctional cross-linkers agents. This technique was previously assayed for the immobilization of acylase and chymotrypsin (Honda et al. 2005, 2006, Yamaguchi et al. 2009) and has been adapted here for the immobilization of laccase. During this process, the internal surface of the microchannel is covered by a cylindrical substrate membrane, composed of the cross-linked polymerized enzyme product formed during the reaction. The structure of this type of microreactor prevents high

pressure in comparison with packed-bed microreactors, and this carrier-free immobilization method would avoid the interactions between the enzyme and the carrier (Cao et al. 2003).

To sum up, the main goal of this work was to explore the emerging and novel technology of the microreactors and, at the same time, develop a simple, versatile and inexpensive method to prepare laccase-immobilized microreactors aiming to enhance the applicability of laccases and extend their use to those fields where microreactors are used as efficient and potent tools. Besides, the immobilized microreactors were characterized with respect to pH, temperature and stability of the biocatalyst under different conditions and also a kinetic study under continuous flow conditions was performed to elucidate the kinetic behavior during operation. Finally, the microreactors were applied for the continuous biotransformation of different model compounds and compared with the efficiency attained by conventional reactor alternatives.

6.2. Materials and methods

6.2.1. Materials, chemicals and enzyme

Glutaraldehyde (GA), paraformaldehyde (PA), triethylamine (TEA) and phenyl isothiocyanate (PITC) were obtained from Wako Pure Chemical. Poly(L)-lysine hydrobromide, the anti-inflammatories: diclofenac (DCF) and naproxen (NPX), the estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) and the mediators: 1-hydroxibenzotriazole (HBT) and syringaldehyde (SA) were purchased from Sigma-Aldrich. Stock solutions of the target compounds were prepared in methanol (HPLC grade, 99.8%, Wako). 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was purchased from KPL. Laccase from *Trametes versicolor* was also purchased from Sigma-Aldrich. All the other chemicals were of analytical grade.

Poly(tetrafluoroethylene) (PTFE) microtubes of 500 µm inner diameter (i.d.) and 1.59 mm outer diameter (o.d.), PTFE adapter and heat-shrink tubing were purchased from Flon Chemical. Silica-fused microcapillaries (100 µm i.d., 350 µm o.d.) and stainless-steel T-shaped connectors (Union Tee, SUS-316) were provided by GL Science Co.

6.2.2. Preparation of laccase-immobilized microreactors

Laccase-immobilized microreactors were prepared by adapting the procedure described for the immobilization of chymotrypsin and acylase (Honda et al. 2005,

2006). The method was based on the formation of a cylindrical polymeric membrane on the inner wall of microtubes. A scheme for microreactor preparation is shown in Figure 6.1.

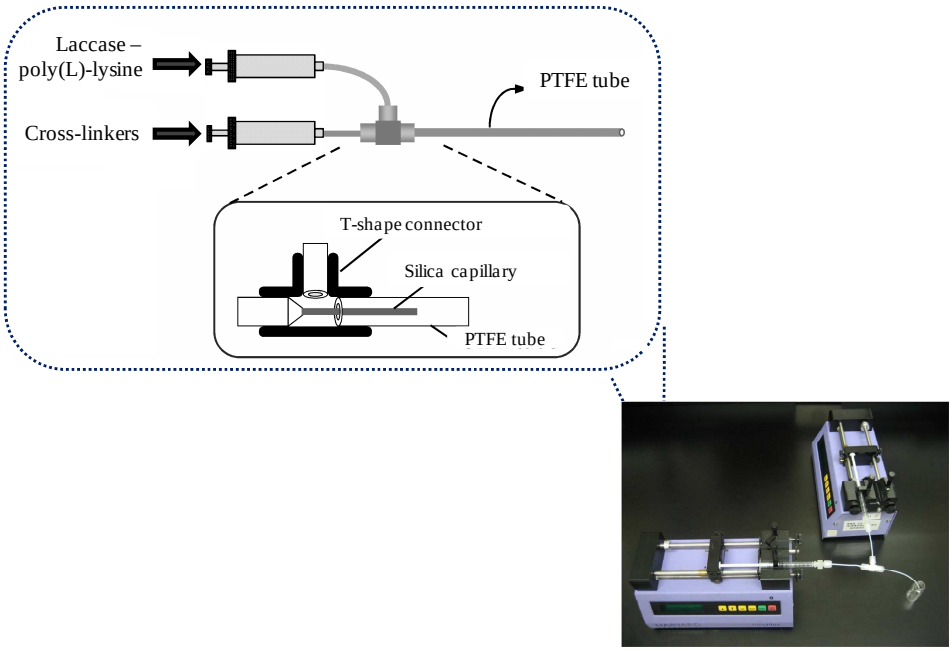

Figure 6.1. Preparation of laccase-immobilized membrane on the inner wall of a PTFE microtube.

A mixed solution of laccase and poly(L)-lysine at concentrations of 0.5 and 1 mg/mL, respectively, was prepared in a 50 mM phosphate buffer solution (pH 8). On the other hand, stock solutions of the cross-linkers: GA (25%, v/v) and PA (20%, v/v), were mixed in the same buffer at a ratio of 1/16 (v/v) which was previously optimized (Honda et al. 2005, Yamaguchi 2011).

For a simple and inexpensive preparation, a commercially available PTFE microtube (500 µm i.d. and 13 cm length, 26 µL of total volume) was used for the microreactor preparation. The PTFE tube was supplied with the solutions of laccase-poly(L)-lysine and cross-linkers for 3 h at 4ºC at flow rates of 0.50 and 0.75 µL/min, respectively, by means of Pico Plus 1 mL syringe pumps (Harvard Apparatus) and through respective PTFE tubes which were connected to the system by means of a T-shape and female luer connectors. Moreover, the injection of the cross-linkers solutions was performed through the central region of the microtube by means of a fused silica microcapillar which was set in the T-

shape connector and placed in the concentric position of the PTFE microtube, aiming to form a concentric laminar flow.

After 3 h of polymerization, the injection of both solutions was stopped and the microtube, containing now the immobilized-laccase layer, was disconnected from the laccase-poly(L)-lysine and cross-linkers inlets. Then, the obtained microreactor was rinsed with 1 M Tris-HCl (pH 8), which simultaneously quenched active aldehyde groups remaining on the membrane, by passing the solution at 20 µL/min for 10 min. Afterwards, the microreactor was treated with 50 mM $NaBH_4$ in borate buffer (pH 9) at 20 µL/min for 10 min in order to reduce the resulting Schiff base. Thereafter, the microreactor was extensively washed by circulating phosphate buffer (50 mM, pH 8) at a flow rate of 20 µL/min for 10 min. Finally, the laccase-immobilized microreactor was filled with phosphate buffer (50 mM, pH 8) and stored at 4°C until its use.

6.2.2.1. Effect of the cross-linkers concentration on the laccase immobilization

The effect of the cross-linkers concentration on the immobilization was studied by preparing the microreactors as previously described, with a GA/PA ratio of 1/16 (v/v). The concentrations tested for GA and PA were: 0.125:2, 0.25:4, 0.376:6, 0.5:8, 0.562:9 and 0.625:10 (v/v ratio).

The immobilization efficiency was calculated as the percentage of immobilized protein referred to the total protein used for the preparation of the laccase-immobilized microreactor, determined by amino acid analyses. In addition, the catalytic efficiency of each microreactor was determined by its continuous performance with ABTS as substrate to measure its laccase activity. The relative activity was calculated as the ratio between the laccase activity detected in each microreactor at different cross-linker concentrations and the maximum activity attained

6.2.3. Protein estimation by amino acid analysis

The amount of immobilized protein was determined by quantitative amino acid analysis. After washing the microreactor with distilled water, it was cut in small fragments (5 mm), mixed with 400 µL of HCl (6 M) and incubated at 110ºC for 24 h to promote protein hydrolysis. The liquid phase was evaporated by using a rotary-evaporator and the obtained solid was washed three times with 1 mL of distilled water. The solid was suspended in 500 µL of distilled water and freeze-dried overnight. The neutralization was then conducted by mixing the product

with 30 µL of a solution containing ethanol, distilled water and TEA (2:2:1 v/v), and freeze dried for 2 h. The product was reacted to PITC by mixing the solid with 30 µL of a solution of ethanol, TEA, distilled water and PTIC (7:1:1:1, v/v) at room temperature for 20 min and later dehydrated. Finally, the obtained solid was re-suspended in 1 mL of potassium acetate buffer (60 mM, pH 6) containing 6% (v/v) of acetonitrile.

The PTIC-labeled amino acids were analyzed by high performance liquid chromatography (HPLC) system equipped with a UV detector and a Wakosil-PTC column (4.0 mm x 20 mm, Wako Pure Chemical). The operational conditions for the analysis were: 50 µL injection volume, wavelength of 254 nm, 40ºC and eluent solutions of acetonitrile of 6% and 60% (v/v) in potassium acetate buffer (60 mM, pH 6) in isocratic conditions of 30:70% at a flow rate of 1 mL/min. The amount of immobilized laccase was determined according to the amino acid composition of the laccase standard.

6.2.4. Continuous laccase activity assay

The enzymatic activity of the laccase-immobilized microreactors was evaluated by the oxidation of 50 µM ABTS under standard conditions (50 mM potassium acetate buffer pH 5 and 30ºC) in a continuous flow. The microreactor was washed with 50 mM potassium acetate buffer (pH 5) at a flow rate of 20 µL/min for 2 min. Then, the solution of ABTS prepared in the same buffer was injected at a flow rate of 10 µL/min with a hydraulic residence time (HRT) of 2.6 min. After operating the reactor during two times the HRT, a sample from the outlet was withdrawn and the absorbance was measured at 405 nm (ε = 36,800 1/(M·cm)). The enzymatic activity of each microreactor was determined as the concentration of substrate oxidized per residence time (µM/min). The microreactor was washed prior to reuse with 50 mM phosphate buffer (pH 8) at a flow rate of 20 µL/min for 10 min and stored at 4°C.

6.3. Results and discussion

6.3.1. Preparation of laccase-immobilized microreactors

The preparation of the laccase-immobilized microreactors (Figure 6.1) consisted basically on the injection of two different solutions into a microtube, one containing laccase and poly(L)-lysine and the other containing the cross-linker agents. This procedure enabled the subsequent formation of an enzyme-polymeric membrane on the inner wall of the microchannel by means of cross-linking

polymerization in a concentric laminar flow. The immobilization mechanism is based on the formation of cross-linked enzyme aggregates (CLEAs) through the reaction of the aldehyde groups of the cross-linkers with the amino groups of the laccase-poly(L)-lysine mixture (Figure 6.2). Although this procedure has been previously applied to different enzymes in batch experiments (Cabana et al. 2007a, Matijošytė et al. 2010), in this work the reaction took place inside a microchannel and the solutions were continuously added in laminar flow. As a result, the cylindrical membrane, consisting on the cross-linked polymerized enzyme, covered the internal surface of the microchannel.

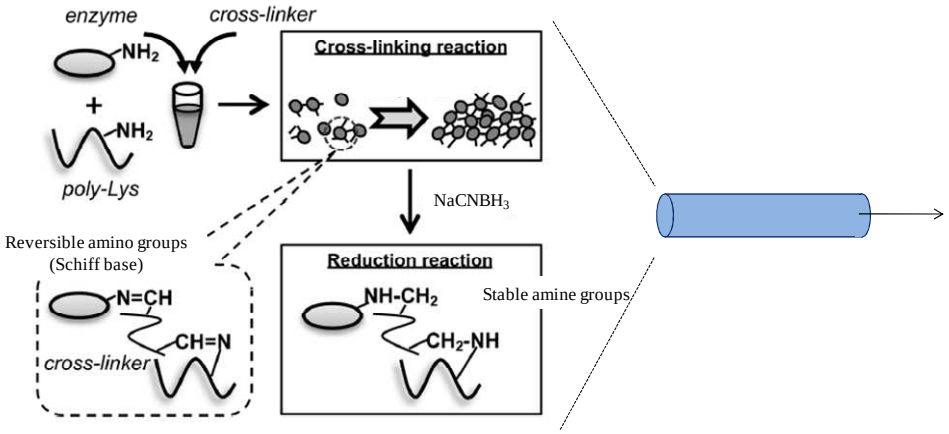

Figure 6.2. Scheme of the cross-linking reaction which takes places inside microchannels.

The formation of the membrane is deemed to be attributed to the parabolic velocity profile in the microtube due to the laminar flow regime (calculated Reynolds number was lower than 2,100), as indicated in Figure 6.3. According to that profile, the flow velocity in the central region of the microtube reaches its maximum and thus, when the complex begins to polymerize would be unable to have sufficient reaction time to render into insoluble aggregates due to the shorter residence time in such area. However, the velocity in the region close to the inner wall markedly decreases, being difficult for the products polymerizing in such area to drift forward in the direction of the flow.

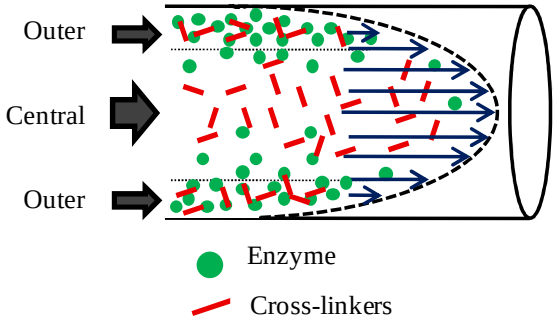

Figure 6.3. Parabolic velocity profile characteristic of the laminar flow inside the microtube.

These factors would lead to the formation of enzyme aggregates on the inner wall as compared to that in the central region of the microchannel, which would be also favored by the fact that the enzyme-poly(L)-lysine solution is injected through the outer area whereas cross-linkers are introduced through the central region by means of the microcapillar (Figure 6.1). Consequently, CLEAs of laccase are formed and laid on the internal surface, covering the inner wall of the microchannel and resulting in a polymeric cylindrical membrane. This phenomenon was verified by confocal microcopy, depicting a cylindrical membrane with a thickness of 40-60 µm (Figure 6.4).

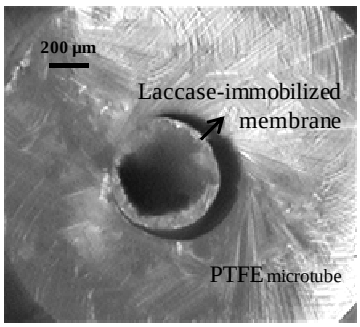

Figure 6.4. Confocal acquisition of the sectional view of the laccase-immobilized microreactor.

When a suspension of the reagents was prepared batchwise for the subsequent injection through the PTFE tube, no membrane was observed in the inner wall. Thus, it was concluded that the polymerization in the microfluid allowed the formation of a membrane structure on the inner wall of the microtube.

Furthermore, when a PTFE tube was flowed with the laccase-poly(L)-lysine solution without the cross-linkers, no enzymatic activity was detected. This indicated that laccase activity of the microreactors corresponded to the immobilized enzyme in the CLEA-based membrane.

Although there are slight differences between the batchwise formation of CLEAs and the CLEA-based microreactor, the preparation and operation of the microreactor is easier. For instance, the batchwise procedure requires a series of operations and washing procedures such as mechanical agitation, centrifugation and removal of the supernatant, while such steps are simply substituted by pumping in the microreactor system, leading to a simpler, more efficient and accurate procedure.

The method presented for the preparation and performance of a laccase-immobilized microreactor is straightforward and inexpensive and high pressure is not required. Moreover, the enzymatic immobilization takes place on site, which means a reduction in time and effort as well as prevention from possible losses of enzymatic activity related to multi-step procedures. Furthermore, this carrier-free method avoids the interactions between the enzyme and the support materials, avoiding the reduction of specific and volumetric activity of the biocatalyst (Fernández-Fernández et al. 2012).

6.3.1.1. Optimum conditions for the immobilization

The efficient formation of enzyme aggregates is essential for the successful preparation of the microreactor in which laccase is immobilized by cross-linking polymerization. The poor immobilization of electronegative enzymes due to the relatively low content of lysine residues has been reported, leading to inefficient aggregation (Honda et al. 2005). Thus, the use of a cationic polymer was recommended in order to promote the formation of an enzyme-enzyme complex prior to the cross-linking reaction (Cao et al. 2003, Honda et al. 2006). In the current work, poly(L)-lysine was selected as the coupling agent at a concentration of 1 mg/mL. Some authors have previously reported the use of albumin to improve the formation of co-aggregates of enzymes with low lysine residues content; however, that method could be inefficient to enhance stability (Matijošytė et al. 2010).

In preliminary experiments, the possibility of using a single cross-linker for the preparation of the microreactor was investigated. The use of high concentrations of GA, required for significant cross-linking yields, led to the obstruction of the tube. In the case of using only PA, the resulting membrane

formed was very fragile and gradually detached when washing the microreactor. Hence, the combination of GA and PA was concluded to be a key factor in the successful formation of a stable CLEA-based membrane.

The effect of the cross-linkers concentration on the immobilization efficiency and laccase activity has also been studied. Different GA:PA ratios: 0.125:2, 0.25:4, 0.376:6, 0.5:8, 0.562:9 and 0.625:10 (v/v) were evaluated. It is well known that enzymes may be inactivated by chemical modifiers, including cross-linking agents. On the other hand, the degree of cross-linkage is dependent on the amount of cross-linkers used. These effects were demonstrated by the results depicted in Figure 6.5.

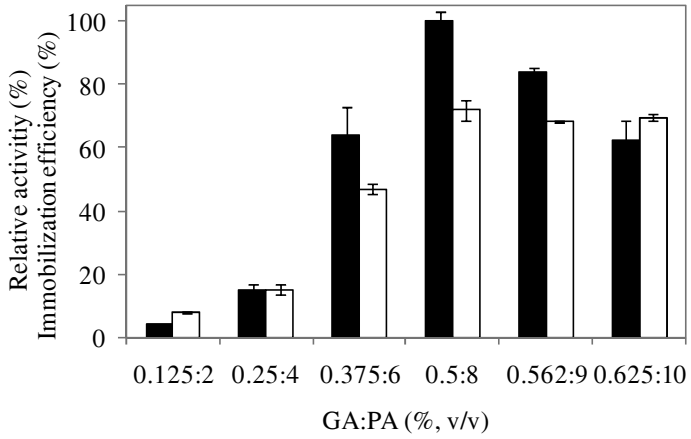

Figure 6.5. Effects of cross-linkers (GA and PA) concentration on laccase activity (black bars) and immobilization efficiency (white bars) of the microreactors. Data show the mean value of two independent experiments with standard deviations.

As expected, the progressive increase of GA and PA concentrations from 0.125 and 2% to 0.5 and 8% resulted in a significant improvement of immobilization yield and laccase activity. However, relative enzymatic activities decreased to 84% (37.8 μM/min) and 62% (27.9 μM/min) when higher concentrations were used, while immobilization efficiencies remained at 68-72%. This means that the maximum values for both immobilization yield (72%) and laccase activity (45 μM/min) were achieved at GA and PA concentrations of 0.5 and 8%, respectively. Thus, these conditions were selected for the preparation of the microreactors for the following experiments.

6.3.2. Biochemical characterization of laccase-immobilized microreactors

In order to investigate the effect of the immobilization on the behavior of the biocatalyst and examine the optimum operational conditions of the microreactors, the activity and stability of laccase-immobilized microreactors were investigated over a wide range of conditions.

The effect of pH on the laccase activity of the prepared microreactors was investigated in the range pH 2-7 in citrate-phosphate-borate buffer (50 mM), while the effect of temperature was tested at 20-70ºC (Figures 6.6.A and B). Results obtained for free laccase from *Trametes versicolor*, already shown in Chapter 2, are also represented in order to facilitate the comparison.

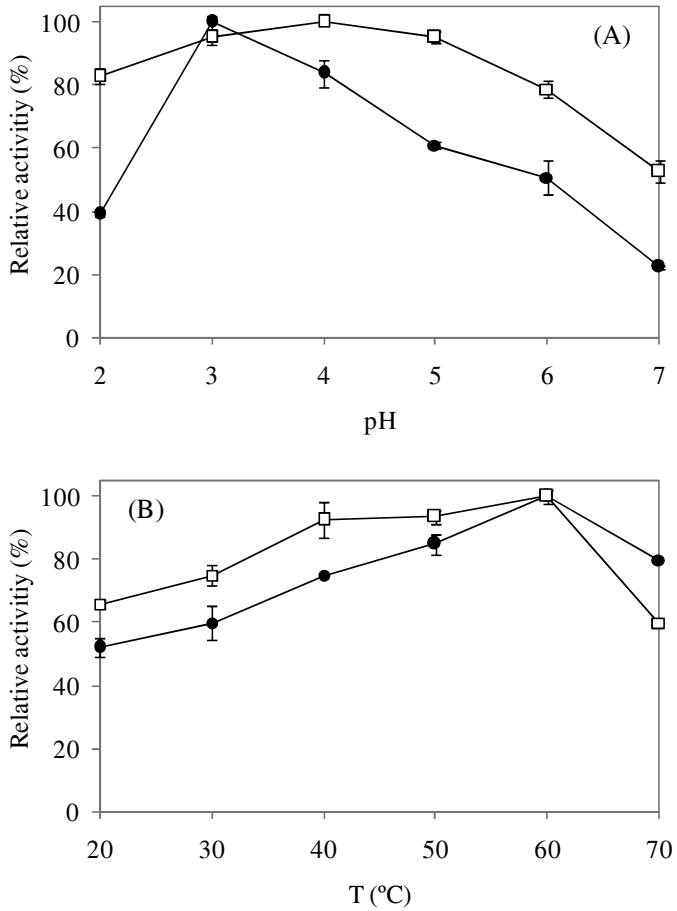

Figure 6.6. Effects of pH (A) and temperature (B) on the activity of free (●) and laccase-immobilized microreactors (□).

It was observed that the relative activity of the laccase-immobilized microreactor was significantly higher than that of free *Trametes versicolor* laccase in a pH range of 4 to 7. The improvement was not so significant when evaluating the effect of temperature, although the relative activity of the immobilized laccase was higher under all the conditions tested. The broader range of optimum activity against pH and temperature after immobilization might be caused by the modification of amino groups in the laccase and its restricted mobility (Honda et al. 2006), besides the buffering action of poly(L)-lysine matrix in the microenvironment of the enzyme (He et al. 1992).

Nevertheless, the efficiency of an enzyme-catalyzed process depends on its tolerance to inactivation over time under certain environmental conditions, commonly temperature and pH. In the current work, the effects of pH and temperature on enzyme stability of laccase-immobilized microreactors for 24 h at different conditions in the absence of substrate: pH 2 to 7 at 30ºC and 20 to 70ºC at pH 7. For this purpose, the corresponding buffer solution was continuously fed at a flow rate of 1 µL/min and the operation was stopped at different incubation times to transfer the microreactors to standard conditions in order to determine the residual laccase activity. The results after 4 h of operation are shown in Figure 6.7.A and B, where the results for free enzyme are also depicted for comparison.

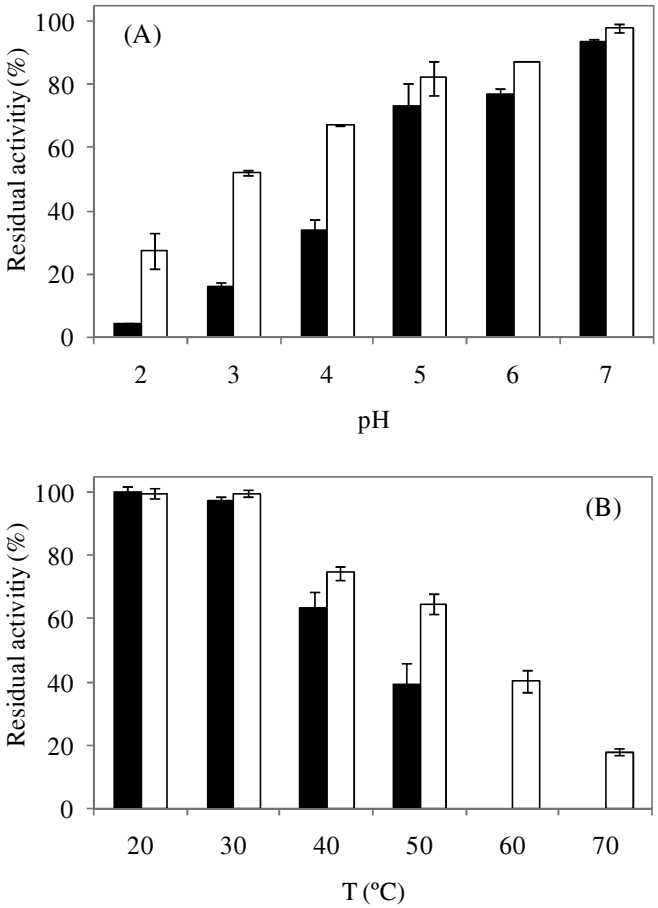

Figure 6.7. pH (A) and thermal (B) stability of free laccase (■) and laccase-immobilized microreactors (□).

It was demonstrated the improved stability of the laccase-immobilized microreactors in comparison with free enzyme. In this sense, free laccase only retained 5-30% after incubation at pH 2-4 and it was completely inactivated after only 4 h of incubation at high temperatures (60 and 70ºC), while the microreactors exhibited 25-70% and 20-40% of their initial activities under identical conditions. Furthermore, free laccase activity dropped more rapidly than that of immobilized laccase during 24 h of incubation (data not shown). This could be explained by the fact that cross-linking prevents the unfolding of laccase (Fernández-Lafuente et al. 1995). These findings agree those of Cabana et al. (2007a) for CLEAs of *Coriolopsis polyzona* laccase as well as Honda et al. (2006)

and Yamaguchi et al. (2009) for the immobilization of acylase and protease in microreactors.

The stability against different chemical inactivating agents was also assessed by circulating the buffer solution (sodium acetate buffer, pH 5) containing the different compounds through the microreactors at 1 µL/min and determining the residual activities at different incubation times under standard conditions. Aiming to compare the results with those for free enzyme, stability in the presence of these compounds was also evaluated for free laccase by performing batch experiments as those with *Myceliophthora thermophila* laccase in Chapter 3. The results are shown in Table 6.1.

Table 6.1. Residual activity (%) of free laccase and laccase-immobilized microreactors after incubation with the different inactivating agents evaluated.

Denaturant	Laccase-immobilized microreactor	Free laccase
NaN_3 (30 µM)	47.8 ± 0.8	30.2 ± 1.2
$CoCl_2$ (10 µM)	79.3 ± 5.3	44.5 ± 2.2
$CaCl_2$ (10 µM)	72.7 ± 0.9	56.6 ± 1.5
Methanol (25%, v/v)	63.5 ± 1.3	58.4 ± 4.2
Acetone (25%, v/v)	63.6 ± 0.7	44.3 ± 1.1

Laccase-immobilized microreactors presented significant stability against the organic solvents tested, probably due to the immobilization-based conformational rigidity which allows laccase to avoid the conformational collapse, responsible for inactivation not only by pH and heat but also organic solvents (He et al. 1992). Chymotrypsin-immobilized microreactors, prepared by a similar procedure, showed higher resistance to urea and dimethyl sulfoxide (DMSO) (Honda et al. 2005) and microreactors with immobilized acylase were also demonstrated to be efficient in the presence of N,N-dimethylformamide (DMF) (Honda et al. 2006).

Aiming to check the stability in long-term operation, a microreactor was operated under continuous flow of 500 µM ABTS at pH 7 and 30ºC for 12 days. Under all the flow rates tested (0.5, 2.5 and 10 µL/min), the residual activity was approximately 92% after the experiment. In addition, a microreactor was

subjected to high pressure with a flow rate of 5 mL/min of phosphate buffer. Neither the destruction of the membrane nor enzyme detachment from the microtube was observed, which indicated that the membrane has sufficient mechanical strength for microfluidic system applications. It was also demonstrated that the microreactors retained their initial activity after 3 months of storage at 4ºC.

6.3.3 Continuous flow kinetics of laccase-immobilized microreactors

In continuous flow kinetics, the investigation seeks to determine the variability of kinetic parameters with the flow rate (Jones et al. 2004, Matosevic et al. 2011). For this purpose, enzyme kinetics in continuous flow reaction systems are usually investigated using the Lilly-Hornby model (Lilly et al. 1966). This model enables the estimation of kinetic parameters for immobilized enzyme reactors and more specifically, the quantification of diffusional or mass transfer limitations which may be masking the true kinetics of the immobilized enzyme. The model is an adaption of the standard Michaelis-Menten model for enzyme kinetics and is described in Equation (6.1).

$$f \cdot [A]_0 = \frac{C}{Q} + K_{m(app)} \cdot \ln(1-f) \qquad (6.1)$$

where f is the fraction of substrate converted during the reaction, Q is the flow rate, $[A]_0$ is the initial concentration of substrate, C is the reaction capacity of the microreactor and $K_{m(app)}$ is the apparent Michaelis constant.

Thus, a kinetic study under continuous flow conditions was conducted in order to evaluate the effect of flow rate on the value of $K_{m(app)}$ of the laccase-immobilized microreactors at 30ºC. ABTS was used as substrate in the range 25-500 µM in phosphate buffer (50 mM, pH 7) and the inlet flow rate was varied in the range 2.5-20 µL/min (HRTs 10.4-1.3 min). The results obtained are shown in Figure 6.8.A. As expected, the increase in the flow rate implied lower conversion of ABTS. Moreover, the highest levels of initial ABTS concentration were shown to be more affected by the flow rate. From these data, linear plots of $f \cdot [A]_0$ versus -ln(1-f) were obtained by fitting experimental data to the Equation (6.1) (Figure 6.8.B). The slope of the straight-lines increased with the flow rate, which suggests that the apparent kinetics of laccase immobilized microreactor is significantly affected by mass transfer (Matosevic et al. 2011).

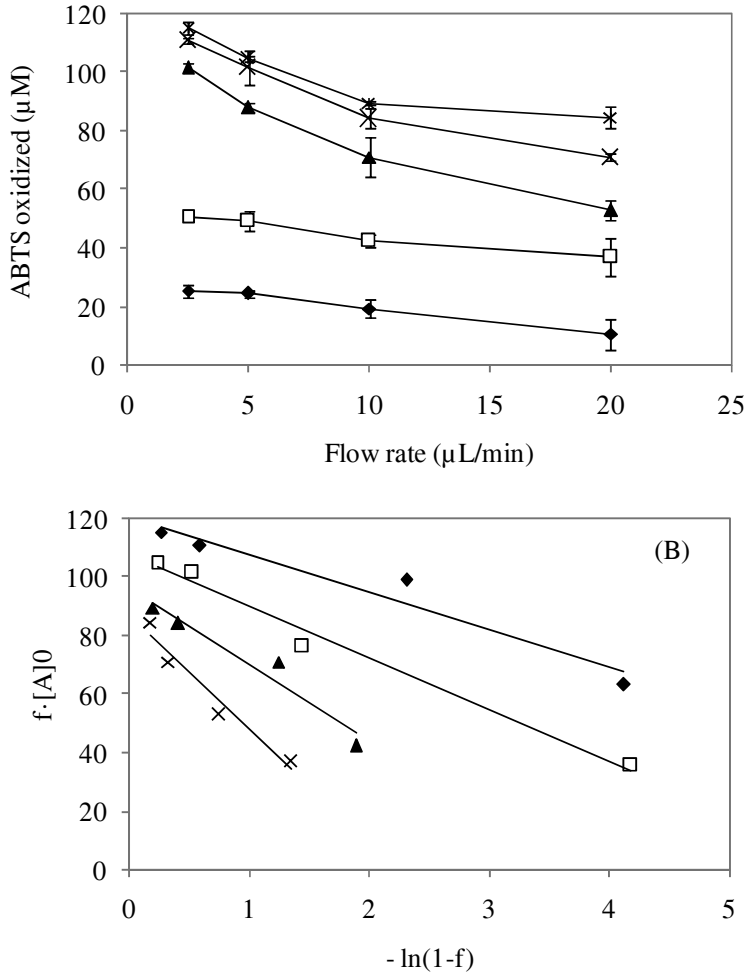

Figure 6.8. (A) Flow rate and concentration effects on the oxidation of ABTS in laccase-immobilized microreactors under continuous flow conditions. Initial concentrations of ABTS tested were: 25 (◆), 50 (□), 100 (▲), 250 (×) and 500 µM (∗). (B) Analysis of the kinetic data collected using Equation (6.1) model. Symbols correspond to: 2.5 (◆), 5 (□), 10 (▲) and 20 µL/min (×) of continuous flow rate. Solid lines fitted by linear regression.

The values of $K_{m(app)}$ and the corresponding correlation coefficient (R^2) are shown in Table 6.2.

Table 6.2. Apparent Michaelis constant ($K_{m(app)}$) and correlation coefficient from the Lilly-Hornby model (Equation (6.1)).

Flow rate (µL/min)	$K_{m(app)}$ (µM)	R^2
2.5	12.8 ± 1.2	0.971
5	17.7 ± 2.2	0.992
10	26.4 ± 4.2	0.970
20	39.1 ± 1.1	0.977

The increase of the $K_{m(app)}$ with the flow rate is indicative of mass transfer limitations, commonly found in fast enzymatic reactions, although it was not excessively marked: when increasing the flow rate from 2.5 to 20 µL/min, $K_{m(app)}$ was only 3.05-fold higher. It has been reported that a catalyzed-reaction in microchannel might be affected by the transfer of the substrate through a difussional layer surrounding the immobilized enzyme, which is dependent on the flow rate, also determining the conversion rate of the immobilized enzyme-catalyzed reaction (Matosevic et al. 2011).

6.3.4. Application of laccase-immobilized microreactors

In order to demonstrate the efficiency as well as the operational stability of laccase-immobilized microreactors, this technology was applied for the continuous biotransformation of five model compounds. Three estrogens (E1, E2 and EE2) and two anti-inflammatories (NPX and DCF) were selected as model substrates since their biotransformation by laccases have been previously investigated in this research.

The target compounds were quantified by HPLC using a Waters-2695 system equipped with a dual absorbance detector and a reverse phase column Lichrocart 150-4.6 (Waters-2487) packed with Lichrosphere 100 RP-18 5 µm (Kanto Chemical Co.), by applying the method described in Chapter 2, and residual activities were evaluated under standard conditions after 24 h of operation.

6.3.4.1. Continuous transformation of estrogenic compounds

The elimination of E1, E2 and EE2 (18 µM each, i.e. ~5 mg/L) from the reaction medium by immobilized laccase was proved by operating the microreactors at different flow rates (0.5 to 5 µL/min) to study the effect of HRT (52 to 5.2 min) and therefore, the effect of the feed addition rate (0.35 to 3.5 µmol/(L·min)), as schematized in Figure 6.9.A. The reaction was conducted at pH 7 and 30ºC since these conditions were demonstrated to be the optimal considering the characterization of the biocatalyst. The results are shown in Table 6.3.

As observed, high transformation percentages were found for the highest values of HRT considered. For example, nearly complete eliminations of the three compounds were achieved when working at a HRT of 52 min (removal rates 0.35 µmol/(L·min)) and E1, E2 and EE2 were removed by 95, 99 and 98%, respectively, for a HRT of 26 min. As expected, the removal efficiency decreased with HRT; however, removal yields between 43 and 74% were attained at HRTs of only 17.3 and 10.4 min. E1 was the compound whose removal was mostly influenced by the flow rate, as also observed with immobilized laccase from *Myceliophthora thermophila* on epoxy supports as indicated in Chapters 3. With regard to the residual laccase activity of the microreactors, the biocatalyst retained almost the total initial activity after 24 h of operation under all the conditions investigated.

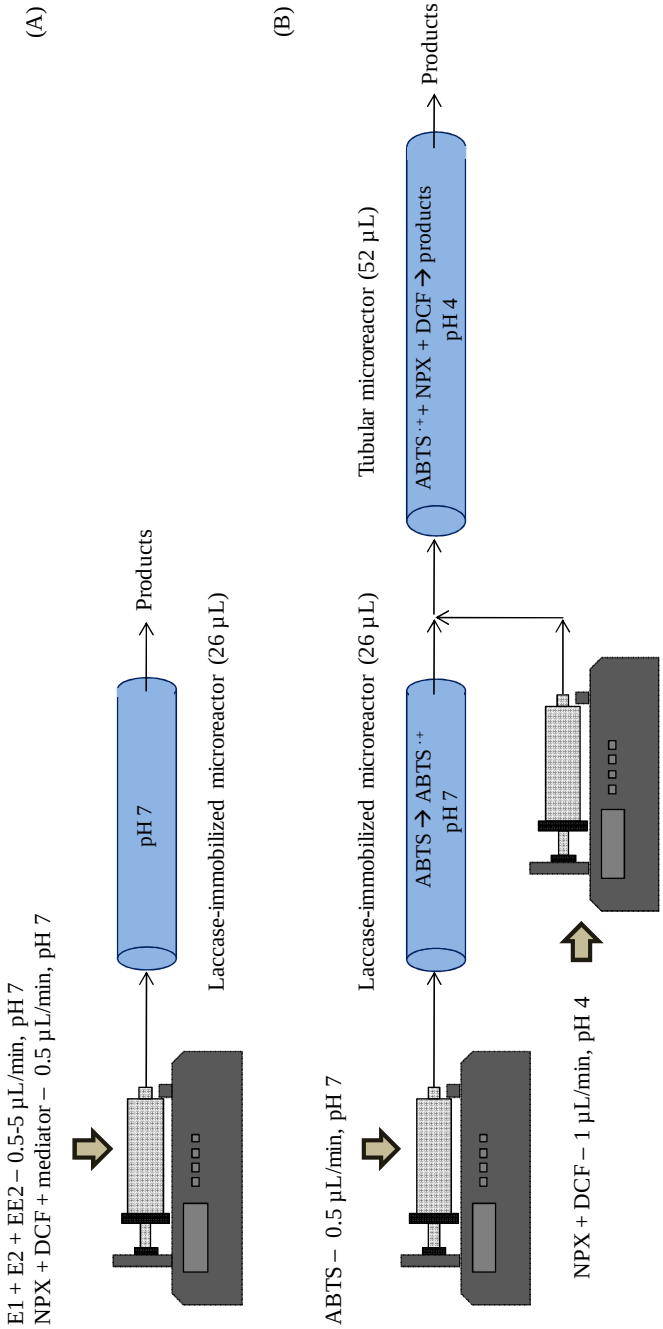

Figure 6.9. Schematic representation of the laccase-immobilized microreactor used for the elimination of estrogens and anti-inflammatory compounds (A) and two-stage microreactor designed for the transformation of anti-inflammatory compounds using ABTS as mediator (B).

Table 6.3. Elimination of estrogens (E1, E2 and EE2) by laccase-immobilized microreactors (Figure 6.9.A) operated under different flow rate conditions.

Flow rate (μL/min)	HRT (min)	Removal percentage (%)			Removal rate (μmol/(L·min))			Residual activity (%)
		E1	E2	EE2	E1	E2	EE2	
0.5	52.0	>99*	>99	>99	0.35	0.35	0.35	98
1.0	26.0	95	>99	98	0.66	0.70	0.67	97
1.5	17.3	56	74	74	0.58	0.77	0.77	97
2.5	10.4	43	52	50	0.74	0.90	0.87	95
5.0	5.2	18	28	25	0.62	0.97	0.87	95

*Concentrations below detection limits.

Great removal yields were found with considerably reduced residence times; in fact, the results obtained were improved when comparing with those found with free or immobilized laccases from *Myceliophthora thermophila*. For instance, removal yields of 75-85% were detected after 1 h of laccase-catalyzed transformation in batch reactors (Chapter 2) and poorer reaction yields were achieved with immobilized biocatalysts (Chapter 3). Conversely, the implementation of the biotransformation in microchannels allowed a noticeable intensification of the reaction even for the immobilized biocatalyst: e.g., for HRTs of only 10.4 min, approximately half of the initial concentration of the substrates was transformed. Hence, the microreactor design was demonstrated to be capable of outperforming the conventional designs.

These results are in agreement with those previously reported by Miyazaki et al. (2004) who reported a 15 times faster process with cucumisin immobilized in a microreactor than the batch reaction. Marques et al. (2012) reported a 100-fold decrease in the residence time required to attain similar yields of enzyme-catalyzed conversion of cholesterol in microchannels in comparison with the traditional stirred tank and plug-flow reactors.

The great performance of the microreaction system may be due to the more rapid mass transfer and the larger area to volume ratio and makes the prepared

laccase-immobilized microreactors an excellent technology to improve and favor the uses of laccases, such as biotransformations and bioanalyses, by means of process intensification and reduction of the system volume (Lin et al. 2010, Roman-Gusetu et al. 2009). Besides, the developed system have significant potential as a platform technology for laccase-immobilized microreactors to be used in the exploration of new applications.

6.3.4.2. Continuous transformation of anti-inflammatory compounds

The removal of NPX and DCF was investigated in two different configurations. First, the laccase immobilized microreactors were applied for the continuous elimination of these compounds (18 µM each, i.e. ~5 mg/L) from the reaction medium at pH 7 and 30ºC (Figure 6.9.A). The flow rate selected was 0.5 µL/min to maintain a HRT of 52 min, which is equivalent to a feed addition rate of 0.35 µmol/(L·min). Moreover, the effect of using laccase mediators was investigated by the addition of 500 µM of HBT, SA or ABTS in the inlet flow. The results are shown in Table 6.4.

It was demonstrated that the use of a mediator is required to attain significant reaction efficiencies since only DCF was oxidized by 25% when no mediator was used; similar conclusions were reported when using free laccase in Chapter 2. HBT and SA provided a slight enhancement of the results, but these compounds are likely to cause biocatalyst inactivation since the residual activity after 24 h of operation with SA was 88% while HBT caused a 25% of inactivation. This inactivation effect was verified through stability assays by incubating the microreactors at 30ºC and continuous flow rate of phosphate buffer (50 mM, pH 7) containing the mediators.

Table 6.4. Elimination of anti-inflammatories (NPX and DCF) in the laccase-immobilized microreactor (Figure 6.9.A) without and with mediators (SA, HBT and ABTS); and elimination in the two-stage microreactor (Figure 6.9.B) using ABTS as mediator.

Configuration	Mediator	Removal percentage (%)		Removal rate (μmol/(L·min))		Residual activity (%)
		NPX	DCF	NPX	DCF	
Single step	—	0	25	0.00	0.09	92
	SA	15	35	0.05	0.12	88
	HBT	30	65	0.11	0.23	75
	ABTS	50	75	0.18	0.26	90
Two steps	ABTS	71	90	0.12	0.15	99

The best reaction efficiency results were found for ABTS: eliminations of 50 and 75% were found for NPX and DCF, respectively, which implied removal rates of 0.18 and 0.26 μmol/(L·min). Additionally, the biocatalyst retained 90% of its initial activity.

Although it has been demonstrated in Chapter 2 the suitability of free laccase from *Trametes versicolor* to remove anti-inflammatories at neutral pH and in batch operation, the use of 1 mM of HBT and reaction periods of 24 h were necessary to attain removal yields of 70 and 98% of NPX and DCF, respectively. Moreover, the transformation yields attained after 52 min (the HRT applied for the transformation in laccase-immobilized microreactors) were only significant (about 50%) in the case of DCF. Hence, the results presented in the current chapter show further evidence of the noticeable improvement on the reaction progress attained with the microtechnology designed in this investigation.

On the other hand, laccases usually present their maximum activity at acid values of pH. In fact, the requirement of reaction media at pH 4 aiming to achieve significant oxidation of recalcitrant compounds such as NPX and DCF was already demonstrated for the free enzyme. Also, the action of the mediator would be improved by acid pH since higher stability and reactivity at low pH values of the radicals formed by the laccase-oxidation of ABTS has been demonstrated

(Majcherczyk et al. 1999). However, it was proved that acid pH leads to an important enzyme inactivation of the laccase-immobilized microreactors (Figure 6.7.A).

To overcome this challenge, a novel two-stage microreactor configuration for the biotransformation of target compounds was designed: the first step consisted of a prepared laccase-immobilized microreactor (26 µL) for the oxidation of the mediator at pH 7; and the second stage was based on a tubular microreactor (PTFE tube of 500 µm i.d. and 26 cm length, 52 µL) coupled to the enzymatic microreactor by means of a T-connector, to perform the chemical transformation of the substrates at pH 4 (Figure 6.9.B). The laccase-immobilized microreactor was fed with ABTS (500 µM in 50 mM phosphate buffer, pH 7) at 0.5 µL/min (HRT 52 min) for its oxidation to $ABTS^{\cdot+}$, responsible specie of the transformation of the substrates in laccase-mediated reactions (Morozova et al. 2007b). The second reactor was fed with both ABTS oxidized solution (0.5 µL/min, HRT 104 min) and a solution of NPX and DCF prepared in potassium acetate buffer (250 mM, pH 4) at a flow rate of 1 µL/min (HRT 52 min). Due to the change of scale from the first to the second reactor, the feed addition rate of ABTS varied from 9.6 to 4.8 µmol/(L·min) in the tubular microreactor. Thus, the concentration of NPX and DCF was 9 µM (feed addition rate 0.17 µmol/(L·min)) with the aim of maintaining the same ratio between addition rates of ABTS and the target compounds used in the first configuration.

With that system, improved removal yields of both compounds were found: NPX and DCF were oxidized by 71 and 90%, respectively, which entails removal rates of 0.12 and 0.15 µmol/(L·min) (Table 6.4). In addition, the inactivation of the biocatalyst by pH was completely avoided. The effect of the ABTS initial concentration was also investigated (data not shown) and it was observed that the concentration could be decreased to 200 µM and still high removal percentages of NPX and DCF could be achieved (55 and 80%, respectively). Solís-Oba et al. (2005) reported the feasibility of oxidized ABTS produced by immobilized laccase and separated by filtration to remove dyes, although both radical cation production and removal processes were only tested batchwise.

Although the presented system should be further studied for its optimum implementation, this novel configuration is a potential tool to expand the applicability of laccases to those fields where adverse pH conditions make this enzyme unsuitable. In addition, this two-stage microreactor technology would present additional benefits: e.g. the inactivation of the immobilized laccase not only by pH but also by the presence of inactivating agents and/or products formed

during reaction would be prevented; both reactors could operate under different conditions (pH, temperature, etc.) to adapt the technology to the requirements of the specific application; the activity of the laccase-immobilized microreactor could be monitored by using an in-line UV detector, instead of the time-consuming off-line analysis required to determine laccase activity of the microreactor when other compounds besides ABTS are present; easy replacement in case of inactivation or microreactor damage; easy scale-up; and potential mediator reuse.

ABTS mediation action

In view of the previous results regarding transformation of NPX and DCF by laccase-mediator system using ABTS in the two steps system reactor configuration, interesting discussion comes out.

As previously reported, oxidation of ABTS takes place in successive stages as shown in Figure 6.10. (Bourbonnais et al. 1998). ABTS undergoes one-electron oxidation to the relatively stable colored radical cation $ABTS^{\bullet+}$, at a redox potential of 0.69 V which almost matches that of laccase. However, further one-electron oxidation of $ABTS^{\bullet+}$ to $ABTS^{+2}$ with redox potential 1.01 V may occur.

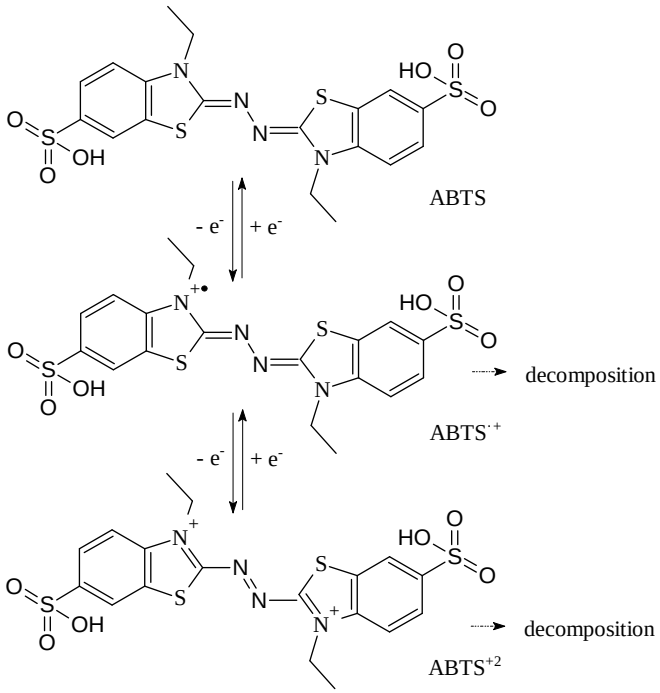

Figure 6.10. Species generated during oxidation of ABTS.

On the other hand, some authors reported that oxidation by cation radical $ABTS^{\bullet+}$ is limited to phenolic groups, whereas $ABTS^{+2}$ is required for the oxidation of non-phenolic compounds (Bourbonnais et al. 1998). In fact, previous authors explained the negligible transformation yields of certain compounds when using ABTS as mediator by the inability of the biocatalyst to perform the oxidation of ABTS beyond the cation radical (Fillat et al. 2012b).

In the current research, NPX and DCF were further transformed by using laccase-immobilized microreactors in the presence of ABTS, and once these are non-phenol structured compounds, $ABTS^{+2}$ could be thought to be generated during the enzymatic mechanism; nevertheless, $ABTS^{+2}$ was reported to be too short-lived (Branchi et al. 2005). From this, different conclusions may be stated: either the short reaction times in the microreactors are enough for the target substrates to be oxidized by $ABTS^{+2}$ formed in the first stage of the reactor, or some other mechanisms take place. Previous authors suggested an enzyme effect which would be capable of protecting the oxidized forms of ABTS, thereby favoring their action in the reaction with the substrates (Branchi et al. 2005). However, the fact that oxidized ABTS was able to react with NPX and DCF in the absence of the enzyme makes to discard that hypothesis. An explanation might be that degradation byproducts of $ABTS^{\bullet+}$ or $ABTS^{+2}$ are the responsible of the transformation of non-phenolic compounds, by their formation *in situ* and subsequent reaction with the substrates (Branchi et al. 2005). Different opinions are reported in literature with regard the formed reaction products of the laccase-catalyzed oxidation of ABTS: some authors sustain the idea that it can be produced only the cation radical, whereas others reported the formation of both cations (Munteanu et al. 2007). Nevertheless, as the oxidation of $ABTS^{\bullet+}$ to $ABTS^{+2}$ might be not probable because of the thermodynamically unfavorable reaction, decomposition products from $ABTS^{\bullet+}$ are those most likely to be involved in the substrates transformation. In addition, other degradation fragments of the oxidized mediator could be radicals and their contribution of radical oxidation routes towards recalcitrant compounds cannot be excluded (Cantarella et al. 2003).

6.4. Conclusions

In this chapter, the emerging microreaction technology was explored aiming to expand and improve the applicability of laccases. With this goal, a novel, inexpensive and efficient method for the preparation of laccase-immobilized microreactors was developed; it was based on the formation of an enzyme-

polymeric membrane on the inner wall of microtubes (500 µm inner diameter) as a result of the cross-linking polymerization reaction between laccase and bifunctional cross-linkers agents (paraformaldehyde and glutaraldehyde).

Under the optimum conditions, an immobilization yield of 72% and an activity of 45 µM/min determined in a continuous-flow assay were detected. The biochemical characterization of the laccase-immobilized microreactors demonstrated their enhanced characteristics: they exhibited a broader range of optimal activity and excellent stability under different conditions of pH, temperature, in the present of inactivating agents and during storage and long-term operation.

The laccase-immobilized microreactors were applied for the biotransformation of model compounds to demonstrate their efficiency and performance: significant reaction yields were obtained even at fairly low residence times. Furthermore, it was designed a novel two-stage bioreactor system for the application on laccase-mediated reactions preventing the biocatalyst from inactivation.

The great performance of the microreactor system, possibly due to the more rapid mass transfer and the larger area to volume ratio, makes the technologies developed in this chapter excellent platforms for increasing laccase uses and improving their catalytic action in several fields such as biotransformations or bioanalyses.

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General conclusions

This Thesis contributes to the development of novel technologies for the oxidation of emerging pollutants: estrogenic and anti-inflammatory compounds were evaluated. The following main conclusions were withdrawn from the work conducted throughout this research:

1. It was demonstrated with the batch experiments conducted the ability of laccase and laccase-mediator systems to remove the selected estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethynilestradiol (EE2), and anti-inflammatories: naproxen (NPX) and diclofenac (DCF). The results obtained from the characterization of the enzymes in terms of pH and temperature impacts served as basis for these biotransformation experiments and discussion of the corresponding results.
2. E1, E2 and EE2 were eliminated by *Myceliophthora thermophila* laccase at both pH 4 and 7. Thus, neutral pH was selected once it is beneficial not only for enzyme stability but also for removal percentages (significant transformation efficiencies were found within only 1 h), and moreover it favors the treatment of wastewaters avoiding their acidification.
3. LYES analysis proved that the elimination of the estrogenic compounds by enzymatic treatment leads to a parallel reduction of the estrogenicity of the medium.
4. The identified as the optimum strategy to attain significant removal efficiencies of NPX and DCF when *Myceliophthora thermophila* laccase is used is the application of laccase-mediator systems under acidic conditions. In this way, the effect of synthetic mediators: 1-hydroxybenzotriazole (HBT) and violuric acid, was evaluated, yielding degradation efficiencies of 60 and 36% for NPX, respectively, whereas almost complete elimination of DCF (below detection limits) was detected.
5. Among the natural mediators assayed aiming to ensure an environmentally friendly and cost-effective technology, syringaldehyde (SA) provided the best results: DCF was removed within only 1 h with 1 mM SA, although, NPX transformation was not affected by the use of these mediators.
6. The use of a high-redox potential enzyme, *Trametes versicolor* laccase, leads to more favorable kinetics due to the increase of ΔE^0 between the substrate and the T1 copper of the biocatalyst. Results proved high removal yields for NPX (70-94%) after 24 h in the presence of HBT at acidic and neutral pH, while DCF was completely transformed at pH 4 within shorter periods, from 30 min to 4 h both in the absence or presence of mediators.

7. Kinetic parameters of the transformation reactions and laccase inactivation coefficients were useful for the estimation and comparison of the results for the different experimental conditions assayed during the anti-inflammatories transformation.
8. Laccase-catalyzed treatment of DCF was proved to result in biotransformation products less toxic than the parent substrate, with a considerable higher aerobic biodegradability.
9. Both common considered routes for the development of continuous enzymatic bioreactors were successfully applied: enzyme immobilization and its retention by a membrane system.
10. *Myceliophthora thermophila* laccase was successfully immobilized by its encapsulation in a sol-gel matrix as well as by covalent bonding to commercial solid epoxy-activated acrylic supports: Eupergit C and Eupergit C 250L.
11. Both immobilization procedures resulted in biocatalysts with enhanced stability and catalytic activity over a wider range of temperature, pH and in the presence of inactivating agents.
12. Different packed bed reactors (PBRs) designed for the application of encapsulated and immobilized laccase on Eupergit supports in continuous processes were successfully utilized to test the potential reusability of the biocatalysts by their application on the removal of Acid Green 27 dye used as model compound in consecutive continuous cycles.
13. The proposed PBRs were found to be feasible for the continuous treatment of E1, E2 and EE2: removal yields of 55-75 and 65-80% for the encapsulated and the covalently immobilized laccase, respectively, were found; 6-14 and 11-22% of elimination was attributed to adsorption of the substrates. Furthermore, the biocatalysts retained about 70 and 84% of their initial activity after 8 h of continuous operation.
14. A fluidized bed reactor (FBR) with laccase immobilized on Eupergit C 250L was developed and operated to avoid the drawbacks generally associated to PBRs: elimination percentages up to 92% were found and a residual activity of 89% was detected after 10-h operation of that system.
15. The FBR was also proved to be effective on the removal of estrogens at concentrations as low as 100 and 10 µg/L, but a hydraulic residence time (HRT) of 150 min was needed to attain significant removal efficiencies and an estrogenicity reduction of 90%. The system was fairly stable for more than 10 days of continuous operation.

16. Fed-batch reactors were found to be an interesting strategy for the removal of estrogens by *Myceliophthora thermophila* laccase as well as useful systems to investigate the influence of various operational parameters affecting the E1 and E2 transformation: laccase activity, substrate frequency addition and aeration/oxygenation, aiming to elucidate the most suitable conditions for the following continuous operation with free enzyme.
17. The designed enzymatic membrane reactor (EMR), based on a continuous stirred tank reactor coupled to an ultrafiltration membrane, permitted the continuous operation with free enzyme by its retention in the system.
18. A three-level Box-Behken factorial design combined with the response surface methodology was successfully applied to study the continuous transformation of E1, E2 and EE2 in a 370-mL EMR. This methodology permitted the evaluation of individual and interrelated effects of the variables analyzed: oxygenation rate, HRT and laccase activity, on the different response variables investigated and optimized: removal rate, removal rate per units of enzyme used and the percentage of estrogenic activity reduction.
19. Laccase activity and HRT showed, as expected, great effects; nonetheless, the relevance of oxygen in the improvement of oxidation reaction kinetics through the rise of the dissolved oxygen above saturation levels was proved.
20. Only 100 U/L were found as optimal to maximize the efficacy of the enzyme: E1 was oxidized by 0.06 mg/(L·h·U), although the removal of estrogenicity was 60%. On the other hand, the highest values assayed (1,000 U/L, HRT 4 h and 60 mg O_2/(L·h)) provided nearly complete detoxification.
21. The real applicability of the developed technology was also demonstrated: a 2-L EMR was operated, for 100 h and with low enzyme requirements, for the treatment of filtered secondary wastewater effluents collected in a municipal wastewater treatment plant containing E1, E2 and EE2 at only100 µg/L as well as at real environmental levels (0.29-1.52 ng/L). High removal yields (80-100%) were attained despite partial inactivation of the enzyme (about 20% within the first hours).
22. It was concluded from the assays performed for the assessment of reaction products the formation of dimers of E1, E2 and EE2, trimers of E2 and EE2, as well as the transformation of E2 into E1, after transformation with *Myceliophthora thermophila* laccase. These findings were corroborated by determination of accurate masses.
23. A tentative identification of reaction products resulted from the transformation DCF catalyzed by *Trametes versicolor* laccase was also

conducted: regarding their spectra, biotransformation products were identified as compounds resulted from decarboxylation reactions.

24. A novel, inexpensive and efficient method for the preparation of laccase-immobilized microreactors was developed, based on the formation of an enzyme-polymeric membrane on the inner wall of microtubes as a result of cross-linking and polymerization reactions.
25. The biochemical characterization of the laccase-immobilized microreactors demonstrated their broader range of optimum pH and temperature and excellent stability under different conditions of pH, temperature, in the presence of chemical inactivating agents and during storage and long-term operation.
26. These microreactor systems were proved to be feasible on the oxidation of the target substrates, estrogens and anti-inflammatories, with significant reduced retention times.
27. It was also proved the performance of a two-stage microreactor system which was designed to prevent the biocatalyst from inactivation when the operation under adverse conditions for the enzyme is required.

Conclusiones generales

Esta Tesis contribuye al desarrollo de nuevas tecnologías para la oxidación de contaminantes emergentes: se evaluaron compuestos estrogénicos y antiinflamatorios. Las conclusiones principales extraídas del trabajo realizado a lo largo de esta investigación son las siguientes:

1. Se demostró mediante los ensayos en discontinuo llevados a cabo la capacidad de los sistemas lacasa y lacasa-mediador para eliminar los compuestos estrogénicos: estrona (E1), 17β-estradiol (E2) y 17α-etinilestradiol (EE2), y los antiinflamatorios: naproxeno (NPX) y diclofenaco (DCF). Los resultados obtenidos a partir de la caracterización de las enzimas en términos de pH y temperatura sirvieron como base para estos experimentos de biotransformación y la discusión de los resultados correspondientes.
2. E1, E2 y EE2 fueron eliminados por lacasa de *Myceliophthora thermophila* tanto a pH 4 y 7. Por lo tanto, se seleccionó pH neutro ya que es beneficioso no sólo para la estabilidad de la enzima sino también para la eliminación de los compuestos (se obtuvieron altas eficacias de transformación en sólo 1 h), y además se favorece el tratamiento de aguas residuales evitando su acidificación.
3. Los análisis LYES demostraron que la eliminación de estrógenos mediante tratamiento enzimático está ligado a una reducción en paralelo de la estrogenicidad del medio.
4. La estrategia óptima para alcanzar niveles significativos de eliminación de NPX y DCF cuando se utiliza lacasa de *Myceliophthora thermophila* fue la aplicación de sistemas lacasa-mediador en condiciones ácidas. Así, se evaluó el efecto de mediadores sintéticos: 1-hidroxibenzotriazol (HBT) y ácido violúrico, obteniéndose porcentajes de degradación de 60 y 36% para NPX, respectivamente, mientras que se detectó una eliminación casi completa de DCF (por debajo de los límites de detección).
5. Entre los mediadores naturales ensayados con el objetivo de garantizar una tecnología respetuosa con el medio ambiente y de bajo coste, el empleo de siringaldehido (SA) proporcionó los mejores resultados: se eliminó DCF en tan solo 1 h con 1mM de SA, aunque, por otro lado, la transformación de NPX no se vio afectado por el uso de estos mediadores.
6. El uso de una enzima de alto potencial redox, lacasa de *Trametes versicolor*, proporcionó una cinética más favorable debido al incremento de ΔE^0 entre el

sustrato y el cobre T1 del biocatalizador. Los resultados demostraron altos rendimientos de eliminación de NPX (70-94%) después de 24 h en presencia de HBT a pH ácido y neutro, mientras que DCF se transformó por completo a pH 4 en tiempos más cortos de 30 min-4 h tanto en ausencia como en presencia de mediadores.

7. Los parámetros cinéticos de las reacciones de transformación y los coeficientes de inactivación enzimática fueron útiles para la estimación y la comparación de los resultados para las diferentes condiciones experimentales ensayadas durante la transformación de antiinflamatorios.
8. Se demostró que los productos de transformación del DCF tras la reacción catalizada por lacasa son menos tóxicos, con una considerable mayor biodegradabilidad aeróbica.
9. Ambas rutas comúnmente consideradas para el desarrollo de reactores enzimáticos operados en continuo fueron aplicadas satisfactoriamente: inmovilización enzimática y su retención mediante un sistema de membrana.
10. Se inmovilizó exitosamente lacasa de *Myceliophthora thermophila* mediante su encapsulación en matrices de *sol-gel*, así como mediante unión covalente sobre soportes comerciales acrílicos con grupos epóxido activados: Eupergit C y Eupergit C 250L.
11. Ambos procedimientos dieron como resultado biocatalizadores con una mejorada estabilidad y actividad catalítica en una amplia gama de temperatura, pH y en presencia de agentes inactivantes.
12. Diferentes reactores de lecho fijo (PBRs, por sus siglas en inglés), diseñados para la aplicación en continuo de la enzima encapsulada e inmovilizada covalentemente, fueron empleados con éxito para probar el potencial reúso de los biocatalizadores mediante su aplicación en la eliminación del tinte Acid Green 27, usado como compuesto modelo, en ciclos continuos consecutivos.
13. Se demostró la eficacia de los PBRs con enzima inmovilizada para la eliminación en continuo de E1, E2 y EE2: se obtuvieron rendimientos de eliminación de 55-75 y 65-80% para la lacasa encapsulada e inmovilizada covalentemente, respectivamente; se atribuyeron porcentajes 6-14 y 11-22% a la adsorción de los sustratos. Además, los biocatalizadores mantuvieron aproximadamente el 70 y el 84% de su actividad inicial después de 8 h de operación.
14. Se desarrolló y operó un reactor de lecho fluidizado (FBR, por sus siglas en inglés) con lacasa inmovilizada en Eupergit C 250L para evitar los

inconvenientes asociados generalmente a los PBRs: se obtuvieron con ese sistema porcentajes de eliminación de hasta 92% y una actividad residual de 89% después de 10 h.

15. Se demostró también la eficacia del sistema basado en el FBR para la eliminación de estrógenos a concentraciones tan bajas como 100 y 10 µg/L, aunque fue preciso un tiempos de residencia hidraúlico (TRH) de 150 minutos para alcanzar niveles de eliminación significativos y una reducción de estrogenicidad del 90%. El sistema fue estable durante más de 10 días de operación.
16. Se encontró que el uso de reactores en operación semi-continua (*fed-batch*) es una interesante estrategia para la eliminación de estrógenos mediante lacasa de *Myceliophthora thermophila*, y fueron además sistemas útiles para investigar la influencia de diversos parámetros operacionales que afectan a la transformación E1 y E2: actividad enzimática, frecuencia de adición de los sustratos y aireación/oxigenación, con el objetivo de dilucidar las condiciones más adecuadas para la operación en continuo con enzima libre.
17. El reactor enzimático de membrana (EMR, por sus siglas en inglés) diseñado, basado en un reactor continuo de tanque agitado acoplado a una membrana de ultrafiltración, permitió la operación en continuo con enzima libre mediante su retención en el sistema.
18. La aplicación de diseño factorial Box-Behken junto con la metodología de superficie de respuesta resultó ser una herramienta útil para el estudio de la transformación de E1, E2 y EE2 en un EMR de 370 mL. Esta metodología permitió la evaluación de los efectos individuales y cruzados de las variables analizadas: oxigenación, TRH y actividad enzimática, sobre las diferentes variables respuesta investigadas y optimizadas: velocidad de eliminación, velocidad de eliminación por unidades de enzima utilizadas y el porcentaje de reducción de actividad estrogénica.
19. La actividad enzimática y el TRH fueron, como se esperaba, los efectos con mayor peso; sin embargo, se comprobó la importancia significativa del oxígeno en la mejora de la cinética de la reacción de oxidación a través del aumento de la concentración de oxígeno disuelto por encima de los niveles de saturación.
20. El óptimo de eficacia de enzima se encontró para una actividad de tan solo 100 U/L: se oxidó E1 a una tasa de 0,06 mg/(L·h·U), aunque la reducción de estrogenicidad fue del 60 %. Por otro lado, los valores más altos ensayados

(1000 U/L, TRH 4 h y 60 mg O_2/(L·h)) proporcionaron una descontaminación completa.

21. Se demostró la aplicabilidad real de la tecnología desarrollada mediante la operación de un EMR de 2 L, durante 100 h y con baja actividad enzimática, para el tratamiento de efluentes secundarios previamente filtrados, recogidos de una planta municipal de tratamiento de aguas, que contienen E1, E2 y EE2 a concentraciones de solo 100 µg/L así como a niveles ambientales (0,29-1,52 ng/L). Se alcanzaron altos rendimientos de eliminación (80-100%) a pesar de la inactivación parcial de la enzima (alrededor de 20% durante las primeras horas).
22. Se concluyó a partir de los ensayos realizados para la evaluación de los productos de reacción la formación de dímeros de E1, E2 y EE2 , trímeros de E2 y EE2, así como la transformación de E2 en E1, después de la transformación catalizada con lacasa de *Myceliophthora thermophila*. Estos hallazgos fueron corroborados mediante la determinación de masas exactas.
23. Se llevó a cabo una identificación tentativa de los productos de reacción de DCF como resultado de la transformación catalizada por lacasa de *Trametes versicolor*: atentiendo a los espectros de los productos detectados, éstos fueron identificados como productos resultantes de reacciones de descarboxilación.
24. Se desarrolló un método novedoso y de bajo coste que resultó ser eficiente para la preparación de microrreactores con lacasa inmovilizada, basado en la formación de una membrana enzimática polimérica en las paredes internas de microtubos como resultado de reacciones de *cross-linking* y polimerización.
25. La caracterización bioquímica de los microrreactores con lacasa inmovilizada demostró su amplio rango de pH y temperatura óptimos, y su excelente estabilidad en diferentes condiciones de pH, temperatura, en presencia de agentes inactivantes y durante almacenamiento y operaciones de larga duración.
26. Estos microrreactores fueron factibles en la oxidación de los sustratos objetivo, estrógenos y antiinflamatorios, con reducidos tiempos de residencia.
27. Se probó también la eficacia de un sistema de microrreactor de dos etapas que fue diseñado para evitar la inactivación del biocatalizador cuando es necesario operar bajo condiciones adversas para la enzima.

Conclusións xerais

Esta Tese contribúe ao desenvolvemento de novas tecnoloxías para a oxidación de contaminantes emerxentes: avaliáronse compostos estroxénicos e antiinflamatorios. As conclusións principais extraídas do traballo realizado ao longo desta investigación son as seguintes:

1. Demostrouse mediante os ensaios en discontinuo levados a cabo a capacidade dos sistemas lacasa e lacasa-mediador para eliminar os compostos estroxénicos: estrona (E1), 17β-estradiol (E2) e 17α-etinilestradiol (EE2), e os antiinflamatorios: naproxeno (NPX) e diclofenaco (DCF). Os resultados obtidos a partir da caracterización das enzimas en termos de pH e temperatura serviron como base para estes experimentos de biotransformación e a discusión dos resultados correspondentes.
2. E1, E2 e EE2 foron eliminados por lacasa de *Myceliophthora thermophila* tanto a pH 4 coma 7. Polo tanto, seleccionouse pH neutro xa que é beneficioso non só para a estabilidade da enzima senón tamén para a eliminación dos compostos (obtivéronse altas eficacias de transformación en só 1 h), e ademais favorece o tratamento de augas residuais evitando a súa acidificación.
3. As análises LYES demostraron que a eliminación de estróxenos mediante tratamento enzimático está ligado a unha redución en paralelo da estroxenicidade do medio.
4. A estratexia óptima para alcanzar niveis de eliminación significativos de NPX e DCF cando se utiliza lacasa de *Myceliophthora thermophila* resultou ser a aplicación de sistemas lacasa-mediador en condicións ácidas. Así, avaliouse o efecto de mediadores sintéticos, 1-hidroxibenzotriazol (HBT) e ácido violúrico, obténdose eficiencias de eliminación de 60 e 36% para NPX, respectivamente, mentres que se detectou unha eliminación case completa de DCF (por debaixo dos límites de detección).
5. Entre os mediadores naturais ensaiados co obxectivo de garantir unha tecnoloxía respetuosa co medio ambiente e de baixo custo, o emprego de siringaldehido (SA) proporcionou os mellores resultados: eliminouse DCF en tan só 1 h con 1mM de SA, aínda que, por outra banda, a transformación de NPX non se viu afectado polo uso destes mediadores.
6. O uso dunha enzima de alto potencial redox, lacasa de *Trametes versicolor*, proporcionou unha cinética máis favorable debido ao incremento de ΔE^0 entre o substrato e o cobre T1 do biocatalizador. Os resultados demostraron

altos rendementos de eliminación de NPX (70-94%) logo de 24 h en presenza de HBT a pH ácido e neutro, mentres que DCF transformouse por completo a pH 4 en tempos máis curtos de 30 min-4 h tanto en ausencia como en presenza de mediadores.

7. Os parámetros cinéticos das reaccións de transformación e os coeficientes de inactivación enzimática foron útiles para a estimación e a comparación dos resultados para as diferentes condicións experimentais ensaiadas durante a transformación de antiinflamatorios.
8. Demostrouse que os produtos de transformación do DCF trala reacción catalizada por lacasa son menos tóxicos, cunha considerable maior biodegradabilidade aeróbica.
9. Ámbalas dúas rutas comúnmente consideradas para o desenvolvemento de reactores enzimáticos operados en continuo foron aplicadas satisfactoriamente: inmobilización enzimática e a súa retención mediante un sistema de membrana.
10. Inmobilizouse exitosamente lacasa de *Myceliophthora thermophila* mediante a súa encapsulación en matrices *sol-gel*, así como mediante unión covalente sobre soportes comerciais acrílicos con grupos epóxido activados: Eupergit C e Eupergit C 250L.
11. Ámbolos dous procedementos deron como resultado biocatalizadores cunha mellorada estabilidade e actividade catalítica nunha ampla gama de temperatura, pH e en presenza de axentes inactivantes.
12. Diferentes reactores de leito fixo (PBRs, polas súas siglas en inglés), deseñados para a aplicación en continuo da enzima encapsulada e inmobilizada covalentemente, foron empregados con éxito para probar a o potencial reúso dos biocatalizadores mediante a súa aplicación na eliminación do tinte Acid Green 27, usado como composto modelo, en ciclos continuos consecutivos.
13. Demostrouse a eficacia dos PBRs con enzima inmobilizada para a eliminación en continuo de E1, E2 e EE2: obtivéronse rendementos de eliminación de 55-75 e 65-80% para a lacasa encapsulada e inmobilizada covalentemente, respectivamente; atribuironse porcentaxes 6-14 e 11-22% á adsorción dos substratos. Ademais, os biocatalizadores mantiveron aproximadamente o 70 e 84% da súa actividade inicial logo de 8 h de operación.
14. Desenvolveuse e operouse un reactor de leito fluidizado (FBR, polas súas siglas en inglés) con lacasa inmobilizada en Eupergit C 250L para evitar os

inconvenientes asociados xeralmente aos PBRs: obtivéronse con ese sistema porcentaxes de eliminación de ata 92% e unha actividade residual de 89% logo de 10 h.

15. Demostrouse tamén a eficacia do sistema baseado no FBR para a eliminación de estróxenos a concentracións tan baixas como 100 e 10 µg/L, aínda que foi preciso un tempo de residencia hidraúlico (TRH) de 150 minutos para acadar niveis de eliminación significativos e unha redución de estroxenicidade do 90%. O sistema foi estable durante máis de 10 días de operación.
16. Atopouse que o uso de reactores en operación semi-continua (*fed-batch*) é unha interesante estratexia para a eliminación de estróxenos mediante lacasa de *Myceliophthora thermophila*, e foron ademais sistemas útiles para investigar a influencia de diversos parámetros operacionais que afectan á transformación E1 e E2: actividade enzimática, frecuencia de adición dos substratos e aireación/osixenación, co obxectivo de dilucidar as condicións máis adecuadas para a operación en continuo con enzima libre.
17. O reactor enzimático de membrana (EMR, polas súas siglas en inglés) deseñado, baseado nun reactor continuo de tanque axitado acoplado a unha membrana de ultrafiltración, permitiu a operación en continuo con enzima libre mediante a súa retención no sistema.
18. A aplicación de deseño factorial Box-Behken xunto coa metodoloxía de superficie de resposta resultou ser unha ferramenta útil para o estudo da transformación de E1, E2 e EE2 nun EMR de 370 mL. Esta metodoloxía permitiu a avaliación dos efectos individuais e cruzados das variables analizadas: osixenación, TRH e actividade enzimática, sobre as diferentes variables resposta investigadas e optimizadas: velocidade de eliminación, velocidade de eliminación por unidades de enzima utilizadas e a porcentaxe de redución da actividade estroxénica.
19. A actividade enzimática e o TRH foron, como se esperaba, os efectos con maior peso; non obstante, comprobouse a importancia significativa do osíxeno na mellora da cinética da reacción de oxidación a través do aumento da concentración de osíxeno disolto por encima dos niveis de saturación.
20. O óptimo de eficacia de enzima atopouse para unha actividade de tan só 100 U/L: E1 oxidouse a unha taxa de 0,06 mg/(L·h·U), aínda que a redución de estroxenicidade foi do 60 %. Por outra banda, os valores máis altos ensaiados (1000 U/L, TRH 4 h e 60 mg O_2/(L·h)) proporcionaron unha descontaminación completa.

21. Demostrouse a aplicabilidade real da tecnoloxía desenvolvida mediante a operación dun EMR de 2 L, durante 100 h e con baixa actividade enzimática, para o tratamento de efluentes secundarios previamente filtrados, recollidos nunha planta municipal de tratamento de augas, que conteñen E1, E2 e EE2 a concentracións de só 100 µg/L así como a niveis ambientais (0,29-1,52 ng/L). Acadáronse altos rendementos altos de eliminación (80-100%) porén da inactivación parcial da enzima (ao redor de 20% durante as primeiras horas).
22. Concluíuse a partir dos ensaios realizados para a avaliación dos produtos de reacción a formación de dímeros de E1, E2 e EE2, trímeros de E2 e EE2 , así como a transformación de E2 en E1, logo da transformación catalizada con lacasa de *Myceliophthora thermophila*. Estes achados foron corroborados mediante a determinación de masas exactas.
23. Levouse a cabo unha identificación tentativa dos produtos de reacción de DCF como resultado da transformación catalizada por lacasa de *Trametes versicolor*: atendendo aos espectros dos produtos detectados, estes foron identificados como produtos resultantes de reaccións de descarboxilación.
24. Desenvolveuse un método novedoso e de baixo custo que resultou ser eficiente para a preparación de microrreactores con lacasa inmobilizada, baseado na formación dunha membrana enzimática polimérica nas paredes internas de microtubos como resultado de reaccións de *cross-linking* e polimerización.
25. A caracterización bioquímica dos microrreactores con lacasa inmobilizada demostrou o seu amplo rango de pH e temperatura óptimos, e a súa excelente estabilidade en diferentes condicións de pH, temperatura, en presenza de axentes inactivantes e durante almacenamento e operacións de longa duración.
26. Estes microrreactores foron factibles na oxidación dos substratos obxectivo, estróxenos e antiinflamatorios, con reducidos tempos de residencia.
27. Probouse tamén a eficacia dun sistema de microrreactor de dúas etapas que foi deseñado para evitar a inactivación do biocatalizador cando é necesario operar baixo condicions adversas para a enzima.

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