UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Ingeniería Química

Degradation of pharmaceutical compounds by ligninolytic fungi

Memoria presentada por

Angélica Iliana Rodarte Morales

Para optar al grado de Doctor por la

Universidad de Santiago de Compostela

Santiago de Compostela, 3 de Febrero de 2012

Título:

Degradation of pharmaceutical compounds by ligninolytic fungi

Serie:

Tesis Doctorales
Grupo de Ingeniería Química y Bioprocesos
Universidad de Santiago de Compostela

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Ingeniería Química

D. Juan Manuel Lema Rodicio, Catedrático de Universidad del Departamento de Ingeniería Química y Dª María Teresa Moreira Vilar, Catedrática de Universidad del Departamento de Ingeniería Química de la Universidad de Santiago de Compostela.

Informan:

Que la presente memoria, titulada **"Degradation of pharmaceutical compounds by ligninolytic fungi"** que, para optar al grado de Doctor en Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Dª Angélica Iliana Rodarte Morales, ha sido realizada bajo nuestra inmediata dirección en el Departamento de Ingeniería Química de la Universidad de Santiago de Compostela.

Y para que así conste, firman el presente informe en Santiago de Compostela, Noviembre de 2011.

D. Juan Manuel Lema Rodicio Dª María Teresa Moreira Vilar

Esta memoria fue presentada el 3 de Febrero de 2012 en la Escuela Técnica Superior de Ingeniería de la Universidad de Santiago de Compostela ante el tribunal compuesto por:

Presidente
Dr. Sixto Malato Rodríguez
Investigador CSIC
Universidad de Almería

1° vocal
Dra. Gloria Caminal Saperas
Departamento de Ingeniería Química
Universitat Autónoma de Barcelona

2° vocal
Dra. Susana Camarero Fernández
Departamento de Biología Ambiental
CSIC Madrid

3° vocal
Prof. Francisco Omil Prieto
Departamento de Ingeniería Química
Universidad de Santiago de Compostela

Secretaria
Dra. Carmen López Díaz
Departamento de Ingeniería Química
Universidad Autónoma de Barcelona

Agradecimientos

Primeramente, quiero agradecer al Consejo Nacional de Ciencia y Tecnología (CONACYT) de México por la beca proporcionada para realizar mis estudios de Doctorado en Ingeniería Química y Ambiental en la Universidad de Santiago de Compostela. También agradezco a los proyectos de la Xunta de Galicia (PGIDIT06PXIB265088PR y GRC 2010/37), al Ministerio Español de Ciencia y Educación (CICYT-CTQ2007-66788/PPQ y CTQ2010-20258) y al Proyecto Europeo (EUI 2008-03703) por el apoyo otorgado para la realización de esta tesis doctoral.

A mis directores de tesis, Dr. Juan M. Lema y Dra. María Teresa Moreira por haber confiado en mí desde el principio, por permitirme realizar mis estudios de doctorado bajo su supervisión, por guiarme durante la planeación y desarrollo de esta investigación, sin ello, esta tesis no hubiera sido posible. Les agradezco de manera especial la supervisión y el tiempo dedicado durante la redacción de esta tesis.

Agradezco también al Dr. Ramón Méndez Pampín y nuevamente al Dr. Juan M. Lema por brindarme la oportunidad de incorporarme en el Grupo de Ingeniería Ambiental y de Bioprocesos del Departamento de Ingeniería Química. También agradezco especialmente al Dr. Gumersindo Feijoo por su disponibilidad, su opinión acertada y su gran optimismo a lo largo de estos 4 años. Al resto de profesores del Biogrup, Dra. Anuska Mosquera, Dr. Jose Luis Campos, Dr. Juan M. Garrido y Dr. Francisco Omil les doy las gracias por transmitirme sus conocimientos durante los cursos de doctorado.

A mis compañeros del grupo de Fermentación: Adriana, Carlos, Lucía, Paula D. y Rober, por recibirme cada mañana con una sonrisa y por el apoyo proporcionado durante el desarrollo de esta tesis ¡Gracias por su confianza y amistad! Estoy muy agradecida con Cecilia Baldini, por su ayuda durante el inicio de mi investigación y por su amistad. También agradezco a las personas que estuvieron en el laboratorio de estancia de investigación: Mayra, Inés y Enrique, gracias por su amistad. Agradezco de manera especial a la Dra. Gemma Eibes y al Dr. Thelmo Lu Chau por toda la ayuda proporcionada durante este tiempo pero sobre todo por su amabilidad y disponibilidad. También quiero dar las gracias a los técnicos del Biogrup: Carmen, Mar, Miriam, Mónica y Ovidio, por transmitime sus conocimientos pero sobre todo el tiempo dedicado al análisis de cada muestra.

Gracias a todos los integrantes del Biogrup que he conocido a lo largo de estos 4 años por toda la ayuda que me proporcionaron y que contribuyó con el desarrollo de mi tesis, pero especialmente por su amistad ¡Gracias a todos y cada uno de ellos!

Quiero agradecer a varias personas que son especiales en mi vida: Jorge y Marivel, gracias por su disponibilidad y su amistad. Gaby y familia, gracias por toda su ayuda y por su amistad. Gracias a Alicia P. por su apoyo y amistad. Carmen Fajardo, fuiste un gran apoyo desde el día en que llegué a Santiago, gracias por todo. A Dafne y Denisse, gracias por cada momento compartido durante estos años, no encuentro la manera de agradecer todo el apoyo que me han proporcionado. Muchas gracias por su confianza, su amistad, pero especialmente por hacer que mi estancia en Santiago de Compostela fuera genial. También agradezco a mis amigas y amigos de Santiago por su amistad. A mis amigas de toda la vida les agradezco por no olvidarse de mí, pero sobre todo por hacerme creer que era capaz de conseguir este sueño que ahora es realidad.

Por último, quiero dedicar especialmente esta tesis a las personas más importantes de mi vida. Siempre estaré completamente agradecida con Alex Vidal por haber llegado a mi vida en un momento tan oportuno, por apoyarme a lo largo de estos años, pero sobre todo por estar siempre allí. Para mis hermanos Gerardo, Alejandro, Jorge, Luis, Laura y Fernando, mis cuñadas, mi cuñado y mis sobrinos, quienes a pesar de la distancia siempre me han apoyado y motivado para conseguir mis metas. A mis padres, Herminio y Alejandrina, por la confianza que han tenido siempre en mí, por no dejarme caer cuando sentía que no lo iba a conseguir, por sus consejos, por creer en mí pero sobre todo gracias por su amor incondicional. Sé que para ustedes fue muy difícil dejarme marchar al otro lado del mundo, sin su apoyo y comprensión no hubiera podido superar esta meta que me planteé con tanta ilusión desde hace muchos años ¡Gracias por ser mis padres!

Nunca desistas de tus sueños
Sigue las señales
Paulo Coelho

Table of contents

Resumen general

Los compuestos farmacéuticos se utilizan ampliamente a nivel mundial con fines terapéuticos y de mejora de calidad de vida en el ámbito de salud humana y animal. Existe un gran número de grupos terapéuticos aplicados para el tratamiento de distintas enfermedades o dolencias: antibióticos, analgésicos, anti-inflamatorios, anticonceptivos, antidepresivos, antineoplásicos, antiepilépticos, anticonvulsivos, compuestos beta-bloqueantes, reguladores lipídicos, medios de contraste para rayos X, tranquilizantes, entre otros. Se ha calculado que el consumo a nivel mundial de este tipo de compuestos supera las 1000 toneladas por año. El consumo de cada grupo terapéutico puede variar dependiendo de si la venta se encuentra restringida a prescripción médica, tal es el caso de los antidepresivos, o bien pueden comprarse libremente, tal es el caso de los anti-inflamatorios. Tras su consumo y/o uso, estos compuestos se liberan al ambiente a través de la orina y heces fecales ya sea en su forma original o bien en forma de metabolitos y productos de degradación. Posteriormente, pueden ser transportados a través del sistema de alcantarillado municipal, distribuyéndose en aguas superficiales o bien en aguas subterráneas, en concentraciones variables desde ng/L hasta µg/L. Su presencia en el medio acuático se debe, en gran medida, al hecho de que los tratamientos convencionales de las estaciones depuradoras de aguas residuales (EDARs) no los eliminan de forma eficaz. Por ello, el agua residual municipal representa la fuente de emisión de compuestos farmacéuticos más relevante. Por el contrario, estos compuestos se pueden detectar en agua potable, si bien existen evidencias de la presencia de algunos de estos compuestos en concentraciones muy bajas, del orden de ng/L, en este medio.

Estos compuestos pueden generar problemas de ecotoxicidad sobre los organismos acuáticos y terrestres debido a sus propiedades fisicoquímicas, su distribución y persistencia, pero principalmente debido al hecho de que estos compuestos han sido diseñados para producir efectos biológicos en los seres vivos incluso en concentraciones bajas. Por ello, existe un riesgo potencial a su exposición, ya que pueden afectar organismos en diversas formas (cambios de sexo en organismos superiores, modificaciones de crecimiento en plantas, deformidades anatómicas, entre otros). Los fármacos son capaces de persistir en el ambiente durante períodos prolongados ya sea en su forma original o como sus productos de degradación, dependiendo de su estructura química así como de sus propiedades fisicoquímicas. Es importante conocer los parámetros característicos de cada uno de ellos para determinar los métodos óptimos para su eliminación eficaz. Las principales propiedades fisicoquímicas a tener en cuenta

que se encuentran potencialmente relacionadas con los mecanismos de eliminación de estos compuestos son: (i) la solubilidad (S), que a su vez dependerá del pH ambiental, presencia de sales y/o metales, temperatura; (ii) el coeficiente de Henry (H), indicador de su volatilidad; (iii) la constante de disociación (pKa), que indica la tendencia que tiene cada compuesto para disociarse; (iv) el coeficiente de partición octanol-agua (K_{ow}), que indica el carácter lipofílico de cada compuesto; (v) el coeficiente de distribución sólido-agua (K_d), que se relaciona con la concentración del compuesto que se encuentra en la fase sólida o líquida; (vi) finalmente la constante de degradación de pseudo-primer orden (K_{biol}), que indica la facilidad de biodegradación que presenta un determinado compuesto en condiciones aeróbicas mediante lodos biológicos.

En los últimos años se han estudiado e implementado diversos métodos fisicoquímicos y biológicos para eliminar estos compuestos en las EDARs así como en el ambiente acuático. Algunos métodos como procesos de coagulación-floculación, flotación, nitrificación-desnitrificación, biorreactores de membrana (MBRs) han demostrado altas eficiencias de eliminación de compuestos farmacéuticos, especialmente aquellos considerados en esta tesis doctoral. Sin embargo, algunos compuestos recalcitrantes tales como fluoxetina, carbamazepina y diazepam requieren procesos avanzados de oxidación (AOPs) para conseguir su eliminación completa. A pesar de la alta eficiencia de estos métodos, la principal desventaja es su elevado coste comparado con el resto de los procesos mencionados. Una alternativa para la eliminación de fármacos podría ser el uso de hongos ligninolíticos de podredumbre blanca (WRF). Estos son un grupo de microorganismos capaces de degradar la lignina, uno de los componentes de la madera, con una estructura aromática altamente compleja. Estos hongos secretan enzimas ligninolíticas durante su metabolismo secundario: manganeso peroxidasa (MnP), lignina peroxidasa (LiP), peroxidasa versátil (VP) y Lacasa (Lac), que son responsables de la generación de radicales libres altamente reactivos y con baja especificidad por substrato, lo cual plantea su uso como una alternativa potencial para la eliminación de compuestos altamente biodegradables. En esta tesis doctoral se ha evaluado la degradación de ocho compuestos farmacéuticos pertenecientes a diversos grupos terapéuticos: antibióticos (sulfametoxazol, SMX), antidepresivos (citalopram, CTL; fluoxetina, FLX), anti-inflamatorios (diclofenaco, DCF; ibuprofeno, IBP; naproxeno, NPX), antiepilépticos (carbamazepina, CBZ) y tranquilizantes (diazepam, DZP) por acción de tres cepas de WRF: un hongo anamorfo de *Bjerkandera* sp. R1, *Bjerkandera adusta* y *Phanerochaete chrysosporium*.

En la primera parte del **capítulo 1** se presenta una revisión bibliográfica acerca de los compuestos farmacéuticos donde se incluyen algunos datos relevantes acerca de su consumo y presencia en aguas. Además se explica brevemente las vías de entrada de

estos compuestos al ambiente acuático e incluso se plantean los posibles efectos tóxicos que pueden generar estos compuestos tras su liberación al ambiente. Además se hace una breve descripción de los compuestos pertenecientes a los grupos terapéuticos antes mencionados así como los principales mecanismos de eliminación y sus propiedades fisicoquímicas. Finalmente, se incluyen algunos métodos biológicos y fisicoquímicos que han sido investigados de cara a su degradación. En la segunda parte de este capítulo se hace referencia a la importancia que tienen los hongos ligninolíticos así como a las principales características de las enzimas MnP, LiP, VP y Lac para así entender mejor la capacidad de estos organismos y sus enzimas para la eliminación de compuestos recalcitrantes incluyendo los fármacos considerados. Finalmente, se plantean los objetivos principales de esta tesis doctoral.

En el **capítulo 2** se incluyen los materiales y métodos utilizados. Primeramente, se incluyen las tres cepas de hongos ligninolíticos seleccionadas así como una lista con los compuestos farmacéuticos y los disolventes utilizados. Inicialmente se llevaron a cabo ensayos de inhibición del crecimiento del micelio bajo diferentes concentraciones de mezclas de fármacos para determinar la concentración máxima que estos microorganismos pueden tolerar sin que se vea afectado su crecimiento. También se presentan las condiciones de cultivo en estático, libre en forma de pellets e inmovilizado en espuma de poliuretano, especialmente relevantes en los experimentos en discontinuos; así como la preparación del pre-inóculo para los diferentes tipos de biorreactor. Por ello, se presenta la configuración de los biorreactores empleados: reactores de tanque agitado (RTA) y reactores de lecho fijo (FBR), operados con hongo libre e inmovilizado, así como una breve descripción de los ensayos realizados, incluyendo detalles sobre el sistema de pulsación de oxígeno y la distribución del tiempo de residencia (RTD). Por otra parte, se describen los métodos para extraer los compuestos farmacéuticos con acetonitrilo así como los ensayos de adsorción de fármacos sobre la biomasa de los hongos y/o la espuma de poliuretano. Para la identificación y cuantificación de los compuestos analizados se utilizó cromatografía líquida de alta eficacia con un detector de array de diodos (HPLC-DAD) así como cromatografía de gases y espectrometría de masas (GC-MS). Finalmente se enumeran las técnicas analíticas utilizadas para realizar un seguimiento de los perfiles de fermentación durante los ensayos de degradación.

Los primeros experimentos se describen en el **capítulo 3** donde se ha estudiado el efecto de diferentes concentraciones de los compuestos farmacéuticos seleccionados sobre el crecimiento del micelio de las tres cepas de WRF cultivadas en placa para determinar la concentración máxima de ensayo. Los resultados de este capítulo permitieron determinar que los hongos pueden crecer sin problemas de inhibición a

concentraciones sobre 1 mg/L. Adicionalmente se analizó la capacidad de eliminación de los fármacos en ensayos en discontinuo utilizando tres tipos de cultivo diferentes como son los cultivos en estático, con pellets y con hongo inmovilizado en espuma de poliuretano. Los cultivos con pellets presentaron los porcentajes de eliminaciones más altos, más del 99% para DCF, IBP, NPX, CBZ y 57% para DZP. Los ensayos de degradación de fármacos indican que incluso niveles de actividad bajos de enzima pueden ser suficientes para eliminar eficazmente estos compuestos. Estos resultados fueron la base de un experimento posterior realizado en un RTA de 0.75 L, que se aplicó en la degradación de cinco compuestos farmacéuticos diferentes (DCF, IBP, NPX, CBZ, DZP) mediante el hongo anamorfo y *P. chrysosporium*. Finalmente se llevó a cabo un ensayo de degradación de 24 h así como un experimento en fed-batch con pulsos diarios de farmacos para analizar la eliminación de los tres anti-inflamatorios bajo la acción del anamorfo de *Bjerkandera* sp. R1. Se determinó un tiempo de residencia hidráulico (HRT) adecuado para eliminar estos compuestos de 24 h o incluso inferior (se logró la eliminación completa de IBP en sólo 4 h). Los compuestos anti-inflamatorios se eliminaron de forma total por ambas cepas en el RTA, mientras que CBZ y DZP se eliminaron parcialmente (desde 15% hasta 55%). El comportamiento de los pellets dentro del reactor varió de una cepa a otra, debido a que el hongo anamorfo presentó un crecimiento excesivo de biomasa. Debido a esto, se descartó la utilización de dicho hongo para posteriores experimentos en fed-batch y continuo en biorreactores con el fin de evitar problemas de compactación y taponamiento del lecho. Para los experimentos posteriores de esta tesis se seleccionó *P. chrysosporium*.

En el **capítulo 4** se describen las condiciones de operación en fed-batch y continuo en un RTA con pellets de *P. chrysosporium* para la eliminación de DCF, IBP, NPX, CBZ y DZP. Uno de los objetivos es evaluar si los reactores en fed-batch o en continuo lograban mantener o incluso superar los porcentajes de degradación obtenidos en experimentos previos. Así, en un primer experimento se estudió el efecto del nivel de oxígeno sobre la degradación de los tres anti-inflamatorios así como el efecto sobre la morfología de los pellets durante una operación fed-batch en el reactor durante 30 días. En un reactor se alimentaron pulsos de oxígeno con diferentes frecuencias mientras que en el otro se aplicó un flujo de aire en continuo. Los resultados obtenidos durante este capítulo demostraron que los niveles altos de oxígeno no tienen un efecto significativo sobre la degradación de los fármacos, ya que los porcentajes de eliminación bajo el flujo de aire en continuo y pulsos de oxígeno fueron similares (>99% para DCF e IBP y más de 77% para NPX). Sin embargo, los pulsos de oxígeno favorecieron el control del crecimiento excesivo de la biomasa mejorando así la operación y estabilidad del RTA durante períodos de tiempo mayores. A pesar de esto, durante los siguientes ensayos se consideró un flujo de

aire en continuo como sistema de aireación del reactor debido a los menores costes de operación. Posteriormente, se utilizó un RTA con pellets de la cepa seleccionada para eliminar los tres anti-inflamatorios y el antiepiléptico CBZ durante una primera etapa en operación fed-batch (26 días). A continuación, el biorreactor se operó en continuo hasta el día 70. En un experimento adicional, se operó en continuo un RTA durante 50 días para la eliminación de los tres anti-inflamatorios, incluyendo los compuestos recalcitrantes (CBZ y DZP). Así, se demostró que la degradación de los fármacos se favoreció cuando el RTA inició su operación en modo fed-batch durante algunas semanas consiguiendo eliminaciones completas de los tres anti-inflamatorios y significativas de CBZ (hasta un 63%). Cuando el reactor cambió su operación a modo continuo el hongo fue capaz de mantener la degradación parcial o total de estos compuestos durante más de 50 días alcanzando los siguientes porcentajes de eliminación: DCF (34% - 90%); IBP (65% - 95%); NPX (17% - 94%); CBZ (5% - 94%). Los resultados de la operación en continuo demostraron que *P. chrysosporium* fue capaz de eliminar los anti-inflamatorios pero con tiempos de residencias superiores. Además, se consiguió eliminar un 53% de CBZ, sin embargo no fue posible eliminar DZP.

En el **capítulo 5** se describen las condiciones de operación en FBRs utilizando *P. chrysosporium* previamente inmovilizado en espuma de poliuretano para el tratamiento en continuo de DCF, IBP, NPX, CBZ y DZP durante 100 días. Para ello, se estudió el efecto del nivel de oxígeno sobre la degradación de los fármacos así como sobre el control del crecimiento del micelio mediante dos sistemas de aireación: flujo de aire en continuo y pulsos de oxígeno. Los resultados de este experimento demostraron que el hongo inmovilizado fue capaz de eliminar fácilmente los compuestos anti-inflamatorios durante 100 días de operación (78% - 99%), mientras que se consiguió eliminar parcialmente CBZ (hasta el 90%) y DZP (hasta el 72%) bajo ambas condiciones de aireación (aire y oxígeno). Se obtuvieron porcentajes de eliminación más altos para los compuestos recalcitrantes durante la primera mitad de operación (hasta el día 40). Posteriormente, la eficiencia de eliminación disminuyó en ambos reactores. Es importante mencionar que la degradación de DZP se favoreció cuando había niveles altos de oxígeno dentro del biorreactor; mientras que los porcentajes mayores de eliminación de CBZ se lograron bajo flujo de aire en continuo. En un segundo experimento se procedió a analizar la degradación en continuo de estos compuestos farmacéuticos en un RTA con hongo inmovilizado durante 50 días. Este reactor mantuvo una operación estable, sin problemas de taponamiento durante el período establecido alcanzando altos porcentajes de eliminación para los anti-inflamatorios. Sin embargo estos porcentajes fueron menores que los conseguidos en los FBRs. La eliminación de los compuestos recalcitrantes CBZ y DZP también fue menor

cuando se utilizó el RTA bajo esta configuración: menos del 61% y menos del 40%, respectivamente.

En el **capítulo 6** se incluye una revisión bibliográfica de los principales productos de degradación de los tres anti-inflamatorios utilizados (DCF, IBP y NPX). Según las referencias previas, los principales productos de degradación de DCF, IBP y NPX son 4-hidroxi-diclofenac, 1-hidroxi-ibuprofeno y 6-O-desmetil-naproxen, respectivamente. Se estudiaron los cromatogramas obtenidos por GC-MS en diversos ensayos de degradación para determinar la presencia de compuestos que pudieran corresponder con sus posibles metabolitos. Para ello, se han estudiado las posibles estructuras de los compuestos originales y sus posibles metabolitos después de sufrir una derivatización. Posteriormente, se evaluaron las posibles rutas de fragmentación de estos compuestos, que deberían estar relacionados con los iones de cuantificación mayoritarios detectados en los cromatogramas obtenidos durante los experimentos. Tras analizar los cromatogramas, se pudo observar la presencia de diversos picos con iones de cuantificación que podrían corresponder con los principales productos de degradación. Sin embargo, no se ha podido identificar inequívocamente ni cuantificar su presencia ya que sería necesario disponer de los compuestos patrón que no están comercialmente disponibles.

Los resultados obtenidos en esta tesis doctoral demuestran que los hongos ligninolíticos tienen la capacidad para eliminar eficazmente productos como los anti-inflamatorios, antibióticos y anti-depresivos, mientras que compuestos altamente recalcitrantes como FLX, CBZ y DZP también se eliminaron, aunque de forma parcial. La determinación de los productos de degradación y/o metabolitos es un aspecto de especial relevancia de cara a plantear las posibles rutas de degradación de estos compuestos.

Resumo xeral

Os compostos farmacéuticos son utilizados amplamente a nivel mundial con fins terapéuticos e de mellora de calidade de vida no ámbito de saúde humana e animal. Existe un gran número de grupos terapéuticos aplicados para o tratamento de distintas enfermidades ou doenzas: antibióticos, analxésicos, anti-inflamatorios, anticonceptivos, antidepresivos, antineoplásicos, antiepilépticos, anticonvulsivos, compostos beta-bloqueantes, reguladores lipídicos, medios de contraste para raios X, tranquilizantes, entre outros. Calculouse que o consumo a nivel mundial deste tipo de compostos supera as 1000 toneladas por ano. O consumo de cada grupo terapéutico pode variar dependendo de se a venda atópase restrinxida a prescrición médica, tal é o caso dos antidepresivos, ou ben poden comprarse libremente, tal é o caso dos anti-inflamatorios. Tras o seu consumo e/ou uso, estes compostos libéranse ao ambiente a través dos ouriños e feces fecais xa sexa na súa forma orixinal ou como metabolitos e produtos de degradación. Posteriormente, poden ser transportados a través do sistema de rede de sumidoiros municipal distribuíndose en augas superficiais ou ben en augas subterráneas en concentracións dende ng/L ata µg/L. A súa presenza no medio acuático débese, en gran medida, ao feito de que os tratamentos convencionais das estacións depuradoras de augas residuais (EDARs) non os eliminan de forma eficaz. Por iso, a auga residual municipal representa a fonte de emisión de compostos farmacéuticos máis relevante. Pola contra, estes compostos pódense detectar en auga potable, aínda que existen evidencias da presenza dalgúns destes compostos en concentracións moi baixas, da orde de ng/L, neste medio.

Estes compostos poden xerar problemas de ecotoxicidad sobre os organismos acuáticos e terrestres debido ás súas propiedades fisicoquímicas, a súa distribución e persistencia, pero principalmente debido ao feito de que estes compostos foron deseñados para producir efectos biolóxicos nos seres vivos mesmo en concentracións baixas. Por iso, existe un risco potencial á súa exposición, xa que poden afectar organismos en diversas formas (cambios de sexo en organismos superiores, modificacións de crecemento en plantas, deformidades anatómicas, entre outros). Os fármacos son capaces de persistir no ambiente durante períodos prolongados xa sexa na súa forma orixinal ou como os seus produtos de degradación, dependendo da súa estrutura química así como das súas propiedades fisicoquímicas. É importante coñecer os parámetros característicos de cada un deles para determinar os métodos óptimos para a súa eliminación eficaz. As principais propiedades fisicoquímicas a ter en conta e que se atopan

potencialmente relacionadas cos mecanismos de eliminación destes compostos son: (i) a solubilidade (S), que á súa vez dependerá do pH ambiental, presenza de sales e/ou metais, temperatura; (ii) o coeficiente de Henry (H), indicador da súa volatilidade; (iii) a constante de disociación (pKa), que indica a tendencia que ten cada composto para disociarse; (iv) o coeficiente de partición octanol-auga (K_{ow}), que indica o carácter lipofílico de cada composto; (v) o coeficiente de distribución sólido-auga (K_d), que se relaciona coa concentración do composto que se atopa na fase sólida ou líquida; (vi) finalmente a constante de degradación de pseudo-primeira orde (K_{biol}), que indica a facilidade de biodegradación que presenta un determinado composto en condicións aeróbicas mediante lodos biolóxicos.

Nos últimos anos estudáronse e implementáronse diversos métodos fisicoquímicos e biolóxicos para eliminar estes compostos nas PTAR así como no ambiente acuático. Algúns métodos como procesos de coagulación-floculación e flotación, nitrificación-desnitrificación, biorreactores de membrana (MBRs) demostraron altas eficiencias de eliminación dos compostos farmacéuticos, especialmente os compostos considerados nesta tese doutoral. Con todo, algúns compostos recalcitrantes tales como fluoxetina, carbamazepina e diazepam requiren procesos avanzados de oxidación (AOPs) para conseguir a súa completa eliminación. A pesar da alta eficiencia destes métodos, a principal desvantaxe é o seu elevado custo comparado co resto dos procesos mencionados. Unha alternativa para a eliminación de fármacos podería ser o uso de fungos ligninolíticos de podremia branca (WRF). Estes son un grupo de microorganismos capaces de degradar a lignina, un dos compoñentes da madeira, cunha estrutura aromática altamente complexa. Estes fungos secretan encimas ligninolíticas durante o seu metabolismo secundario: manganeso peroxidasa (MnP), lignina peroxidasa (LiP), peroxidasa versátil (VP) e Lacasa (Lac), que son responsables da xeración de radicais libres altamente reactivos e con baixa especificidade por substrato, o cal expón o seu uso como unha alternativa potencial para a eliminación de compostos altamente biodegradables. Nesta tese doutoral avaliouse a degradación de oito compostos farmacéuticos pertencentes a diversos grupos terapéuticos: antibióticos (sulfametoxazol, SMX), antidepresivos (citalopram, CTL; fluoxetina, FLX), anti-inflamatorios (diclofenaco, DCF; ibuprofeno, IBP; naproxeno, NPX), antiepilépticos (carbamazepina, CBZ) e tranquilizantes (diazepam, DZP) por acción de tres cepas de WRF: un fungo anamorfo de *Bjerkandera* sp. R1, *Bjerkandera adusta* e *Phanerochaete chrysosporium*.

Na primeira parte do **capítulo 1** preséntase unha revisión bibliográfica acerca dos compostos farmacéuticos onde se inclúen algúns datos relevantes acerca do seu consumo e presenza en augas. Ademais explícase brevemente as vías de entrada destes compostos ao ambiente acuático e mesmo se expoñen os posibles efectos tóxicos que poden xerar

estes compostos tras a súa liberación ao ambiente. Ademais faise unha breve descrición dos compostos pertencentes aos grupos terapéuticos antes mencionados así como os principais mecanismos de eliminación e as súas propiedades fisicoquímicas. Finalmente, inclúense algúns métodos biolóxicos e fisicoquímicos que foron investigados de cara á súa degradación. Na segunda parte deste capítulo faise referencia á importancia que teñen os fungos ligninolíticos así como ás principais características das encimas MnP, LiP, VP e Lac para así entender mellor a capacidade destes organismos e as súas encimas para a eliminación de compostos recalcitrantes incluíndo os fármacos considerados. Finalmente, exponse os obxectivos principais desta tese doutoral.

No **capítulo 2** inclúense os materiais e métodos utilizados. Primeiramente, inclúense as tres cepas de fungos ligninolíticos seleccionadas así como unha lista cos compostos farmacéuticos e os disolventes utilizados. Inicialmente levaronse a cabo ensaios de inhibición do crecemento do micelio baixo diferentes concentracións de mesturas de fármacos para determinar a concentración máxima que estes microorganismos poden tolerar sen que se vexa afectado o seu crecemento. Tamén se presentan as condicións de cultivo en estático, libre en forma de pellets e inmobilizado en espuma de poliuretano, especialmente relevantes nos experimentos en descontinuos; así como a preparación do pre-inóculo para os diferentes tipos de biorreactor. Por iso, preséntase a configuración dos biorreactores empregados: reactores de tanque axitado (RTA) e reactores de leito fixo (FBR), operados con fungo libre e inmovilizado; así como unha breve descrición dos ensaios realizados, incluíndo detalles sobre o sistema de pulsación de osíxeno e a distribución do tempo de residencia hidráulico (RTD). Por outra banda, descríbense os métodos para extraer os compostos farmacéuticos con acetonitrilo así como os ensaios de absorción de fármacos sobre a biomasa dos fungos e/ou a espuma de poliuretano. Para a identificación e cuantificación dos compostos analizados utilizouse cromatografía líquida de alta eficacia cun detector de array de diodos (HPLC-DAD) así como cromatografía de gases e espectrometría de masas (GC-MS). Finalmente enuméranse as técnicas analíticas utilizadas para realizar un seguimento dos perfís de fermentación durante os ensaios de degradación.

Os primeiros experimentos descríbense no **capítulo 3** onde se estudou o efecto de diferentes concentracións dos compostos farmacéuticos seleccionados sobre o crecemento do micelio das tres cepas de WRF cultivadas en placa para determinar a concentración máxima de ensaio. Os resultados deste capítulo permitiron determinar que os fungos poden crecer sen problemas de inhibición a concentracións sobre 1 mg/L. Adicionalmente analizouse a capacidade de eliminación dos fármacos en ensaios en descontinuo utilizando tres tipos de cultivo diferentes como son os cultivos en estático, con pellets e co fungo inmobilizado en espuma de poliuretano. Os cultivos con pellets

presentaron as porcentaxes de eliminacións máis altos, máis do 99% para DCF, IBP, NPX, CBZ e 57% para DZP. Os ensaios de degradación de os fármacos indican que mesmo niveis de actividade baixos de encima poden ser suficientes para eliminar eficazmente estes compostos. Estes resultados foron a base dun experimento posterior realizado nun RTA de 0.75 L, que se aplicou na degradación de cinco compostos farmacéuticos diferentes (DCF, IBP, NPX, CBZ e DZP) mediante o fungo anamorfo e *P. chrysosporium*. Finalmente levouse a cabo un ensaio de degradación de 24 h así como un experimento en fed-batch con pulsos diarios de farmacéuticos para analizar a eliminación dos tres anti-inflamatorios baixo a acción do anamorfo de *Bjerkandera* sp. R1. Determinouse un tempo de residencia hidráulico (HRT) adecuado para eliminar estes compostos de 24 h ou mesmo inferior (logrouse a eliminación completa de IBP en só 4 h). Os compostos anti-inflamatorios eliminároнse de forma total por ambas as cepas no RTA, mentres que CBZ e DZP eliminároнse parcialmente (desde 15% ata 55%). O comportamento dos pellets dentro do reactor variou dunha cepa a outra, debido a que o fungo anamorfo presentou un crecemento excesivo de biomasa. Debido a isto, descartouse a utilización do devandito fungo para posteriores experimentos en fed-batch e continuo en biorreactores co fin de evitar problemas de compactación e taponamiento do leito. Para os experimentos posteriores desta tese seleccionouse P. chrysosporium.

No **capítulo 4** descríbense as condicións de operación en fed-batch e continuo nun RTA con pellets desta cepa para a eliminación de DCF, IBP, NPX, CBZ e DZP. Un dos obxectivos adicionais é avaliar se os reactores en fed-batch ou continuo lograban manter ou mesmo superar as porcentaxes de degradación obtidos en experimentos previos. Así, nun primeiro experimento estudouse o efecto do nivel de osíxeno sobre a degradación dos tres anti-inflamatorios así como o efecto sobre a morfoloxía dos pellets durante unha operación fed-batch no reactor durante 30 días. Nun reactor alimentáronse pulsos de osíxeno con diferentes frecuencias de pulsación mentres que no outro biorreactor aplicouse un fluxo de aire en continuo. Os resultados obtidos durante este capítulo demostraron que os niveis altos de osíxeno non teñen un efecto significativo sobre a degradación dos fármacos, xa que as porcentaxes de eliminación baixo o fluxo de aire en continuo e pulsos de osíxeno foron similares (>99% para DCF e IBP e máis de 77% para NPX). Con todo, os pulsos de osíxeno favoreceron o control do crecemento excesivo da biomasa mellorando así a operación e estabilidade do RTA durante períodos de tempo maiores. A pesar disto, durante os seguintes ensaios considerouse un fluxo de aire en continuo como sistema de aireación do reactor debido aos menores custos de operación. Posteriormente, utilizouse un RTA con pellets da cepa seleccionada para eliminar o tres anti-inflamatorios e o antiepiléptico CBZ durante unha primeira etapa en operación fed-batch (26 días). A continuación, o biorreactor operouse en continuo ata o día 70. Nun

experimento adicional, operouse en continuo un RTA durante 50 días para a eliminación do tres anti-inflamatorios, incluíndo os compostos recalcitrantes (CBZ e DZP). Así, demostrouse que a degradación dos fármacos se favoreceu cando o RTA iniciou a súa operación en modo fed-batch durante algunhas semanas conseguindo eliminacións completas dos tres anti-inflamatorios e significativas de CBZ (ata un 63%). Cando o reactor cambiou a súa operación a modo continuo o fungo foi capaz de manter a degradación parcial ou total destes compostos durante máis de 50 días alcanzando as seguintes porcentaxes de eliminación: DCF (34% - 90%); IBP (65% - 95%); NPX (17% - 94%); CBZ (5% - 94%). Os resultados da operación en continuo demostraron que *P. chrysosporium* foi capaz de eliminar os anti-inflamatorios pero con tempos de residencias superiores. Ademais, conseguiuse eliminar un 53% de CBZ, con todo non foi posible eliminar DZP. Estes resultados demostran que os pellets de *P. chrysosporium* teñen a capacidade de eliminar eficazmente compostos como DCF, IBP e NPX, así como eliminar parcialmente compostos altamente recalcitrantes como CBZ e DZP, mesmo con baixas actividades encimáticas.

No **capítulo 5** descríbense as condicións de operación en FBRs utilizando *P. chrysosporium* previamente inmobilizado en espuma de poliuretano para o tratamento en continuo de DCF, IBP, NPX, CBZ e DZP durante 100 días. Para iso, estudouse o efecto do nivel de osíxeno sobre a degradación dos fármacos así como sobre o control do crecemento do micelio mediante dous sistemas de aireación: fluxo de aire en continuo e pulsos de osíxeno. Os resultados deste experimento demostraron que o fungo inmobilizado foi capaz de eliminar facilmente os compostos anti-inflamatorios durante 100 días de operación (78% - 99%), mentres que se conseguiu eliminar parcialmente CBZ (ata o 90%) e DZP (ata o 72%) baixo ambas as condicións de aireación (aire e osíxeno). Obtivéronse porcentaxes de eliminación máis altos para os compostos recalcitrantes durante a primeira metade de operación (ata o día 40). Posteriormente, a eficiencia de eliminación diminuíu en ambos os reactores (aire e osíxeno). É importante mencionar que a degradación de DZP favoreceuse cando había niveis altos de osíxeno dentro do biorreactor; mentres que as porcentaxes maiores de eliminación de CBZ lográronse baixo fluxo de aire en continuo. Nun segundo experimento procedeuse a analizar a degradación en continuo destes compostos farmacéuticos nun RTA con fungo inmobilizado durante 50 días. Este reactor mantivo unha operación estable, sen problemas de taponamiento durante ó período establecido alcanzando altas porcentaxes de eliminación para os anti-inflamatorios. Con todo estas porcentaxes foron menores que os conseguidos nos FBRs. A eliminación dos compostos recalcitrantes CBZ e DZP tamén foi menor cando se utilizou o RTA baixo esta configuración: menos do 61% e menos do 40%, respectivamente.

No **capítulo 6** inclúese unha revisión bibliográfica dos principais produtos de degradación dos tres anti-inflamatorios utilizados (DCF, IBP e NPX). Segundo as referencias previas, os principais produtos de degradación de DCF, IBP e NPX son 4-hidroxi-diclofenac, 1-hidroxi-ibuprofeno e 6-O-desmetil-naproxen, respectivamente. Estudáronse os cromatogramas obtidos por GC-MS en diversos ensaios de degradación para determinar a presenza de compostos que puidesen corresponder cos seus posibles metabolitos. Para iso, estudáronse as posibles estruturas dos compostos orixinais e os seus posibles metabolitos despois de sufrir unha derivatización. Posteriormente, avaliáronse as posibles rutas de fragmentación destes compostos, que deberían estar relacionados cos iones de cuantificación maioritarios detectados nos cromatogramas obtidos durante os experimentos. Tras analizar os cromatogramas, púidose observar a presenza de diversos picos con iones de cuantificación que poderían corresponder cos principais produtos de degradación. Con todo, non se puido identificar inequivocamente nin cuantificar a súa presenza xa que sería necesario dispor dos compostos patrón que non están comercialmente dispoñibles.

Os resultados obtidos nesta tese doutoral demostran que os fungos ligninolíticos teñen a capacidade para eliminar eficazmente produtos como os anti-inflamatorios, antibióticos e anti-depresivos, mentres que compostos altamente recalcitrantes como FLX, CBZ e DZP tamén se eliminaron, aínda que de forma parcial. A determinación dos produtos de degradación e/ou metabolitos é un aspecto de especial relevancia de cara a expor as posibles rutas de degradación destes compostos.

General summary

Pharmaceutical compounds are used worldwide for therapeutic purposes in human and animal health. Particularly for pharmaceutical compounds, there are a number of therapeutic groups applied for the treatment of several diseases or illnesses: antibiotics, analgesics, anti-inflammatories, contraceptives, antidepressants, antineoplastics agents, antiepileptics, anticonvulsants, beta-blocker compounds, lipid regulators, X-ray contrast media, tranquilizers, among others. It is estimated that the worldwide consumption of pharmaceuticals exceed 1000 tons per year. The consumption of each therapeutic group may vary depending on whether the sale is restricted to a medical prescription, such is antidepressants, or can be purchased freely in the counter as the case of anti-inflammatory drugs. After their consumption and/or use, these compounds are released into the environment via urine and faeces either in its original form as well as metabolites and degradation products. Then, these compounds can be transported through the municipal drainage system and distributed in surface water and groundwater in variable concentrations ranging from ng/L to µg/L. Their presence in the aquatic environment is mainly due to the fact that conventional treatments in the sewage treatment plants (STPs) do not remove these compounds efficiently. Therefore, municipal wastewater is considered as the most relevant emission source of pharmaceutical compounds. By contrast, these compounds can be detected in drinking water, although there are evidences of the presence of some of these compounds in very low concentrations, in the order of ng/L.

These compounds may cause ecotoxicity problems on aquatic and terrestrial organisms due to their physicochemical properties, distribution and persistence, but mainly due the fact that these compounds have been designed to produce biological effects on living organisms even at low concentrations. Therefore, a potential risk to exposure exists, since some of these compounds could affect organisms in various forms (sex changes in higher organisms, changes in plants growth, anatomical deformities, among others). Pharmaceutical compounds are able to persist in the environment for long periods of time either in its original form or as its degradation products, depending on their chemical structure as well as their physicochemical properties. It is important to know the particular parameters of each compound to determine the optimal methods for their efficient removal. The main physicochemical properties which are potentially related to the removal mechanisms are listed below: (i) solubility (S), which in turn depends on pH, presence of salts and/or metals, temperature; (ii) Henry coefficient (H), related to

volatilization processes; (iii) dissociation constant (pKa), which indicates the dissociation trend of each compound; (iv) octanol-water partition coefficient (K_{ow}), indicative of the lipophilic character of each compound; (v) solid-water distribution coefficient (K_d), related to the concentration of the compound found in the liquid or solid phase; (vi) finally, the pseudo-first order degradation constant (K_{biol}), indicating the biodegradability by aerobic activated sludge.

In recent years several physicochemical and biological methods have been studied and implemented for the removal of these compounds in STPs. Some methods such as coagulation-flocculation and flotation, nitrification-denitrification, membrane bioreactors (MBRs), among others, have shown high removal efficiencies of pharmaceutical compounds, especially the ones considered in this doctoral thesis. However, some recalcitrant compounds such as fluoxetine, carbamazepine and diazepam require advanced oxidation processes (AOPs) to achieve complete removal. Despite the high efficiency of these methods, the main disadvantage is their high costs when compared with the other processes. An alternative for the elimination of pharmaceutical compounds could be the use of white rot fungi (WRF). These are a group of microorganisms capable of degrading lignin, a component of wood, with a highly complex aromatic structure. These fungi secrete ligninolytic enzymes (LMEs) during their secondary metabolism: manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP) and Laccase (Lac), which are responsible for the generation of highly reactive free radicals with low substrate specificity, which suggests their use as a potential alternative for removal of highly biodegradable compounds. In this doctoral thesis the removal of eight pharmaceutical compounds belonging to several therapeutic groups has been evaluated: antibiotics (sulfamethoxazole, SMX), antidepressants (citalopram, CTL, fluoxetine, FLX), anti-inflammatory (diclofenac, DCF, ibuprofen, IBP, naproxen, NPX), anticonvulsants (carbamazepine, CBZ) and tranquilizers (diazepam, DZP) by three WRF strains: an anamorph of *Bjerkandera* sp. R1, *Bjerkandera adusta* and *Phanerochaete chrysosporium*.

The first part of **chapter 1** provides a literature review about pharmaceutical compounds which includes some relevant information about their consumption and occurrence in the environment. Also it is explain the pathways of these compounds into the aquatic environment and even the toxic effects generated after the release of these compounds into the environment. In addition, a brief description of the pharmaceutical compounds belonging to the therapeutic groups mentioned above is included as well as the main removal mechanisms and the physicochemical properties of each compound. Finally, some biological and physicochemical methods which have been investigated for their removal are included. The second part of this chapter refers to the importance of the

ligninolytic fungi as well as the main features of the enzymes MnP, LiP, VP and Lac to achieve a better understanding of the ability of these microorganisms and their enzymes for the removal of several recalcitrant compounds. Finally, the main objectives of this thesis are presented.

In **chapter 2** the materials and methods used. Firstly, the three strains of WRF and a list of the selected pharmaceuticals as well as the chemical solvents used are detailed. Initially experiments to assess mycelium growth inhibition under different concentrations of mixtures of pharmaceuticals were conducted to determine the optimal concentration which does not affect fungal growth. Also the culture conditions for static, free pellets and immobilized fungus in polyurethane foam, particularly relevant during batch experiments, are presented; as well as the preparation of the pre-inoculum required for the experiments in bioreactors. Therefore, the configurations of two different types of bioreactors are presented: stirred tank reactors (STRs) and fixed-bed reactors (FBRs) operated with free and immobilized fungus. Moreover, a brief description of the experiments carried out, including details about the oxygen pulsation system and the residence time distribution (RTD), is included. On the other hand, methods for pharmaceuticals extraction with acetonitrile as well as adsorption assays on fungal biomass and/or polyurethane foam are described. For the identification and quantification of the analyzed compounds, both high performance liquid chromatography equipped with a diode array detector (HPLC-DAD) and gas chromatography-mass spectrometry (GC-MS) were used. Finally, a list of the analytical techniques used to perform a monitor of the fermentation profiles during the degradation assays is included.

The first experiments are described in **chapter 3** where the effect of different concentrations of the selected pharmaceuticals of the mycelium growth of three strains of WRF cultured in agar plates was assessed with the objective of determining the threshold concentration. The results of this chapter indicated that concentrations lower than 1 mg/L allowed fungal growth with no remarkable inhibition. Additionally, the ability for pharmaceutical removal during batch assays was studied using static, free pellets and immobilized fungus in polyurethane foam. Free pellets cultures attained the highest removal percentages, above 99% for DCF, IBP, NPX, CBZ, and 57% of DZP. The degradation experiments pointed out that low level of enzyme activity may be enough to effectively remove these compounds. These results were the basis for a subsequent experiment conducted in STR of 0.75 L, which was applied for the degradation of five different pharmaceuticals (DCF, IBP, NPX, CBZ and DZP) by the anamorph fungus and *P. chrysosporium*. Finally, 24 h time-course and fed-batch experiments were carried out to evaluate the removal of the three anti-inflammatory drugs under the action of the anamorph of *Bjerkandera* sp. R1. A hydraulic residence time (HRT) of 24 h or even less (a

complete removal of IBP was achieved in only 4 h) was considered as sufficient for a major operation. Anti-inflammatory compounds were totally removed in the STR by both fungal strains, while CBZ and DZP were partially degraded (from 15% up to 55%). The behaviour of free pellets within the STR may vary from one fungal strain to another, since the anamorph fungus presented excessive biomass growth. Because of this, the use of this fungus was discarded for the following experiments in fed-batch and continuous bioreactors in order to avoid possible compaction and/or clogging problems. The fungus P. chrysosporium was selected for all futher experiments.

Chapter 4 describes the operational conditions in fed-batch and continuous in a STR with fungal pellets for removal of DCF, IBP, NPX, CBZ and DZP. The main objective was to assess whether the fed-batch or continuous bioreactors were able to maintain or even enhance the degradation rates obtained in previous experiments. Thus, in a first experiment the effect of the oxygen level on the degradation of the three anti-inflammatories as well as on pellet morphology were studied during a fed-batch operation in a STR for 30 days. Oxygen pulses were added in a reactor using different pulse frequencies while in the other one a continuous air flow was applied. The results obtained in this chapter showed that high oxygen levels have no significant effect on the degradation of the considered drugs, since the removal percentages under continuous air flow and oxygen pulses were similar (>99% for DCF and IBP; >77% for NPX). However, oxygen pulses favour pellets morphology by control of excessive fungal growth, improving the operation and stability of the STR for longer periods of time. Despite this, during the following experiments, a continuous air flow was considered as an aeration system of the STR since it implies lower operational costs. Thereafter, the removal of the three anti-inflammatories and the anticonvulsant CBZ were conducted in a STR with free pellets of the selected strain in fed-batch mode (26 days). Subsequently, the bioreactor was operated continuously until day 70. In an additional experiment, a continuous STR was operated for 50 days for removal of the three anti-inflammatories, including the recalcitrant compounds (CBZ and DZP). Thus, the degradation of these drugs was enhanced when the STR began its operation in fed-batch mode for few weeks, with complete removals for the three anti-inflammatory drugs and significant for CBZ (up to 64%). When the bioreactor was operated in a continuous mode, the fungus was able to maintain the degradation of these compounds for more than 50 days with the following degradation percentages: DCF (34% up to 90%); IBP (65% up to 95%); NPX (17% up to 94%); CBZ (5% up to 94%). The results of the continuous operation demonstrate that *P. chrysosporium* was able to eliminate the anti-inflammatory drugs but after a longer residence time. In addition, the drug CBZ was partially removed (53%) whereas the removal of DZP was negligible.

In **chapter 5** the application of immobilized *P. chrysosporium* in polyurethane foam was considered for the continuous removal of DCF, IBP, NPX, CBZ and DZP for 100 days. The effect of the oxygen level on the degradation of the drugs as well as on the fungal growth was analyzed considering two aeration systems: a continuous air flow and oxygen pulsation. The results of this experiment showed that the immobilized fungus was able to easily remove the anti-inflammatory compounds (78% - 99%) for 100 days of operation, while partial removal for CBZ (up to 90%) and DZP (up to 72%) under both aeration conditions (air and oxygen) was obtained. The highest removal percentages for recalcitrant compounds were achieved during the first half of the operation (up to day 40). Subsequently, the removal efficiency decreased in both reactors. It is noteworthy that the degradation of DZP was favoured at high levels of oxygen; while the highest removal percentage for CBZ was achieved under continuous air flow. In a second experiment the continuous degradation of these pharmaceutical compounds in STR with immobilized fungus for 50 days was analyzed. This bioreactor maintained a steady operation for the established period, with high removal percentages for the anti-inflammatory compounds. However, these percentages were lower than those achieved in the FBRs. The elimination of CBZ and DZP was also lower in the STR under this configuration: less than 61% and less than 40%, respectively.

Chapter 6 includes a review of the available literature related with the major degradation products of the three anti-inflammatory compounds used (DCF, IBP and NPX). According to literature, the major degradation products of DCF, IBP and NPX are 4-hydroxy-diclofenac, 1-hydroxy-ibuprofen and 6-O-desmethyl-naproxen, respectively. The chromatograms obtained by GC-MS during several degradation assays were analyzed in order to determine the presence of peaks that could correspond to the potential metabolites of the anti-inflammatories. Therefore, the possible chemical structures of the parent compounds as well as their metabolites after derivatization were evaluated. Later, the possible fragmentation pathways of these compounds were evaluated. Such patterns would be related to the identification of the major ions detected in the chromatograms obtained during the experiments. After analyzing the chromatograms, the presence of different quantification ion peaks could correspond with the major degradation products.However, a clearly identify or quantify of their presence was not possible, since it would be necessary the standard compounds which are not commercially available.

The results obtained in this doctoral thesis demonstrate that ligninolytic fungi have the ability to effectively remove compounds such as anti-inflammatories, antibiotics, anti-depressants such as CTL, while highly recalcitrant compounds as FLX, CBZ and DZP were also eliminated, albeit partially. The determination of degradation products and/or

metabolites is an aspect of special relevance facing the possible degradation routes of these compounds.

Chapter 1

General introduction

Summary

This chapter provides an extensive description of the environmental issues generated by the release of pharmaceutical compounds in the environment. These compounds have probably been present in water and the environment for as long as humans have been using them. The drugs that we take are not entirely absorbed by our bodies, and are excreted and passed into wastewater and surface water. Data about their consumption and occurrence as well as the way how these compounds are released into the aquatic environment are detailed. With advances in technology that improved the ability to detect and quantify these chemicals, we can now begin to identify what effects, if any, these chemicals have on human and environmental health and from this point, to design the best strategy for their removal. A brief description of the therapeutic groups of the considered pharmaceuticals in this research study: antibiotics (sulfamethoxazole), anti-depressants (citalopram and fluoxetine), anti-inflammatories (diclofenac, ibuprofen and naproxen), antiepileptics (carbamazepine) and tranquilizers (diazepam) is presented, including the main physicochemical properties of each target compound (chemical structure, solubility, Henry coefficient, pseudo-first order degradation, dissociation constant, octanol-water partition coefficient and the solid-water distribution coefficient). These characteristics are of major relevance as they may be indirect indicators of their potential removal by different technologies according to mechanisms such as sorption, volatilization and biodegradation. The development of alternative approach for their degradation apart from physicochemical and biological treatments may be based on the use of white-rot fungi (WRF) and their lignin modifying enzymes (LMEs). The rationale behind this approach may be justified by the fact that these biocatalysts are capable to degrade lignin and other recalcitrant compounds such as organic pollutants: polycyclic aromatic hydrocarbons (PAHs), dyes, chlorinated and phenolic compounds. This section establishes the framework of the research developed in this doctoral thesis and aims to defend why WRF may be a feasible option to be taken into account for the removal of pharmaceutical compounds.

Outline

1.1. Pharmaceutical compounds

1.1.1. Pharmaceutical compounds in the environment: consumption, sources and fate

Pharmaceutical compounds comprise a wide group of organic compounds used for human and veterinary medicine. Pharmaceuticals include prescription and over-the-counter (OTC) drugs used for treatment of several diseases and can also be applied to illicit drugs. There are more than 3000 compounds of this type, worldwide used, belonging to several therapeutic classes such as antibiotics, analgesics, anti-inflammatory, beta blockers, contraceptives, lipid regulators, antidepressants, antineoplastic agents, tranquilizers, anticonvulsants, among others (Fent et al., 2006). All these compounds have complex molecules with different physicochemical and biological properties and they 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) those drugs that might have low persistence, 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).

A large consumption of these compounds by the population is evidenced, contributing with the continuous release of the parent compound and/or their metabolites. The quantity of pharmaceuticals consumed depends on their therapeutic group; for example, anti-inflammatory drugs along with antibiotics are the groups of medicines most commonly used. Therefore, significant concentrations of these compounds have been detected in the environment (Daughton and Ternes; 1999; Ikehata et al., 2006; Kümmerer, 2009a and 2009b; Ziylan and Ince, 2011). The consumption of several compounds may vary between the different countries (Table 1-1). In 2005, Spain was ranked in the eighth position in the pharmaceutical market in Europe (Gros et al., 2010). The consumption data corresponds to the annual sales or consumption, including prescribed drugs and over-the-counter (OTC) drugs used for diagnosis (or a mixture of both). However, internet sales were not included; therefore, the accurate value of consumption of pharmaceuticals is uncertain but probably higher than some of the data reported (Fent et al., 2006).

Table 1-1. Consumption of several pharmaceutical compounds in different countries

Therapeutic group and compound	Consumption per year
Anti-inflammatory	
Acetylsalicylic acid	836 t in Germany (2001)[2]
Diclofenac	6.1 t in Austria (2004)[1]; 81.7 t in Germany (2004)[2]; 4.5 t in Switzerland (2004)[2]; 9.6 t in Finland (2004)[3]; 940 - 1000 t (Global consumption)[5,6] ; 26 t in England 2000)[7]; 4.4 t in Australia 1998)[7]
Ibuprofen	6.7 t in Austria (2004)[1]; 259.8 t in Germany (2004)[2]; 25 t in Switzerland (2004)[2]; 70 t in Finland (2004)[3]; 162 t in England (2000)[7]; 14.2 t in Australia (1998)[7]
Naproxen	1.7 t in Switzerland (2004)[2]; 6.7 t in Finland (2004)[3]; 35 t in England (2000)[7]; 22.8 t in Australia (1998)[7]
Paracetamol	622 t in Germany (2001)[2]
Antiepileptic	
Carbamazepine	88 t in Germany (2001)[2] ; 6.3 t in Austria (2004)[1]; 40 t in England [4]; 1000 t (Global consumption)[5,6]
Antibiotic	
Sulfamethoxazole	0.9 t in Austria (2004)[1]
Tranquilizer	
Diazepam	0.12 t in Austria (2004)[1]

(1) Clara et al., 2005; (2) Fent et al., 2006; (3) Lindqvist et al., 2005; (4) Vogna et al., 2004; (5) Zhang et al., 2008; (6) Zhang and GeiBen, 2010; (7) Ziylan and Ince, 2011.

Undoubtedly in the last century, the use of pharmaceuticals aided to improve both the quality and length of life (Kummerer, 2007), but also contributed to the spread of pharmaceutical compounds either from direct disposal or from the effluent of STPs. In 1976, Garrison *et al.* were the first to report the occurrence of clofibric acid (the active metabolite from certain lipid regulators) in treated wastewater in USA at concentrations ranging from 0.8 µg/L to 2 µg/L (Daughton and Ternes, 1999; Nikolaou et al., 2007). Nowadays, the presence of several pharmaceuticals in the environment is a fact and it has been confirmed in various countries, such as Germany (Ternes, 1998), Brazil (Stumpf et al., 1999), Italy (Andreozzi et al., 2003; Zucatto et al., 2006), Spain (Carballa et al., 2004; Suárez et al., 2009; Gonzalez-Alonso et al., 2010; Gros et al., 2010), Canada (Lishmann et al., 2006), 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 several pharmaceuticals -more than 80-

could be detected at levels of µg/L in sewage, surface and groundwaters (Heberer, 2002; Castiglioni et al., 2006).

After their administration, drugs are absorbed, distributed, metabolized and finally excreted through urine and faeces, either as active compound and/or its metabolites; even those pharmaceutical compounds used in ophthalmology and skin problems may suffer an absorption process, with a final destination in the blood circulation system (Adler et al., 2006; Mückter, 2006). Since pharmaceutical compounds are drugs for human and/or animal use, two main metabolic pathways after their administration can be distinguished (Figure 1-1).

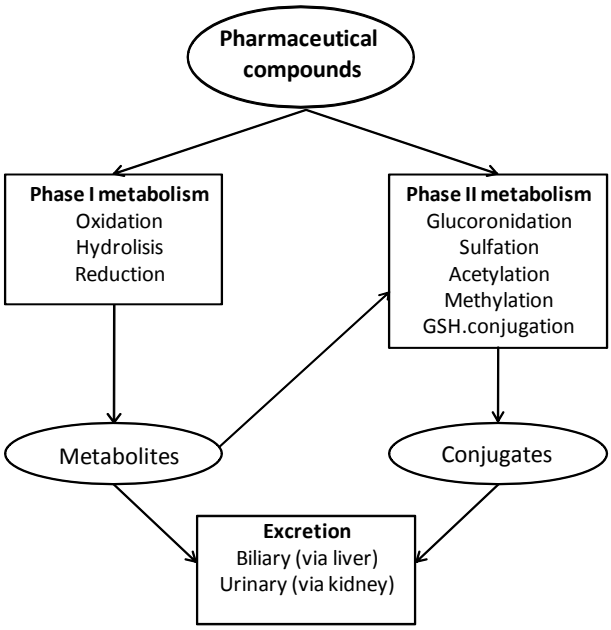


Figure 1-1. Metabolism of the drugs in the human and/or animal body (Adapted from Daughton and Ternes, 1999; Pérez and Barceló, 2007).

In phase I several chemical reactions such as hydrolysis, oxidation, reduction, alkylation and dealkylation may occur, meanwhile the conjugates, mainly glucoronides and sulphonamides, are formed during phase II (Pérez and Barceló, 2007; Suárez et al., 2008). It is noteworthy that a fraction of the pharmaceutical compounds pass through the body unchanged. The degree to which a compound is transformed in the body essentially depends on its pharmacology, structure and mechanism of action. In Table 1-2 the percentages of bioavailability and unchanged excretion of a few drugs are reported. Moreover, excretion percentages of a wide range of pharmaceuticals can be found in literature (Bound and Voulvoulis, 2005; Jjemba, 2006).

Table 1-2. Bioavailability to the body and unchanged excretion of some pharmaceuticals (Bound and Voulvoulis, 2005; Jjemba 2006)

Therapeutic group	Pharmaceutical compound	Bioavailability to the body (%)	Unchanged excretion (%)
Anesthetics	Lidocaine	35	<10
Antibiotic	Amoxicillin	83 - 100	80 - 90
	Ampicillin	25 - 75	30 - 60
	Trimethoprim	90 - 97	60
	Sulfamethoxazole	80 - 90	15
Anti-depressants	Fluoxetine	72	2.5 - 11
	Phenobarbitone	70 - 90	25
Antiepileptic	Carbamazepine	60 - 85	1 - 3
Antihypertensive	Bezafibrate	14 - 95	5 - 45
Anti-inflammatory	Diclofenac	-	15
	Ibuprofen	85 - 100	1 - 8
	Morphine	20 - 33	71.6
Biosulphonates	Alendronate	0.9 - 1.8	40 - 60
Gastrointestinal	Ranitidine	50 - 100	30 - 70
Tranquilizer	Diazepam	85 - 100	1

Since the main pathway of human drugs is ingestion, excretion and disposal, municipal wastewater is considered as the largest emission source of pharmaceuticals due to the high concentrations of these compounds found in STP influents (Figure 1-2). A significant contribution of pharmaceuticals in the aquatic ecosystem is expected to be caused by manufacturing plants and effluents from hospitals. In fact, concentrations of several drugs have been detected in a range between 5 µg/L - 50 µg/L in hospital effluents in the north of Spain (Suárez et al., 2009) and up to 840 µg/L in STP influents close to drug manufacture plants in India (Fent et al., 2006; Ternes et al., 2006; Larsson et al., 2007). Another way by which pharmaceuticals can reach the environment is through out-of-date or unwanted medications. In fact, they are often improperly disposed of with other household waste, contaminating directly the wastewater or the ground water via leachates (Zuccato et al., 2006). Both application to fields and subsequent runoff and direct application in aquaculture are reported as the main sources of veterinary pharmaceuticals in environment (Nikolaou et al., 2007).

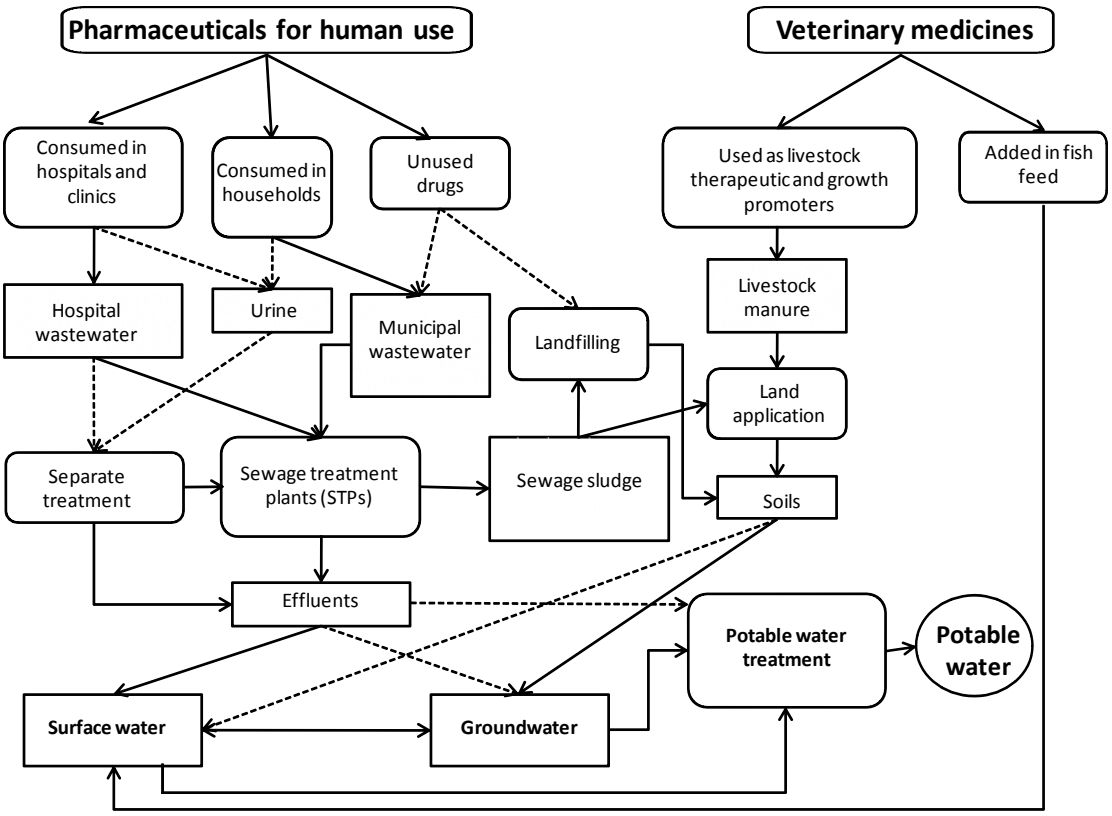


Figure 1-2. Main sources and routes of pharmaceutical compounds into the aquatic environment (Adapted from Ikehata et al., 2006).

Pharmaceuticals have been detected in drinking water, groundwater, sewage and fertilizer used in agriculture in concentrations in the range of nanograms per liter (ng/L) to micrograms per liter (µg/L) (Clara et al. 2005; Jjemba, 2006; Ternes et al., 2006; Zucatto et al. 2006; Larsson et al. 2007; Kim and Tanaka, 2009). In Table 1-3 the environmental concentration of the considered pharmaceuticals in this doctoral thesis are shown as well as their concentration in STP effluents, surface water and other exposure sites.

Although the concentrations in the environment are very low, some of these compounds are considered recalcitrant; as they are not easily removed in STPs (Ternes et al. 2006; Kosjek et al. 2007). Most of these compounds and their metabolites are hardly detected by traditional analytical methods and for their accurate detection, the use of sophisticated equipments such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS-MS) are required (Larsen et al., 2004).

Table 1-3. Environmental concentration of the selected pharmaceuticals

Compound	Concentration			
	Environmental concentrations	Concentration in STPs effluents	Concentration in surface waters	Other exposure sites where pharmaceuticals have been detected
CTL	ng/L - µg/L[7] 0.34 ng/L[12]	80 ng/L[1]	3 - 120 ng/L[9]	770 - 840 µg/L in STPs influent near to 90 bulk drug manufactures in India [13]
FLX	n.a.	0.01 - 0.099 µg/L[1,12]	0.012 - 44 ng/L[9,12]	0.1 - 10 ng/g in fish tissues[1]
SMX	ng/L - µg/L[3,7,10,13,16]	0.6 - 0.9 µg/L[2,10,11] 10 - 127 ng/L[19,24]	n.d. - 0.15 µg/L[11,24]	
DCF	ng/L - µg/L[3,7,10,13,16]	0.17 - 2.5 µg/L[5,6,7] 1 - 49.1 ng/L[6,8]	0.002 - 33.9 µg/L[5,10,11,23] ng/L[18]	
IBP	ng/L - µg/L[3,7,10,13,16]	0.01 - 5.7 µg/L[2,5,7] 121.2 ng/L[23]	0.1 - 33.9 µg/L[5,11,23] 13 - 34 ng/L[17,22,24]	
NPX	ng/L - µg/L[3,7,10,13,16]	n.d. - 12.5 µg/L[2,5,7]	0.002 - 33.9 µg/L[5,23] 135.2 ng/L[22]	
CBZ	ng/L - µg/L[3,7,10,13,16] 1 – 2 µg/L[10]	0.15 - 6.3 µg/L[5,6,7,11] 1 - 2100 ng/L[8,21,24]	1.1 µg/L[4] 23.1 - 1160 ng/L[9,15,18,22,24]	30 ng/L in drinking water and groundwater in Europe, USA and Canada[4,18]
DZP	ng/L - µg/L[3,7,10,13,16]	B.D.L - 0.66 µg/L[1,5,7,24] 1 ng/L - 1 µg/L[6]	n.d.[5, 24]	

n.a. (non available); n.d. (not detected); B.D.L. (below detection limit); (1) Calisto and Esteves, 2009; (2) Carballa et al., 2004; (3) Clara et al., 2005; (4) Cunningham et al., 2010; (5) Daughton and Ternes (1999); (6) Esplugas et al., 2007; (7) Fent et al., 2006; (8) Gagnon et al., 2008; (9) González-Alonso et al., 2010; (10) Ikehata et al., 2006; (11) Jjemba, 2006; (12) Kwon and Armbrust (2005, 2006); (13) Larsson et al., 2007; (14) Lindqvist et al., 2005; (15) Moldovan et al., 2007; (16) Reif et al., 2008; (17) Rodríguez et al., 2003; (18) Vogna et al., 2004; (19) Yang et al, 2011; (20) Zhang and GeiBen, 2010; (21) Zhang et al., 2008; (22) Zhang et al., 2007; (23) Ziylan and Ince, 2011; (24) Zuccato et al., 2006.

1.1.2. Therapeutic classes of the selected pharmaceuticals

Nowadays, there are a wide range of pharmaceutical compounds on the market for the treatment of several diseases in humans and animals. These compounds belong to different therapeutic classes such as antibiotics, analgesics, anti-inflammatory, β-blockers, contraceptives, lipid regulators, antidepressants, antineoplastic agents, tranquilizers, anticonvulsants, antihypertensive, diuretics, among others (Fent et al., 2006; Gros et al., 2010). For research purposes of this doctoral thesis, 8 different pharmaceutical compounds (more details in section 1.1.3.) have been chosen and they belong to the following therapeutic classes:

Antibiotics

These compounds are characterized as a chemotherapeutic agent that inhibits the growth of several microorganisms (bacteria, fungi or protozoa). Therefore, they are used for the treatment of bacterial and viral infections in humans and animals. They are divided into different sub-groups such as β-lactams, quinolones, tetracyclines, macrolides, sulphonamides and others (Kümmerer, 2009a). Most of these drugs are obtained total or partially from certain microorganisms, but some of them could be synthetic such as sulphonamides. Although concentrations of antibiotics in the environment are generally below 1 mg/L, the major issue about their use and release is the resistance among the bacterial pathogens, which is worsened by the fact that it can be irreversibly safeguarded, even in the absence of continued selective pressure (Daughton and Ternes, 1999). Their persistence in the environment may affect the capacity of biological treatment to remove organic matter and nutrients in STPs. Compounds such as amoxicillin, cephradine, penicillin, azithromycin, erythromycin, roxithromycin, lincomycin, sulfadiazine, sulfathiazole, sulfamethoxazole, trimethoprim, tetracycline, spectinomycin are representative of this therapeutic group (Alexy et al., 2004; Adler et al. 2006; Ikehata et al.; 2006 Dantas et al., 2008).

Anti-depressants

This group of drugs is also called selective serotonin reuptake inhibitors (SSRIs) and they are extensively used for the treatment of anxiety, clinical depression, obsessive-compulsive disorders and even bulimia. Serotonin is a biogenic amine present in the nervous system and plays the role of a neurotransmissor, regulating hormonal and neural mechanisms such as food intake and sexual behaviour in mammalians. In aquatic organisms, it is involved in a wide array of reproductive functions (spawning, oocyte maturation, parturition, etc), behaviours and reflexes (heartbeat rhythm, feeding/biting, swimming motors patterns, beating of cilia, induction of larval metamorphosis, aggression) and release of neurohormones (Daughton and Ternes, 1999; Fent et al., 2006). Within this group of drugs, fluoxetine, fluvoxamine, paroxetine, sertraline and citalopram have been identified in STP effluents (Fent et al., 2006).

Antiepileptics and tranquilizers

Medical treatment of seizures has been carried out since 1857 and several compounds such as potassium bromide and phenolbarbital with antiepileptic potency were used for the treatment of epileptic patients. Epilepsy is the second most common central nervous system (CNS) disease, produced by the excessive discharge of a group of neurons (Mückter, 2006). Antiepileptic compounds are used for the treatment of trigeminal

neuralgia and schizophrenia; since they act on the CNS by blocking the voltage-dependent sodium channels of excitatory neurons, reducing cerebral activity (Fent et al., 2006). Another class of drugs acting on CNS is the benzodiazepine family (tranquilizers), which binds the GABA (γ-aminobutyric acid) receptor in a specific site of the γ-sub-unit, amplifying the inhibitory effects of this neurotransmissor and resulting in a diminished neuronal activity (Fent et al., 2006). Within benzodiazepines available, only clonazepam, diazepam and lorazepam have been extensively used in humans and small animals such as cats, dogs and foals (Mückter, 2006).

Anti-inflammatories

Non-steroidal anti-inflammatory drugs (NSAIDs) have analgesic, anti-inflammatory and antipyretic effects and they are usually used for mitigate symptoms of arthritis, bursitis, gout, swelling, stiffness and joint pain (Adler et al., 2006; Esplugas et al., 2007). Their molecular target is the ciclooxygenase enzyme, which catalyzes the synthesis from arachidonic acid of different prostaglandins, which are molecules implicated in a wide range of processes (inflammation and pain, regulation of blood flow in kidney or coagulation). The long-term treatment often leads to side effects, like renal damages or renal failure, gastric or liver damages, triggered by the non-specific prostaglandin inhibition (Fent et al., 2006). There are many different types of NSAIDs widely used without prescription with an estimated annual consumption of several hundred tons in developed countries (Ziylan and Ince, 2011). These compounds have been detected in effluents from STPs in concentrations of 1 μg/L (Fent et al., 2006). The following compounds: diclofenac, ibuprofen, indomethacin, naproxen, mefenamic acid, piroxicam, acetylsalicylic acid and salicylic acid belong to this group (Ikehata et al., 2006).

1.1.3. Physicochemical properties of pharmaceutical compounds

Pharmaceuticals are not a homogenous group since they vary widely in their molecular weight, structure, functionality, etc. The chemical structure of the pharmaceuticals can be used for an alternative classification of these compounds; mainly for the active substances within sub-groups of medicines (antibiotics such as β-lactams, cephalosporins, penicillins or quinolones). These compounds may be treated as groups; however, even smaller changes in their structure may affect their solubility or polarity (Kümmerer, 2009c). The knowledge of physicochemical properties of pharmaceuticals not only explains their bioavailability in animal systems, but is also a helpful tool for the rationalization of the complex processes that take place throughout the STPs and for the choice of the analytical procedures to detect them. The following physico-chemical properties: solubility (S), Henry coefficient (H), pseudo-first order degradation constant (K_{biol}), dissociation constant

(pKa), octanol-water partition coefficient (log K_{ow}) and solid-water distribution coefficient (K_d) are considered of major relevance to evaluate a potential effective removal/degradation treatment.

Solubility (S)

The majority of pharmaceuticals found in the aquatic environment present low or slight solubility (Jjemba, 2006). The solubility is referred to the maximum amount of solute that dissolves in water and/or other organic solvents at a given temperature. This process increases the system entropy degree and the compatibility of intermolecular forces of attraction, breaking bonds solute-solute and solvent-solvent; thereby, promoting the formation of intermolecular bonds between the solute and solvent (Jjemba, 2006). Environmentally solubility is closely related to several factors such as pH, existing metals, presence of salts or complex agents, extreme temperatures, among others. The solubility of the selected pharmaceuticals is shown in Table 1-4.

Table 1-4. Solubility of selected pharmaceuticals (The Merck Index, 2006; Suarez et al., 2008)

Therapeutic class	Compounds	Solubility
Antibiotic	SMX	---
Antidepressants	CTL	Methanol, dimethyl sulfoxide (freely soluble); isotonic saline (soluble); ethanol (sparingly soluble); ethyl acetate (slightly soluble); heptane (insoluble).
	FLX	Methanol, ethanol (>100 mg/mL); ethyl acetate (2.0-2.5 mg/mL); toluene, cyclohexane, hexane (0.5-0.67 mg/mL)
Anti-inflammatory	DCF	Deionized water, pH 5.2 (>9 mg/mL); methanol (>24 mg/mL); acetone (6 mg/mL); acetonitrile, cyclohexane, chlorhydric acid (<1 mg/mL); phosphate buffer, pH 7.2 (6 mg/mL)
	IBP	Water (relatively insoluble); organic solvents (easily soluble)
	NPX	Water (insoluble); 25 parts of ethanol (96%); 20 parts of methanol, 15 parts of chloroform, 40 parts of ether (soluble).
Antiepileptic	CBZ	Water (practically insoluble); alcohol, acetone, propylene glycol (soluble).
Tranquilizer	DZP	Water (practically insoluble); chloroform, dimethylformamide, benzene, acetone and alcohol (soluble).

Henry coefficient (H)

The Henry's law express the ratio between the fraction of compounds present in the air (C_{air}, µg/m^3 air) and those dissolved in water (C_{water}, µg/m^3 wastewater) at equilibrium (Equation 1-1). The Henry coefficient allows foreseeing the losses by evaporation of such compounds, estimating the portion transferred from the water phase into the air and it increases with temperature (Himmelblau, 1996; Joss et al., 2006a).

$$H = \frac{C_{air}}{C_{water}} \qquad \text{[Equation 1-1]}$$

Dissociation constant (Ka)

The dissociation constant (Ka) is a measure of the acid strength of a compound. This constant describes its tendency to be ionized or dissociated at a known pH. It is defined as the equilibrium constant of the proton transfer reaction, but is usually expressed as its negative decadal logarithm (pKa) (Equation 1-2). The ionization is strongly dependent on pH and leads to the formation of charged species, governing the electrostatic interactions with the surface of the microorganisms (Figure 1-3) (Joss et al., 2006a; Suárez et al., 2008).

$$AH \leftrightarrow A^- + H^+$$
$$K_a = \frac{[H^+]\cdot[A^-]}{[AH]} \qquad \text{[Equation 1-2]}$$
$$pK_a = -\log K_a = pH - \log \frac{[A^-]}{[AH]}$$

Octanol-water partition coefficient (K_{ow})

The value of log K_{ow} is an indicator of the lipophility of molecules. K_{ow} is defined as the ratio between the concentration of a compound in organic phase (*n*-octanol), [$C_{octanol}$] (g/L) and that in water [C_{water}] (g/L) (Equation 1-3). Hydrophobic compounds present high values of log K_{ow}, therefore are more easily adsorbed on soils, sediments, minerals and dissolved organic material (Figure 1-3). In contrast, hydrophilic compounds show lower log K_{ow}, leading to enhanced bioavailability in the environment (Jjemba, 2006).

$$K_{OW} = \frac{[C_{octanol}]}{[C_{water}]} \qquad \text{[Equation 1-3]}$$

Solid-water distribution coefficient (K_d)

The sorptive behaviour of pharmaceuticals in the environment is better summarized with the solid-water distribution coefficient (K_d). This parameter expresses the ratio between

the concentrations of a given compound in the solid and liquid phases at equilibrium conditions (Equation 1-4).

$$K_d = \frac{[C_{sorbed}]}{SS \cdot [C_{soluble}]} \quad \text{[Equation 1-4]}$$

where [C_{sorbed}] represents the pharmaceutical concentration in the sludge (µg/L); [$C_{soluble}$], the dissolved concentration of the compound (µg/L) and *SS* the suspended solid concentration (gSS/L) (Suárez et al., 2008).

Pseudo-first order degradation constant (K_{biol})

Joss et al. (2006b) have determined pseudo first-order degradation kinetics (K_{biol}) for a large number of compounds. They performed batch experiments for 48 h with fixed biomass concentrations (0.5 g VSS/L), where the selected pharmaceuticals were spiked at a concentration of 3 µg/L. The biological degradation could be expressed as follows in Equation 1-5:

$$-\frac{dC}{dt} = K_{biol} C_0 SS \quad \text{[Equation 1-5]}$$

where *C* (µg/L) is the concentration of the considered compound (and C_0 its initial concentration in the solution), *t* is the time, SS (gSS/L) the suspended solids concentration and K_{biol} (L/gSS·d) is the degradation rate constant of the pollutant. According to the values established, three groups of compounds can be differentiated: (i) hardly biodegradable compounds (K_{biol} < 0.11 L/gSS·d); (ii) moderately biodegradable compounds (K_{biol} > 0.11 and < 10 L/gSS·d); (iii) highly biodegradable compounds (K_{biol} > 10 L/gSS·d) (Joss et al., 2006a).

1.1.4. Mechanisms of removal of pharmaceuticals

Sorption

One factor that may influence on the removal efficiency of wastewater is the ability of pharmaceuticals to interact with solid particles: both natural (clay, silt, microorganisms) or added to the medium (charcoal, coagulants) because this characteristic facilitates their removal by physicochemical (sedimentation, flotation, etc) or biological (biodegradation) processes (Carballa et al., 2004). Solubility, pKa and K_{ow} have been usually used to predict the sorption of compounds in the environment. This process is considered as an important removal mechanism in STPs. The solid-liquid distribution coefficient (K_d) takes into account two main sorption mechanisms: adsorption and absorption (Larsen et al., 2004) (Figure 1-3).

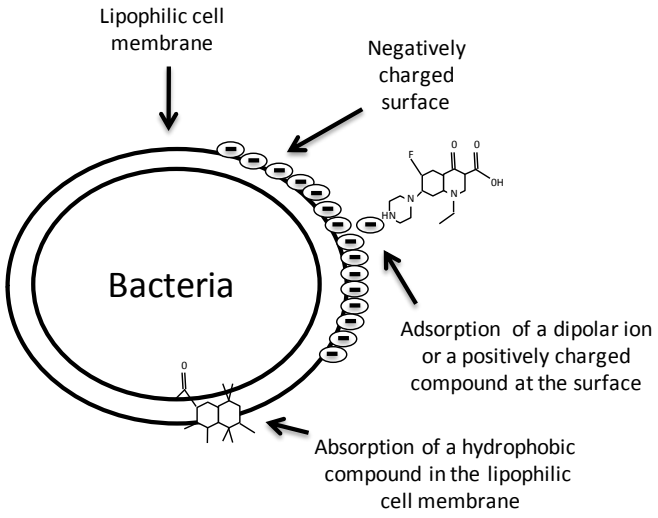


Figure 1-3. Sorption processes of the pollutants onto particles (Adapted from Larsen et al., 2004).

Absorption process refers to hydrophobic interactions of the aliphatic and aromatic groups of a compound with the lipophilic cell membrane of the microorganisms or the lipid fractions of the suspended solids. This mechanism is characterized by the octanol-water partition coefficient (K_{ow}), which is also an indicator of the lipophilicity of the compound. In contrast, adsorption process is related to electrostatic interactions of positively charged groups of chemicals with the negatively charged surfaces of the microorganisms; this process is associated to the dissociation constant (Ka) (Jjemba, 2006).

Volatilization

The volatile fraction of a specific compound depends on the air flow that is in contact with the wastewater, the type of aeration supply and the Henry coefficient (H). Pharmaceutical compounds with low volatility are usually distributed in the environment through an aqueous transport or through the food chain (Fent et al., 2006). It assumes particular relevance for compounds with high H values which subsequently are subject to volatilization.

Biodegradation

There are several factors that may control the biological removal of pharmaceutical compounds in STPs: concentration, chemical structure and physicochemical properties (see section 1.1.5.), especially their K_{biol}. Also, biodegradability of pharmaceuticals can be influenced by different environmental factors, such as the type or adaptation of the sludge (Joss et al., 2006b).

1.1.5. Main characteristics of the selected pharmaceuticals

The following pharmaceutical compounds belonging to different therapeutic groups were selected: antibiotics (sulfamethoxazole, SMX), antidepressants (citalopram, CTL; fluoxetine, FLX), anti-inflammatories (diclofenac, DCF; ibuprofen, IBP; naproxen, NPX), antiepileptic (carbamazepine, CBZ) and tranquilizers (diazepam, DZP). These compounds represent a wide range of commonly prescribed therapeutic classes, most of them included in the top 300 prescription from the Rx-List of Food and Drugs Administration (FDA, 2005), with different physicochemical properties and potential severe effects on aquatic environment (like antibiotics and anti-depressants). A brief description of each of the selected compounds is included below.

Sulfamethoxazole (SMX)

Sulfamethoxazole (4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide) is one of the classes of broad-spectrum antibiotics most widely used and effective against nearly all gram-positive and many gram-negative bacteria. This compound is commonly used together with trimethoprim (TMP) in a combination product known as *Bactrim* or *Septra* (Drillia et al., 2005). The chemical formula of this compound is $C_{10}H_{11}N_3O_3S$ and it is classified as a polar molecule (Figure 1-4). This antibiotic is highly soluble in water (610 mg/L) and it is fairly dissociated at pH 7 (pKa 5.6-6.0). Concerning its Henry's coefficient ($2.6 \cdot 10^{-10}$ $\mu g \cdot m^{-3}$ air/$\mu g \cdot m^{-3}$ wastewater), its potential volatilisation is likely to be negligible in STPs. Other features are a significant value of log K_d (2.4), which is related to potential sorption; however, as it has a low log K_{ow} value (0.9), SMX can be considered as an hydrophilic compound. Finally, according to the biological removal in STPs, a low K_{biol} value (<0.1 L/gSS·d) classifies this compound as hardly biodegradable (Joss et al., 2006a; Joss et al., 2006b; Suárez et al., 2008).

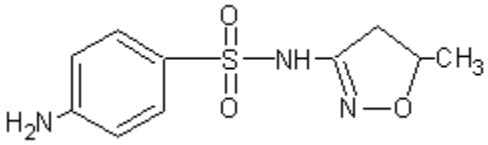


Figure 1-4. Chemical structure of SMX (Daughton and Ternes, 1999).

Citalopram (CTL) and fluoxetine (FLX)

The two compounds, CTL (RS)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile)) and FLX (N-methyl-1-(4-trifluoromethyl)phenoxy) benzene-propanamine) are antidepressants (Figure 1-5). Both are widely prescribed worldwide for their potential as SSRIs and they are commercially known by the name *Prozac*. There are a few studies about their degradation (Kwon and Armbrust, 2005 and

2006; Redshaw et al., 2008; Paterson and Metcalfe, 2008; Calisto and Esteves, 2009). The chemical formula of CTL and FLX are $C_{20}H_{21}FN_2O$ and $C_{17}H_{18}F_3NO$, respectively. The former is considered as a hydrophobic molecule due to its high log K_{ow} (2.9-3.7) with rather low solubility in water (31 mg/L), and potentially adsorbed on the sludge due to its low log K_d (2.0). The CTL molecule has a low Henry's coefficient ($1.1 \cdot 10^{-9}$ $\mu g \cdot m^{-3}$ air/$\mu g \cdot m^{-3}$ wastewater) which is indicative of negligible volatilisation. Fluoxetine is a more polar compound with a log K_{ow} value of 1.6, solubility in water of 60 mg/L and smaller log K_d than CTL (0.7). This compound shows a higher Henry's coefficient ($3.6 \cdot 10^{-6}$ $\mu g \cdot m^{-3}$ air/$\mu g \cdot m^{-3}$ wastewater). Finally, both show remarkable pKa values, respectively 9.6 and 10.1 (Joss et al., 2006a; Joss et al., 2006b; Suárez et al., 2008).

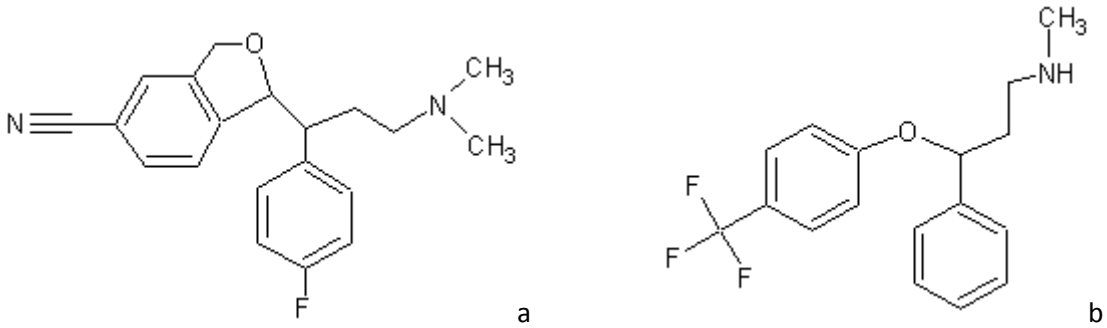


Figure 1-5. Chemical structure of CTL (a) and FLX (b) (Daughton and Ternes, 1999).

Carbamazepine (CBZ)

Carbamazepine (5H-dibenz(b,f)azepine-5-carboxamide) is a widely spread used anticonvulsant. Its chemical formula is $C_{15}H_{12}N_2O$ and its chemical structure is shown in Figure 1-6. More than 72% of orally administered CBZ is absorbed, while the rest is unchanged and discharged to the environment (Zhang et al., 2008). Therefore, due to its recalcitrant behaviour and the high prescription volume, CBZ is often detected into the aquatic environment (Cunningham et al., 2010). CBZ is a positively charged molecule which may favour its sorption and it is moderately soluble in water (17.7 mg/L), quite hydrophobic (log K_{ow} 2.4-2.9), with a high pKa value (7.0) and with low log K_d constants (0.1). The main feature of these substances is the very low value of K_{biol}: <0.01 L/g·SS·d. As most of the pharmaceuticals compounds, CBZ has a low Henry's coefficient ($4.4 \cdot 10^{-9}$ $\mu g \cdot m^{-3}$ air/$\mu g \cdot m^{-3}$ wastewater) which is associated to negligible volatilisation (Joss et al., 2006a; Joss et al., 2006b; Suárez et al., 2008).

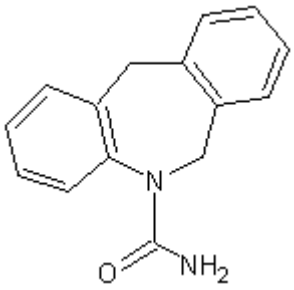


Figure 1-6. Chemical structure of CBZ (Daughton and Ternes, 1999).

Diazepam (DZP)

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), known as *Valium*, is recommended to relieve anxiety, depression and insomnia due to its tranquilizer properties (Fent et al., 2006). Its chemical formula is is $C_{16}H_{13}ClN_2O$ and is a neutral molecule (Figure 1-7) moderately soluble in water (50 mg/L). Due to its log K_{ow} value (2.8-3.0), DZP can be considered as a quite hydrophobic compound. Moreover, DZP has a low K_d constant (1.3), a low pKa value (3.3-3.4) and a low Henry's coefficient ($1.5 \cdot 10^{-7}$ $\mu g \cdot m^{-3}$ air/$\mu g \cdot m^{-3}$ wastewater). The main feature of this substance is the very low value of K_{biol} (~ 0) (Joss et al., 2006a; Joss et al., 2006b; Suárez et al., 2008).

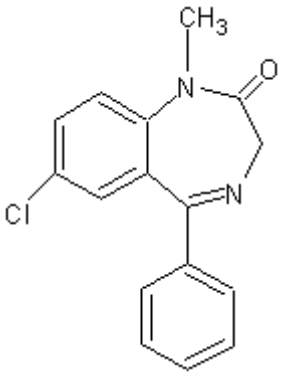


Figure 1-7. Chemical structure of DZP (Daughton and Ternes, 1999).

Diclofenac (DCF), ibuprofen (IBP) and naproxen (NPX)

Three compounds of this type were considered for this study: DCF (2-((2,6-dichlorophenyl)amino)-benzeneacetic acid-Na), IBP (α-methyl-4-(2-methylpropyl)-benzeneacetic acid) and NPX ((S)-6-methoxy- α-methyl-2-naphthaleneacetic acid). The chemical formula for DCF is $C_{14}H_{11}Cl_2NO_2$, for IBP is $C_{13}H_{18}O_2$ and for NPX is $C_{14}H_{14}O_3$. These compounds are characterized by the presence of the carboxylic group in their structure; thus, they are acid compounds (Figure 1-8) with pKa of 4.5 for DCF, 3.2 for NPX and 3.1-4.0 for IBP. The main physicochemical properties of these compounds are:

moderate solubility in water: 2.4, 21 and 16 mg/L for DCF, IBP and NPX, respectively and low log K_d constants: 1.2, 0.9 and 1.1. These compounds have K_{ow} values in a range between 3.1 and 4.5. Their low Henry's coefficient values (from $1.9 \cdot 10^{-10}$ up to $6.1 \cdot 10^{-6}$ $\mu g \cdot m^{-3}$ air/$\mu g \cdot m^{-3}$ wastewater) are not indicative of potential volatilisation. Regarding their pseudo first-order degradation constant, IBP is considered as a highly biodegradable compound due to the high value of K_{biol} (9-35 L/g·SS·d); NPX is moderately biodegradable (K_{biol} 0.4-1.9 L/g·SS·d) while DCF is hardly biodegradable (K_{biol} <0.1 L/g·SS·d) (Joss et al., 2006a; Joss et al., 2006b; Suárez et al., 2008).

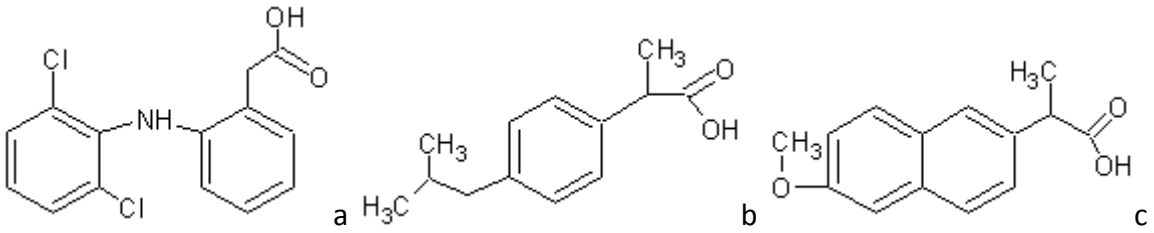


Figure 1-8. Chemical structure of DCF (a), IBP (b) and NPX (b) (Daughton and Ternes, 1999).

1.1.6. Ecotoxicity of pharmaceutical compounds in the environment

Pharmaceuticals are present at relatively low levels in the environment, but their discharge via STPs is continuous (De Lange et al., 2006). Indeed, risk for acute toxic effects is unlikely but chronic environmental toxic effects cannot be excluded (Péry et al., 2008). There is evidence that some pharmaceuticals are able to persist in the environment for long periods of time, causing sex changes in higher organisms, modifications in plant growth and anatomical deformities in a wide variety of organisms (Jjemba, 2006). Moreover, aquatic organisms are important targets as they are exposed via wastewater effluents over their whole life (Fent 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 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 treatment of humans must be accompanied by an environmental risk assessment (ERA) (EMEA, 2005; Fent et al., 2006; Nikolaou et al., 2007).

The negative impact caused by the release of pharmaceuticals into the environment depends on several factors: their physiological activity, their physicochemical properties, their wide distribution and the significant fact that these compounds were

designed to produce biological effects in living organisms, even at low concentrations (Carballa et al., 2008, Suarez et al., 2008). Therefore, it is important to determine the potential exposure of pharmaceutical compounds that are excreted to the environment (Mückter, 2006). There is an alternative classification in four groups of pharmaceutical compounds depending on their possible environmental contamination and the effects that can generate: (i) hormonally active compounds, found in STPs effluents in low concentrations (ng/L). This type of compounds poses a health risk due to its high efficiency at low concentrations and their potential effect as endocrine disruptors; (ii) antineoplastics and cytostatics, characterized for their obvious cytotoxicity, carcinogenesis, mutagenesis and embryotoxic properties. These drugs are considered as highly reactive drugs; (iii) anti-infective agents, providing greater resistance to antibiotics, which in turn creates a risk to the human population; (iv) halogenated compounds such as X-ray contrast media, which present high resistance to biodegradation due to their great mobility and persistence in the environment and food chain (Ikehata et al. 2006; Mückter, 2006).

Negative effects of pharmaceuticals on target species (humans or animals receiving treatment) have been extensively studied during the product development (Crane et al., 2006). The information collected in the clinical phases about the molecular target and the mode of action of drugs in mammalians could be very useful to predict the possible effects in other organisms, because receptors, biochemical pathways and enzymes are conserved during the evolution (De Lange et al., 2006; Fent et al., 2006). Nevertheless, even if a mechanism is shared among mammalians, lower vertebrates and invertebrates, it could produce different outcomes and, as a consequence, also different side effects. Furthermore it is necessary to consider the unexpected responses mediated via pathways or receptors not occurring or not affected in mammals (Crane et al., 2006).

Toxicity of pharmaceuticals has generally been investigated with standardized tests assessing the effects of acute exposure, whilst chronic effects were often improperly calculated from relative short-term exposure (Crane et al., 2006; Fent et al., 2006). Additionally, the endpoint usually evaluated is the organism survival, which is estimated with the LC_{50} (concentration required to kill half the members of a tested population), while sub-lethal effects such as alterations in reproduction strategy, changes in growth rate, behaviour modifications or genetic mutations, that can occur at lower concentrations, are often overlooked (Flaherty and Dodson, 2005). Another important factor, only rarely investigated, is the influence of environmental parameters such as pH on toxicity. Such studies would be of importance for acidic pharmaceuticals that may induce different toxicities depending on speciation at different ambient pH (Fent et al., 2006).

Since drugs are ordinarily found in complex mixtures of unrelated chemicals with a range of pharmacological activities, aquatic toxicity of pharmaceuticals mixtures can be unpredictable and complex compared to individual pharmaceutical effects, suggesting additive and synergic effects (Zuccato et al., 2006). Furthermore, the consequences of pharmaceuticals exposure triggered on organisms from lower trophic levels should not be overlooked, because their presence is critical for the community homeostasis (Daughton and Ternes, 1999) and they may have direct effects on human health.

Maybe the most remarkable case of pollutants toxicity in non-target organisms is the dramatic poisoning of the vulture populations of India and Pakistan (Oaks et al., 2004), 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 diclofenac 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 diclofenac was widely used in these regions as veterinary drug for its analgesic, anti-inflammatory and antipyretic properties. In addition, renal diseases in mammalians and birds (chickens, cranes and quail) caused by NSAIDs are well-known. For instance, 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).

Carlsson et al. (2006) investigated the acute effects of some β-blockers in two crustaceans species (*Ceriodaphnia dubia* and *D. magna*), a sediment living amphipod (*Hyalella azteca*) and the japanese rice fish (*Orzyas latipes*). These compounds belong to a class of drugs used for the treatment of cardiovascular diseases such as hypertension, coronary artery disease or arrhythmias (Daughton and Ternes, 1998; Fent et al., 2006; Ikehata et al., 2006). A reduction in the reproduction of the *H. azteca* species was observed at an LC_{50} of 0.1 mg/L; while for *C. dubia* this value was of 0.24 mg/L. After 4-week exposure to propanolol, the number of produced and hatched eggs by fish was reduced. Additionally, alterations in plasma estradiol and testosterone levels were observed.

The toxic effect of different putative SSRIs (among them, fluoxetine and citalopram) has been analyzed in the last years. Fong et al. (2003) studied the effects of low concentration of different antidepressants in zebra mussle *Dreissena polymorpha* and

fingernail clam *Sphaerium striatinum* finding that this class of compounds takes part in several reproductive processes, inducing spawning in mussel and parturition in clam. Furthermore, using two fish species (juvenile rainbow trout and adult male guppies) the effect of waterborne citalopram was investigated by Holmberg et al. (2011); no effect of the drug was observed after 3-7 days of exposure. It cannot be excluded that the exposure time was too short for the drug to have a significant effect; however in another research study, the antidepressant fluoxetine was detected in japanese medaka tissues (*O. latipes*) after only five hours of exposition to waterborne fluoxetine (Paterson and Metcalfe, 2009). Besides, low concentrations of fluoxetine (10 µg/L) may cause effects over the reproduction of aquatic invertebrates species (*D. magna* and the snail *Potamopyrgus antipodarum*) causing much more pronounced effects in the second generation of the exposed individuals (Péry et al., 2008). This compound is responsible for the reduction of ovarian estrogen levels and reduced the number of spawned eggs in zebrafish (Lister et al., 2009). The chronic exposure of *D. magna* to fluoxetine was considered by Flaherty and Dodson (2005) resulting in increased fecundity of the crustacean. They also found that the mixture of fluoxetine and clofibric acid led to significant deformities, including malformed carapaces and swimming setae. Moreover, when the concentration of clofibric acid was increased, mortality of *D. magna* augmented appreciably.

Antibiotics exposure may have negative reproductive in the aquatic environment since a high mortality rate for *nauplii* (free-swimming first stage of the larva of certain crustaceans) and toxic effects on reproduction of *D. magna* was caused by an exposure to this type of compounds (Kümmerer, 2009c). However, not only the aquatic environment can be affected by the release of drugs. Pharmaceutical compounds that end up in arable land can be available for plant uptake affecting their growth and development. The impact depends on the type of agents, dosage, sorption, bioaccumulation and mobility in soil. The effects of therapeutic agents on plants in soil seem to differ between compounds and plant species. For instance, pinto beans (*Phaseolus vulgaris* L.) were negatively affected by oxytetracycline and chlortetrachycline leading to a reduction in nodulation, fresh weight and a reduced uptake of Ca, K and Mg. Moreover, the growth of radish and wheat was unaffected by the presence of both antibiotics although the uptake of nutrients was enhanced (Jjemba, 2002).

1.1.7. Removal of pharmaceutical compounds from wastewater by physicochemical and biological technologies

Pharmaceutical compounds that are released into the environment are subject to various factors such as distribution, absorption, metabolism and mobility. When these compounds are transformed in the environment, it is commonly referred as degradation (Jjemba,

2006). In the recent years different physicochemical and biological processes applied for the removal of pharmaceuticals have been investigated as most of these compounds are resistant to conventional treatments (Joss et al. 2006; Kosjek et al., 2007). A detailed description of several research studies carried out previously for the degradation of the selected pharmaceuticals is shown in the following section.

Removal of pharmaceutical compounds in sewage treatment plants (STPs)

STPs are mainly designed for the removal of organic matter and nutrients such as nitrogen and phosphorous, typically present in high concentrations. In contrast, the presence of pharmaceutical compounds in STPs represents only a very small fraction (Larsen et al., 2004). Some of the most important pharmaceutical compounds found in STPs belong to different therapeutic groups such as antibiotics, lipid regulators, anti-inflammatories, antiepileptics, tranquilizers, X-ray media contrast and contraceptives (Larsen et al., 2004). Most of these compounds come from domestic discharges, hospitals, industries and municipal STPs (Carballa et al., 2004). Despite the dilution, degradation and sorption processes of pharmaceuticals can be found in treated wastewater effluents, surface waters and groundwater (Zuehlke et al., 2007).

Usually, STPs consist of a three stage process including: (i) Pre-treatment: consisting of various physical and mechanical operations, such as screening, sieving, blast cleaning and grease separation. It allows the removal of voluminous items, sand and grease; (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; (iv) Tertiary treatment, designed to remove remaining unwanted nutrients (mainly nitrogen and phosphorous) through high performance biological or chemical processes.

Flowing through the STP, these compounds can run into four different major routes (Lishman et al., 2006; Nikolaou et al., 2007; Suárez et al., 2008; Klavarioti et al., 2009): (i) degradation to lower molecular weight compounds, or mineralization to CO_2 and H_2O; (ii) sorption on suspended solids (sludge/biosolids) and subsequent removal as sludge, provided that the compound entering the plant or the product of biologically mediated transformation is lipophilic; (iii) cleavage of conjugates yielding the active parent compound; (iv) release to the receiving water either as the original compound or as a degradation product. However, the main removal processes of these compounds are

based on solid sorption, volatilization, photo-oxidation and chemical and/or biological degradation. Among the processes previously mentioned, biological degradation and sorption are relevant for the removal of pharmaceuticals (Joss et al., 2006a; Suarez et al., 2008).

Several pharmaceutical compounds have been studied throughout the different units of STPs and the results showed that compounds such as DCF, DZP, CBZ and roxithromycin (ROX) were not detected since they were below detection limit (BDL). During secondary treatment, all the compounds were partially removed (35% up to 75%). The final removal efficiencies were from 40% up to 65% for the anti-inflammatory drugs and near 60% for E2 and SMX (Carballa et al., 2004). On the other hand, Kosjek et al. (2007) carried out the removal of anti-inflammatory drugs including IBP, NPX, DCF as well as ketoprofen (KPF) and clorfibric acid (CLOFI) in a pilot STP, finding that these compounds were degraded above 87%, excepting DCF (up to 59%) and CLOFI (less than 30%).

The degradation efficiency of each pharmaceutical in STPs is clearly affected by the nature of the drug, but also by parameters such as the treatment process considered, the age of the activated sludge (solids retention time, SRT) and environmental conditions (season, pH, redox condition, etc.) (Larsen et al., 2004; Zuccato et al., 2006). The overall removal efficiencies for some pharmaceuticals are shown in Table 1-5.

Table 1-5. Overall removal efficiencies for some pharmaceuticals in STPs

Compounds	Removal efficiency (%)	Sorption	Biodegradation
SMX	0 - 99	-	-
DCF	0 - 80*	Medium	None - medium
IBP	60 - 99.7	None	High
NPX	0 - 98	-	-
CBZ	0 - 58	None	None

* Mainly in the scope of 21% up to 40%. (Hofmann et al., 2007; Suárez et al., 2008; Zhang et al., 2008; Sipma et al., 2010)

Coagulation-flocculation and flotation

These processes were designed to improve the removal of suspended solids and colloids from wastewaters. In general, these processes of coagulation-flocculation and flotation, significantly removed certain compounds: tranquilizers (DZP) and anti-inflammatory drugs (DCF); whereas other compounds, such as anti-epileptics (CBZ) or certain anti-inflammatory drugs (IBP and NPX), are very remained after treatment (Ikehata et al.

2006). In a specific study, when a coagulant was used, the removal of the total suspended solids (TSS) was enhanced from 40% up to 90% (Suárez et al., 2009). However, compounds such as DZP, DCF and NPX were slightly removed (below 25%) and CBZ and IBP were not degraded. During the flotation phase, the removal percentages were improved in all the cases (Carballa et al., 2005). In other study of Suárez et al., (2009) more than 60% of anti-inflammatory drugs were removed; despite this, the following compounds were not removed: CBZ, DZP, ROX, SMX and also iopromide (IPM).

Other physicochemical processes

Generally, removal of pharmaceutical compounds is enhanced with the increase of sludge age due to fortification of slowly growing bacteria as well as the establishment of diverse biotic community with broad physiological capabilities. Biological systems with high sludge age have a correlation with treatment efficiency; therefore, this process could be applied for the removal of pharmaceutical compounds (Yu et al., 2009). These authors demonstrated a satisfactory and steady removal of SMX and NPX (64% - 96%) and almost complete removal of IBP (>94%). On the other hand, the removal of CBZ, DZP, CTL, IBP, DCF and NPX was analyzed in a nitrifying-denitrifying process. Removal in a range between 68% and 82% for NPX and IBP was achieved, respectively; meanwhile more recalcitrant compounds such as CBZ, CTL and DCF were removed in less than 10% (Suárez et al., 2005). Moreover, the application of anaerobic digestion of sludge attained high removal percentages in the case of antibiotics and the anti-inflammatory drug NPX. The rest of the compounds were partially degraded (20%-60%), except for CBZ which was not removed (Carballa et al., 2007). Also, low removal efficiencies (11% and 18%) of CBZ were achieved in a combined anaerobic digester-constructed wetland pilot plant treating urban wastewater by Reyes-Contreras et al. (2011).

Membrane bioreactors (MBR)

This technology uses membranes to retain the solids used in the secondary treatment and was designed to substitute the second clarifier in the conventional activated sludge (CAS) treatment. The most important features of a MBR are that the SRT can be significantly increased and accurately controlled and that it makes feasible working with high biomass concentrations (Clara et al., 2005). As a result, biodiversity rises inside the reactor since the slowly growing microorganisms can develop themselves achieving complete adaptation to pharmaceuticals and removal rate of lipophilic substances improves since a larger quantity of sludge is available for sorption. The main disadvantages of the MBR are the large energy demand and high investments (Larsen et al., 2004; Reif et al., 2008). The removal of eight pharmaceutical compounds have been carried out in a STP where a MBR

was operated at pilot scale at different retention times (Clara et al., 2005). The results showed that CBZ was not removed, while compounds such as bisphenol A (BPA) (an endocrine disrupting chemical, EDC) or IBP were degraded to a large extent (>90%). On the other hand, Reif et al. (2008) found that compounds such as the anti-inflammatory drugs IBP and NPX were not adsorbed, showing a high removal (above 84% for both). The most recalcitrant compounds were DCF and CBZ with final removal lower than 9%, results in agreement with those reported by Clara et al., (2005). This system achieves complete retention of suspended solids; however, it implies high costs of operation and maintenance as well as high energy consumption.

Membrane filtration and activated carbon demonstrated remarkable removal efficiencies for compounds including IBP, NPX and BPA; however, degradation was limited for other compounds, such as CBZ and DCF (<9%) (Clara et al. 2005; Von-Gunten et al. 2006; Reif et al. 2008). The removal of several persistent compounds in a sequential membrane bioreactor (SMBR) with the addition of powdered activated carbon (PAC) was studied by Serrano et al. (2011). PAC has been extensively used to promote the sorption of organic micropollutants. Before the PAC addition into the SMBR, the removal of compounds such CBZ, DZP and DCF were negligible, partial removal of NPX was observed (up to 64%) and high yields of removal for FLX and IBP: 71% - 97%.

Advanced oxidation processes (AOPs)

Advanced oxidation processes (AOPs), based on the use of highly reactive species (for example hydroxyl radicals, OH), have been applied as a treatment alternative (Klavarioti, 2009). Hydroxyl radicals are strong oxidants, capable of mineralize any organic molecule to CO_2 and inorganic ions. Generally OH radicals mediated reactions increase the number of functional groups and the molecule's polarity, two crucial features for the intended properties of pharmaceuticals, leading to disappearance of its original medicinal activity that should be proved by the analysis of oxidation products (Ternes et al., 2003). Among them, it is worth considering ozonation (O_3/H_2O_2), ultraviolet irradiation (UV/H_2O_2), Fenton's reagent (Fe^{2+}/H_2O_2), electrolysis and ultrasounds (Klavarioti et al., 2009).

The degradation of pharmaceutical compounds using AOPs have been studied since the year 2000, with high removals rates (above 90%) for several compounds such as pesticides, anti-inflammatories, antibiotics, the antiepileptic CBZ and also natural/synthetic estrogens (EDCs) (Vogna et al., 2004; Ikehata et al., 2006; Carballa et al., 2007; Esplugas et al., 2007; Hofmann et al., 2007; Dantas et al., 2008; Gagnon et al., 2008; Zylan and Ince, 2011). For instance, heterogeneous catalytic oxidation of the anti-inflammatory DCF was conducted by Hofmann et al. (2007) using H_2O_2 as oxidizing agent, which is easily to handle and low-priced. The catalytic oxidation of this type of pollutants

depends on temperature and pH. In the mentioned study, the degradation rate of DCF was doubled when the temperature was changed from 40°C to 60°C and extreme low pH values were considered.

A couple of studies concerning the degradation of CTL and FLX using UV lamps have been carried out by Kwon and Armbrust (2005 and 2006) finding that the former is barely degraded (0.5%) after 30 days at pH 5 and 7 and only partially at pH 9. On the other hand, the compound FLX is hydrolytic and photolytically stable under the assayed conditions (pH and UV lamp). In contrast, complete abatement of CBZ by ultraviolet irradiation (UV/H_2O_2) was achieved after only 4 min (Vogna et al., 2004). The degradation of IBP was performed by using several AOPs, such as photolysis, UV-Vis(H_2O_2) and Photo-Fenton processes. An evident decrease in the initial concentration of IBP was observed (40% of removal) using Fenton's reagent; on the other hand, the drug was unaffected after a UV-Vis(H_2O_2) photolysis. Higher degradation percentages were observed when using the photo-Fenton reaction, since this process promoted the decarboxylation of the IBP molecule, which led to total decomposition (Méndez-Arriaga et al., 2010). Also, the anti-inflammatory drug IBP was highly mineralized (92%) by a solar photoelectron-Fenton process, an alternative treatment which uses sunlight as a source of UV light (Skoumal et al., 2008).

Certainly ozonation is the most studied AOP for the application of pharmaceuticals removal (Esplugas et al., 2007). Before disinfection, several pharmaceutical compounds including DCF, NPX, IBP, CBZ, CLOFI as well as salicylic acid (SAL), triclosan (TCS) and a metabolite of IBP, 2-hydroxyibuprofen (2-OH-IBP), were found in concentrations between 42 ng/L and 2556 ng/L. Most of these substances were partially eliminated (above 50%) using an ozone dose of 10 mg/L; however, by doubling the dosage, the removal was up to 70%. Instead, using UV light only 25% of DCF and 40% of TCS were removed. The lower removal efficiencies were obtained using the mixture of performic acid (<8%) (Gagnon et al., 2008). Ternes et al. (2003) showed that a low dose of ozone (5-15 mg/L) is appropriate to oxidize pharmaceuticals and estrogens. However, ozonation is still rather expensive, leading to economical limitation for the feasibility of this method (Ternes et al. 2003; Larsen et al. 2004).

Alternative methods for removal of pharmaceutical compounds

It is important to find alternative ways to carry out the degradation of pharmaceutical compounds, since physicochemical technologies such as AOPs are effective in the removal of these compounds but are costly. This thesis proposes the use of white-rot fungi (WRF), a group of microorganisms capable of degrading lignin as well as other pollutants such as polycyclic aromatic hydrocarbons (PAHs), pesticides, polychlorinated biphenyls (PCBs),

synthetic dyes, pharmaceutical compounds and EDCs. (Field et al., 1992; Zeddel et al., 1993; Wesenberg et al., 2003; Ikehata et al., 2006). A description of the main characteristics of the WRF and their potential uses for the removal of organic pollutants is included in the following section of this thesis.

1.2. White-rot fungi (WRF) and their lignin modifying enzymes (LMEs)

1.2.1. Characteristics of the white-rot fungi (WRF)

Although several microorganisms can grow on living and dead trees, filamentous fungi constitute the dominant decomposers of plant fiber. 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. The major component of lignocellulosic materials is cellulose, a macromolecule composed of several glucose units (45% of the dry weight of wood) and represents the most abundant carbon source in the earth. Also, hemicellulose (25%-30% of the dry weight of wood) is a complex polymer formed from residues of different pentoses and hexoses. Finally, lignin provides plant support, impermeability and resistance to microbial attack. Lignin is constituted by dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (p-hydroxyphenyl, H) phenylpropanoid units derived from its corresponding alcohol, p-hydroxycinnamoyl alcohols, which in turn gives a variety of different sub-units including ethers and C-C bonds (Figure 1-9) (Pérez et al., 2002, Martinez et al. 2005; Wong, 2008).

Lignin is the most recalcitrant compound from the wood, due to its complex structure derived from the coupling of three alcohols (p-coumaryl, coniferyl and sinapyl) (Wong, 2008). It also has a high molecular weight and is very insoluble, which hinders its degradation resisting the attack by most microorganisms (Pérez et al., 2002). Under an anaerobic process, the aromatic rings are not completely attacked meanwhile under aerobic conditions, the breakdown of lignin occurs at a very low oxidation rate. Basidiomycetes fungi are the only microorganisms capable to attack and break the lignin molecule under aerobic conditions demonstrating the dissolution (formation of water-soluble fragments) and mineralization (evolution to CO_2) of natural and synthetic lignin by an enzymatic process (Wong, 2008; Sanchez, 2009). Their hyphal growth, their ability to secrete enzymes freely into the environment, particularly from growing tips, and their ability to translocate nutrients through extensive mycelia, give them ecological advantages over bacteria (Sinsabaug and Liptak, 1997; Cabana et al, 2007).

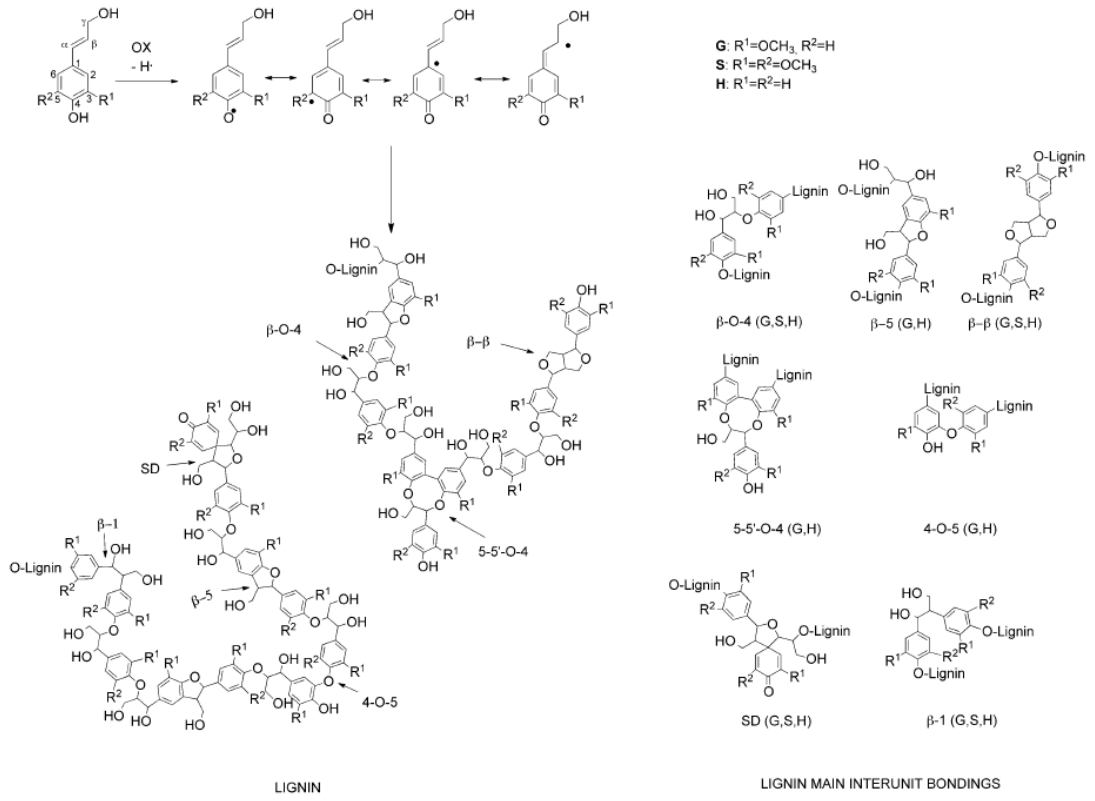


Figure 1-9. Lignin biosynthesis, structure and main lignin-unit linkages (Sette et al., 2011)

A broad range of types of wood decay can result from permutations of host species, fungal species and conditions within the wood. There are four different types of wood-decay fungi, reflected in the overall appearance and decay pattern: (i) Brown rot fungi (BRF) belong exclusively to the class of basidiomycetes and they are able to breakdown cellulose and hemicellulose, however lignin decomposition is limited (Blanchette et al., 1989; Sinsabaug and Liptak, 1997; Schwarze et al., 2000; Schwarze, 2007); (ii) *S*oft-rot fungi (SRF) belong mostly to the classes of ascomycetes and deuteromycetes, although recent researches demonstrated that also basidiomycetes can cause soft-rot. They degrade mostly cellulose and hemicellulose, while lignin results only partially modified (Schwarze et al., 2000; Carlile et al., 2001; Schwarze, 2007); (iii) Stain fungi (SF) some basidiomycetes cause a soft-rot decay pattern while a limited number of ascomycetes fungi can colonize wood through parenchymatic rays and resin channels causing coloration of softwood tissues; however, limited degradation is caused (Martínez et al., 2005); (iv) 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 able to mineralize lignin producing CO_2 and water by two main processes: selective delignification and simultaneous rot. Fungi that use the first strategy leave hemicellulose

and cellulose relatively unchanged during the first step of decay, degrading mostly lignin because their cellulose degrading enzymes operate only at the ends of the cellulose chains; while the latter decay process is referred to a decomposition of the three components of wood at nearly the same rate (Schwarze et al., 2000; Schwarze, 2007).

The term "white-rot" has been traditionally used to describe forms of wood decay where lignin -as well as cellulose and hemicellulose- is broken down, leaving a light, white, rather fibrous residue completely different from the brown powder left by brown rot fungi (Schwarze et al., 2000; Carlile et al., 2001). The main characteristics of WRF are shown in Table 1-6. These microorganisms are a diverse eco-physiological group that includes basidiomycetes and degrading fungi (Wesenberg et al., 2003; Sei et al., 2008). Generally, WRF are unable to use lignin as a sole carbon source but they degrade it in order to remove a physical barrier and gain access to polysaccharides (Sinsabaug and Liptak, 1997; Yeo et al., 2007). Within this group, *Phanerochaete chrysosporium* is the most extensively studied species, although other fungi such as *Bjerkandera adusta*, *Trametes versicolor*, *Pleurotus ostreatus* are also well-known (Schwarze et al., 2000; Schwarze, 2007).

Table 1-6. Characteristic features white rot fungi (Schwarze et al., 2000; Martínez et al., 2005)

WRF	*Simultaneous rot*	*Selective delignification*
Host	Hardwood, rarely softwood. Mainly broad-leaves; seldom in conifers	Hardwood and softwood. Broad–leaves trees, conifers
Fungi	Basidiomycetes and ascomycetes (*Trametes versicolor, Irpex lacteus, Phanerochaete chrysosporium*)	Basidiomycetes (*Ganoderma austral, Phlebia tremellosa*, etc.)
Degradation	Cellulose, lignin and hemicellulose	Initial attack selective for lignin and hemicellulose; later cellulose
Consistency	Brittle fracture	Fibrous (stringy)
Strength	Brittle fracture; great reduction of impact bending strength (at the initial stage); less drastic than in brown rot	Ductile fracture; slight increase in impact bending strength (at the initial stage)
Decay aspect	Bleached appearance, lighter in colour than sound wood, moist, soft, and spongy	

WRF are able to cause two forms of decay, differing in the target of decomposition at the early stage of the process: some fungi adopt selective delignification while others simultaneous rot, according to their enzymatic equipment (Martínez et al., 2005). Selective delignification is characteristic of decay by certain strains of *Ganoderma applanatlum*, *Phellinuts pini*, *P. chrysosporiuim*, *P. tremellosa*, and many others (Blanchette et al., 1989). On the other hand, fungi such as *T. versicolor*, some strains of *Phlebia slubseriialis*, *P. chrysosporium*, and others remove lignin, cellulose and

hemicellulose, since they release enzymes that can degrade all the main constituents of the cell wall (Schwarze et al., 2000; Blanchette et al., 1989).

Delignification is based on the WRF capacity to produce one or more extracellular lignin-modifying enzymes (LMEs), which thanks to their lack of substrate specificity, are also capable of degrading a wide range of xenobiotics also at relatively low concentrations since they are not induced by either lignin or other related compounds (Mester and Tien, 2000). LMEs act by generating free radicals that randomly attack the lignin molecule, breaking covalent bonds and releasing a range of mainly phenolic compounds. Since radicals are highly reactive but short-lived molecules, they can reach only closer lignocellulose material, forming erosion zone around the hyphae (Mester and Field, 1998; Carlile et al., 2001). There are two main types of LMEs: peroxidases and laccases (phenol oxidases). Some fungi are able to produce both types of enzymes, whereas others have only one of them. The main LMEs are lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccases (Lac). In addition, these fungi secrete mediators of high molecular weight increasing the range of compounds potentially biodegradable (Cabana et al, 2007). A detailed description of LMEs and mediators are included in section 1.2.2.

These enzymes are essential for lignin degradation even if the mineralization of this recalcitrant biopolymer requires the contribution of auxiliary systems by themselves unable to degrade lignin (Carlile et al., 2001; Wesenberg et al., 2003; Cabana et al., 2007). For example, synthesis of H_2O_2, a co-substrate of LiP and MnP, is entrusted to glyoxal oxidase, superoxide dismutase (SOD) and aryl alcohol oxidase (AAO), while enzymes such as glucose oxidase, aryl alcohol dehydrogenase (AAD), cellobiose dehydrogenase and cellobiose/quinone oxidoreductase (QR) are involved in feedback circuits and link ligninolysis with cellulose and hemicellulose degradation in nature (Wesenberg et al., 2003; Martínez et al., 2005; Cabana et al., 2007; Wong, 2008).

White-rot fungi start LMEs production during their secondary metabolism, since lignin oxidation provides no net energy to fungi (Wesenberg et al., 2003; Cabana et al., 2007). In particular, LMEs production is caused by nitrogen starvation (Carlile et al., 2001) (Figure 1-10). A culture medium with high nitrogen content permits full utilization of glucose and rapid growth.

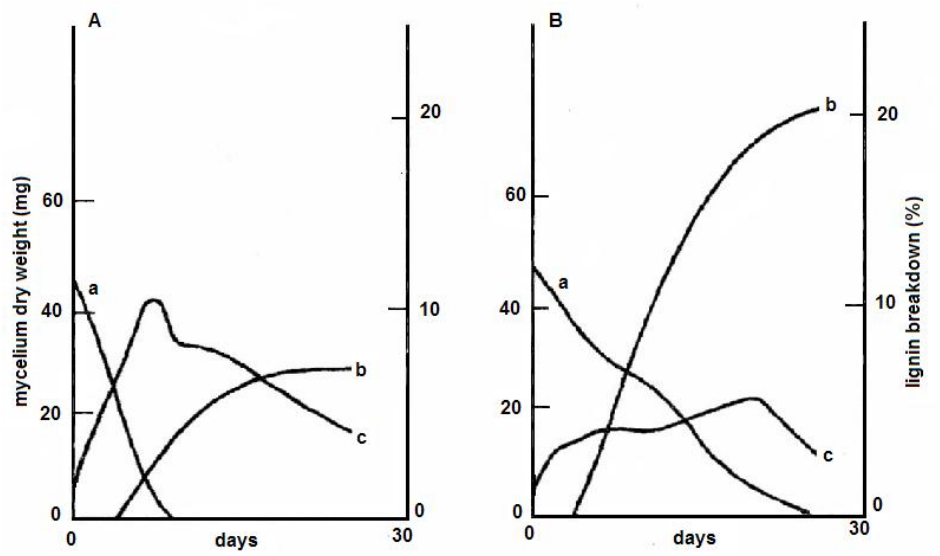


Figure 1-10. Lignin breakdown by *P. chrysosporium* in culture medium with high nitrogen (A) and low nitrogen content (B). Symbols: (a) glucose concentration (initially 56 mM), (b) lignin breakdown, (c) fungal biomass (c) (Carlile et al., 2001).

For *P. chrysosporium* lignin degradation takes place slowly. When the nitrogen source is limiting, glucose utilization and fungal growth are slow. Exhaustion of the nitrogen source is accompanied by a fall in the level of free intracellular amino acids, which induces enzyme synthesis and a high rate of lignin breakdown. Fungi living on cellulose brings into play their ligninolytic activity when nitrogen availability decreases, leading to removal of a lignin coating and subsequent exposition of fresh cellulose and bound nitrogen (Sinsabaug and Liptak, 1997). In this way, hyphae grow up exploiting the new C and N source and lignin decomposition is depressed while N level is adequate (Sinsabaug and Liptak, 1997; Carlile et al., 2001).

1.2.2. Lignin modifying enzymes (LMEs) and their mediators

These enzymes are responsible for generating highly reactive and non specific free radicals which have an effect on lignin degradation (Kersten and Cullen, 2007). The nonspecific nature and the extraordinary potential of the oxidizing peroxidases makes them attractive for the development of bioprocesses such as pulp and bleaching paper as well as for the degradation of recalcitrant compounds. Ligninolytic oxidoreductases include peroxidases, which are able to oxidize non-phenolic lignin, and laccases, whose direct action is restricted to phenolic units of lignin (that represent less than 10% of the total polymer (Martínez et al., 2005). The main lignin-modifying enzymes are described below.

Peroxidases

They are a vast 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. Usually their prosthetic group is protoporphyrin IX, with histidine as axial ligand (proximal His). Other conserved residues present in the distal side of all peroxidases are histidine and arginine. These two aminoacids are directly involved in the reaction with H_2O_2 and the stabilization of the intermediates (Martínez, 2002).

Peroxidases have a typical enzymatic cycle 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 an 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 [Fe^{3+}] 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 with a ferric-superoxo complex [$PFe^{3+}O_2^{\cdot-}$] that can be converted to the native state (spontaneously or by oxidation with a substrate) releasing a superoxide anion ($O_2^{\cdot-}$) (Figure 1-11) (Mester and Tien, 2000; Martínez, 2002; Wong, 2008).

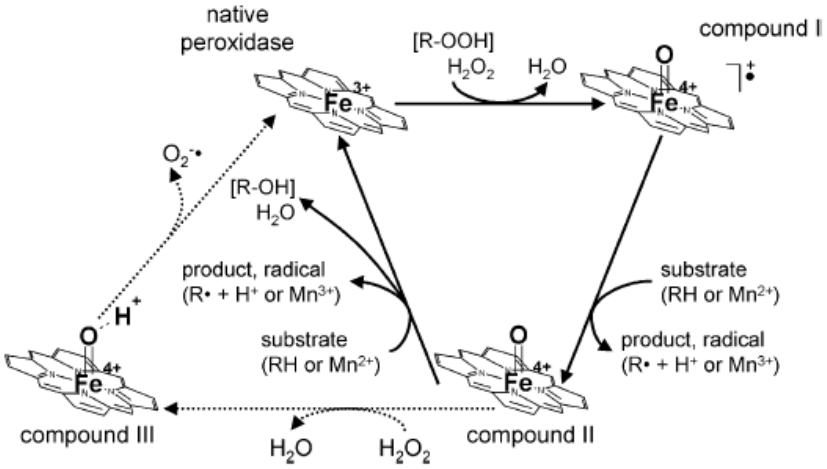


Figure 1-11. Catalytic cycle of peroxidases. (Wesenberg et al., 2003).

Lignin peroxidase (LiP, E.C.1.11.1.14)

It was the first ligninolytic enzyme isolated in 1980's decade from the fungus *P. chrysosporium* (Glenn et al., 1983; Tien and Kirk, 1983). It is a glycoprotein able to catalyze the oxidation of non-phenolic and aromatic compounds with a similar structure to lignin. The main characteristics of this extracellular N-glycosylated enzyme are shown in Table 1-7. LiP shows a classical peroxidase mechanism: it can react with non-phenolic aromatic substrates forming phenoxy radicals, but it is unique in its ability to oxidize substrates of high redox potential (up to 1.4 V) (Wesenberg et al., 2003; Wong, 2008). In fact, it shows the strongest oxidizing properties among peroxidases (E'_0~1.2 V at pH 3.0), that enables it to catalyze the oxidation of non-phenolic aromatic compounds without participation of mediators, forming radical aromatic cations (Martínez, 2002; Wong, 2008). Its high redox potential is due to the heme active site, more electron deficient than in other peroxidases (Wesenberg et al. 2003; Ashger et al. 2008; Wong, 2008).

The LiP isoenzymes are globular glycoproteins with molecular mass between 38 and 47 kDa with a distinctive property of an unusually low pH optimum near pH 3 (Table 1-7) (Wesenberg et al., 2003; Wong, 2008). The substrate interaction site is localized at the surface of the protein. In fact, even if deformation and enlargement of the heme access channels can facilitate the movement of small molecules towards the active site of the protein, the cleft size is too small to permit direct interaction between the ferryl oxygen and the larger substrates of the enzyme (Martínez, 2002; Wong, 2008). Figure 1-12 shows the three-dimensional structure of the enzyme LiP.

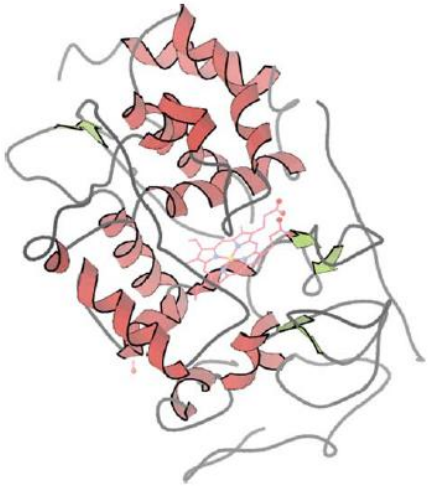


Figure 1-12. Three-dimensional structure of LiP from *P. chrysosporium* (Wong, 2008).

LiP is an enzyme relatively specific to their substrates, being the preferred substrate veratryl alcohol (VA) a natural metabolite (in *P. chrysosporium* produced at the

same time as LiP) that increases the enzyme activity and the rate of lignin degradation (Wong, 2008). The role of LiP in ligninolysis could be the next step after the lignin fragments are released initially by MnP, since it is able to degrade a wide variety of organic compounds with a redox potential above than 1.4 V in presence of H_2O_2: PAHs of three and four rings, PCBs and dyes (Wesenberg et al., 2003, Yu et al. 2006; Ashger et al. 2008; Wong, 2008). The general mechanism of LiP catalyzed reaction consists of two steps (Figure 1-13): (i) a $2e^-$ oxidation of the native ferric enzyme (Fe^{3+}) to yield compound I intermediate that exists as a ferry iron porphyrin radical cation ($Fe^{4+}=O^{\cdot+}$, LiP-I), with the peroxide substrate (H_2O_2) cleaved at the O–O bond; (ii) a two consecutive $1e^-$ reduction of LiP-I by electron donor substrates to the native enzyme. The first $1e^-$ reduction of LiP-I by a reducing substrate, such as VA, yields compound II (Fe^{4+}=O, LiP-I), and a VA radical cation ($VA^{\cdot+}$). A second $1e^-$ reduction returns the enzyme to the ferric oxidation state, completing the catalytic cycle. LiP-I can also return to the native enzyme by a direct $2e^-$ reduction in some cases (Wong, 2008).

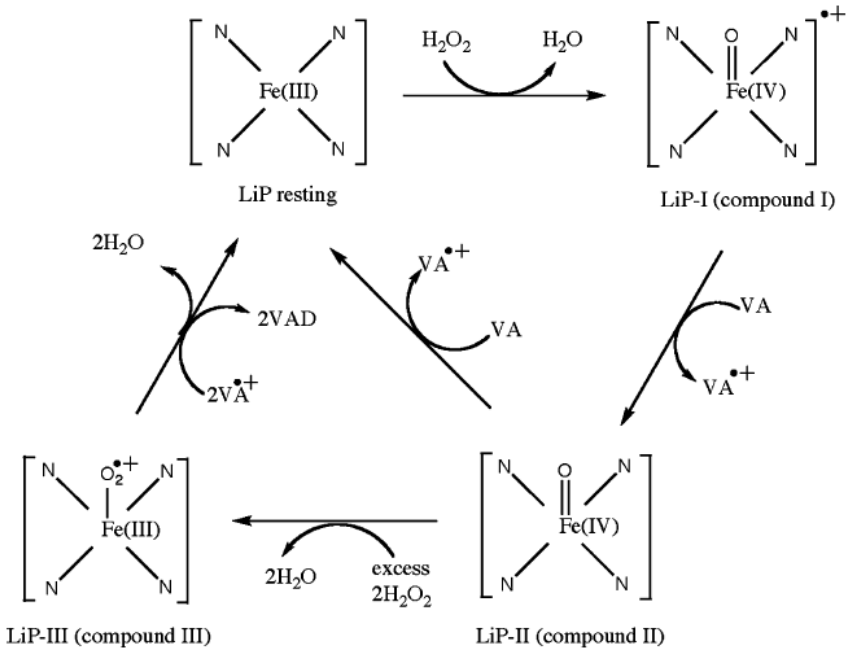


Figure 1-13. Catalytic cycle of LiP (Wong, 2008).

Manganese peroxidase (MnP, E.C.1.11.1.13)

Manganese peroxidase is a extracellular enzyme purified first time from *P. chrysosporium* by Kuwahara et al. (1984) and is considered as the most common ligninolytic peroxidase produced by almost all white-rot basidiomycetes and by various litter-decomposing fungi (Wesenberg et al., 2003). MnP is constituted by extracellular glycoproteins with a prosthetic group with an iron protoporphyrin. The optimal conditions for the secretion of

this enzyme are the use of a substrate with low carbon and nitrogen content, supplemented with Mn^{2+}. MnP is a glycoprotein with molecular weights between 32 and 62.5 kDa (Table 1-7) which structure is shown in Figure 1-14 (Wesenberg et al., 2003; Wong, 2008). This enzyme has a similar catalytic cycle to other peroxidases involving a $2e^-$ oxidation; however, MnP is unique in its ability to oxidize Mn^{2+}, resulting in the formation of diffusible oxidants (Mn^{3+}) capable of penetrating the cell wall matrix and oxidizing mainly phenolic substrates (Sinsabaug and Liptak, 1997; Pérez et al., 2002; Wong, 2008).

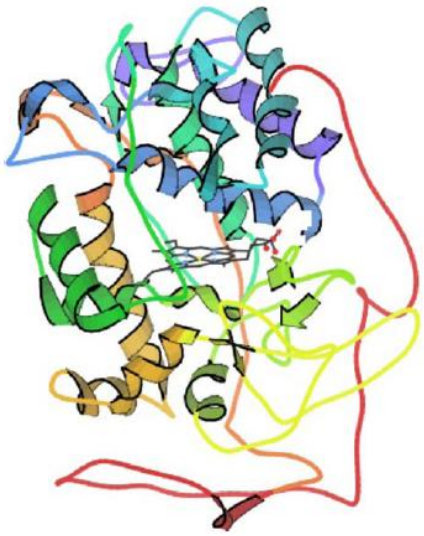


Figure 1-14. Three-dimensional structure of MnP from *P. chrysosporium* (Wong, 2008)

The catalytic cycle is initiated by binding of H_2O_2 to the native (ferric) enzyme and formation of an iron-H_2O_2 complex (Figure 1-15). Subsequent cleavage of the H_2O_2 oxygen-oxygen bond requires a $2e^-$ transfer from the heme resulting in formation of MnP compound I (Fe^{4+}). Afterwards, the O-O bond is heterolytically cleaved and a H_2O molecule expelled. Subsequent reduction proceeds through MnP-II (Fe^{4+}). Mn^{2+} ion acts as one electron-donor for this porphyrin intermediate and is oxidized to Mn^{3+}. The reduction of compound II proceeds in a similar way and another Mn^{3+} is formed from Mn^{2+}, thereby leading to generation of native enzyme and release of the second water molecule.

Like other peroxidases, MnP is sensitive to high concentrations of H_2O_2 that cause reversible inactivation of the enzyme by forming compound III, a catalytically inactive oxidation state but can be rescued by Mn^{3+} (Hofrichter, 2002; Wong, 2008). Ions of Mn^{3+} are quite unstable in aqueous media. To overcome this drawback, they form complexes with organic acids, such as malonic or oxalic acid, secreted by the fungus in significant amounts that attack organic molecules non-specifically at location remote from the enzyme active site (Hofrichter, 2002; Wong, 2008). These chelators could also accomplish

other physiological functions; they enhance the dissociation of Mn^{2+} from the enzyme improving its activity; allow the fungus to control pH; sequester Ca^{2+} ions to increase the pore size of the plant cell wall and facilitate the penetration of the enzyme or react with O_2 to form H_2O_2 useful for the enzyme activity (Wong, 2008).

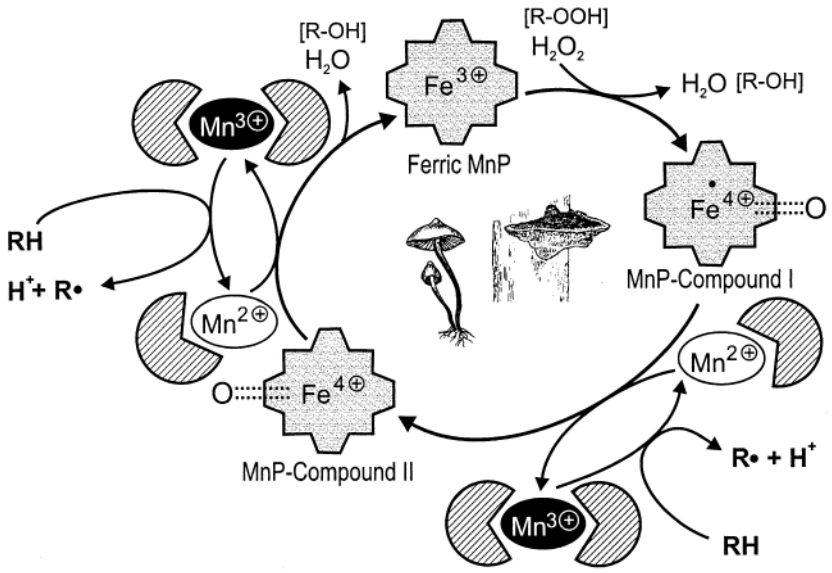


Figure 1-15, Catalytic cicle of MnP (Hofrichter, 2002).

Versatile peroxidase (VP, E.C.1.11.1.16)

The enzyme VP (Figure 1-16) is a peroxidase and is considered a hybrid between MnP and LiP (Wesenberg et al., 2003). It can oxidize not only Mn^{2+} but also VA and phenolic aromatic compounds with a high molecular weight by manganese-independent reactions (Wong, 2008). This enzyme was able to oxidize hydroquinone in the absence of exogenous H_2O_2 when Mn^{2+} is present (Pérez et al., 2002). VP is found in cultures of several fungal strains such as *Pleurotus* and *Bjerkandera* species (Wesenberg et al. 2003; Ashger et al. 2008). It is interesting underline that VP enzyme shows different pH optimal for oxidation of Mn^{2+} (pH 5) and aromatic compounds (pH 3), similar to those of optimal LiP and MnP activity (Martínez, 2002). Molecular characterization of VP enzyme reveals structures closer to the LiP than to the MnP isoenzymes of *P. chrysosporium* (Wong, 2008).

The VP catalytic cycle is similar to that of LiP, MnP and other heme peroxidases. The enzyme catalyzes the electron transfer from an oxidizable substrate, involving the formation and reduction of compound I and II intermediates. Compound I is a two oxidizing equivalent intermediate, with one-oxidizing equivalent resided in the ferryl state of the Fe^{4+} and the second localized as a porphyrin radical cation (Wong, 2008).

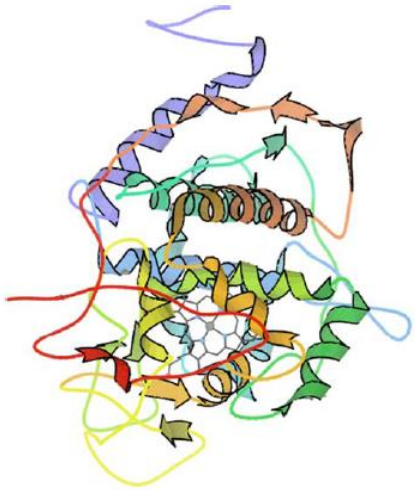


Figure 1-16. Three-dimensional structure of VP from *Pleurotus eryngii* (Wong, 2008)

Phenoloxidases

Laccase (Lac, E.C.1.10.3.2)

The enzyme Lac is part of the ligninolytic system and is a multi-copper oxidase that catalyzes single electron oxidation of four phenolic molecules with the concomitant reduction of oxygen to water (Sinsabaug and Liptak, 1997; Wesenberg et al., 2003). Laccase shows low substrate specificity and can react with diphenols, aryl diamines, aminophenoles. The oxidation reaction of phenolic moieties is usually accompanied by demethylation, decarboxylation and can also result in ring cleavage (Wesenberg et al., 2003). Laccases catalyzed-oxidation depends on the redox potential of the type-I copper, typically ranging between 500 - 800 mV. However, in presence of a mediator, Lac is able to oxidize also non-phenolic molecules (Wong, 2008). A description of the mainly natural and synthetic mediators is included in the following section of this chapter.

This enzyme is ubiquitous in all wood- and litter-transforming basidiomycetes, but Laccase activity has also been found in plants and bacteria. Laccase has been isolated from cultures of *Aspergillus* and thermophilic fungi such as *Myceliophora thermophila* and *Chaemotium thermophilium* (Pérez et al. 2002; Wesenberg et al. 2003; Ashger et al., 2008). In Figure 1-17 the structure of Lac enzyme is shown. Noteworthy that the active site is well conserved (Wong, 2008, Martínez et al., 2005). Laccase contains four copper atoms in its active site: type-I (T1, one Cu atom) that acts as electron acceptor from the substrate, type-II (T2, one Cu atom) copper which forms a trinuclear copper-cluster with the type-III copper (T3, two Cu atoms) where the reduction of O_2 takes place (Martínez et al., 2005; Wong, 2008). Laccases are a group of N-glycosylated extracellular isoenzymes

with monomeric and dimeric protein structure and molecular masses of 60–390 kDa (Table 1-9) (Wesenberg et al., 2003; Wong, 2008).

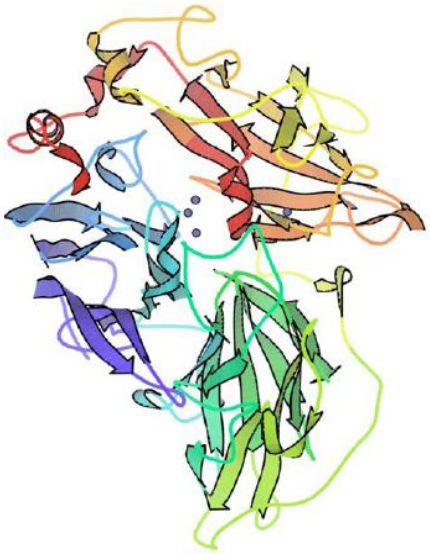


Figure 1-17. Three-dimensional structure of Lac from *T. versicolor* (Wong, 2008)

The catalytic cycle of this class of enzymes, shown in Figure 1-18, is described as follows (Wong, 2008). In the resting state the enzyme is completely oxidized then a total reduction of the metallic centers by four molecules of substrates (each one provides just $1e^-$). T1 site is the primary electron acceptor. Once totally reduced, the enzyme can bind a dioxygen molecule.

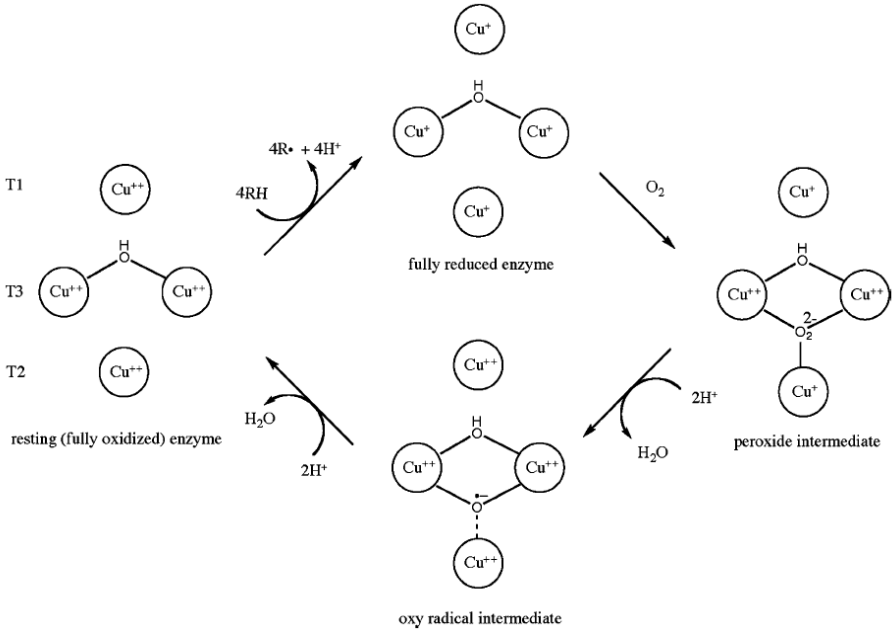


Figure 1-18. Catalytic cycle of Lac (Wong, 2008).

The diffusion of O_2 into the active site is rate limiting, but subsequently the T2/T3 site binds it and rapidly moves to it $2e^-$ from the T3 coppers, resulting in the formation of peroxide intermediate. Afterwards, $1e^-$ is transferred from T1 causing the decay of the peroxide intermediate and the formation of the oxy radical intermediate, through the cleavage of the O-O bond and the release of a water molecule. Finally, the last electron transfer from T2 leads to the ejection of O^{2-} as a second water molecule and closes the cycle.

Several WRF produce the three main LMEs (LiP, MnP and Lac), while others produce only one or two of them. These enzymes are necessary for lignin degradation; however, for its mineralization is necessary the combination of other processes with additional enzymes. These auxiliary enzymes are glyoxal oxidase and superoxide dismutase for intracellular production of H_2O_2, a co-substrate of LiP and MnP, as well as glucose oxidase, alcohol oxidase and cellobiose dehydrogenase aryl involved in the feedback loops and ligninolysis links during the degradation of cellulose and hemicellulose in nature (Wesenberg et al., 2003, Cabana et al., 2007). Table 1-7 compares the main characteristics of MnP, LiP and Lac enzymes.

Table 1-7. Main properties of MnP, LiP and Lac from WRF

	MnP	LiP	Lac
Prosthetic group	Heme	Heme	1 type 1-Cu, 1 type 2-Cu, 2 coupled type 3-Cu
Molecular weight (kDa)	32 - 62.5	38 - 47	59 - 110
pH optimal range	2.6 - 4.5	2 - 5	2 - 8.5
E^0 (mV)	1510	1450	500 - 800
H_2O_2 - regulation	+	+	-
Stability	+++	+	+++
Native mediators	Mn^{2+}; Mn^{3+}	VA, 2Cl-14DMB	
Specificity	Mn^{2+}	Aromatic compounds (including nonphenolics)	Phenolic compounds
Secondary and synthetic mediators	Thiols, unsaturated fatty acids	No	ABTS, HBT, syringaldazine

(-) none; (+) low; (++) medium; (+++) high; (VA) violuric acid; (2Cl-14DMB) 2-chloro-1,4-dimethoxybenzene acid; (3-HAA) 3-hydroxyanthralinic acid; (ABTS) 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); (HBT) 1-hydroxibenzothriazole (Wesenberg et al., 2003).

Main enzymatic mediators

Due to the complexity of lignin molecule and the volume of LMEs excreted by fungi, direct and specific interactions between lignin (or recalcitrant compounds with similar structure) and enzymes are highly unlikely. Therefore, there are redox mediators with low molecular weight which provide high redox potentials (> 900 mV) to attack lignin and are able to migrate within the lignocellulose complex. Several natural mediators such as syringaldehyde, acetosyringone, vanillin, acetovanillone, methyl vanillate and p-coumaric acid by laccase (Cañas and Camarero, 2010) have promoted the oxidation of recalcitrant dyes. Examples of native and synthetic mediators are shown in Table 1-8.

Table 1-8. Main fungal mediators (Wesenberg et al., 2003)

Mediator	Microorganism (Enzyme)
Mn^{3+}	*Phanerochaete chrysosporium* (MnP)
Organic acids (Malonate, oxalate)	*Armillaria mellea, Fomes annosus, Pleurotus ostreatus, Phanerochaete chrysosporium, Phlebia radiata, Nematoloma frowardii* (LiP, MnP)
VA	*Phanerochaete chrysosporium* (LiP)
3-HAA	*Pycnoporus cinnabarinus* (Lac)
2Cl-14DMB	*Trametes versicolor* (LiP)
1-HBT	*Trametes versicolor, Trametes villosa, Pycnoporus cinnabarinus, Botrytis cinérea, Myceliophthora thermophila, Pleurotus ostreatus* (Lac)
Violuric acid	*Trametes villosa, Pycnoporus cinnabarinus, Botrytis cinérea, Myceliophthora thermophila* (Lac)
ABTS	*Trametes versicolor, Coriolopsis gallica, Pleurotus ostreatus* (Lac)

1.2.3. Applications of white-rot fungi (WRF) and lignin modifying enzymes (LMEs)

White-rot fungi and their degrading enzymes have long been studied for biotechnological applications as new potential tools for the biodegradation of harmful chemicals (Lee et al., 2005; Yeo et al., 2007). These microorganisms have a unique lignin degradation system, which is suitable for removal of not only lignin from wood but also various so-called lignin-related pollutants from soil or water (Mester and Tien, 2000). A briefly description of the potential applications of WRF and their LMEs is included below.

Paper pulp biobleaching

Several strains of WRF such as *Coriolus versicolor, P. eryngii, P. radiata, P. chrysosporium, B. adusta*, among others, as well as their enzymes, are able to degrade wood and

lignocellulosic residues. Furthermore, WRFs or LMEs could represent a method for the treatment of waste bleach liquor to remove degraded lignin fragments. The dominant process during the bleaching of pulp is known as Kraft and is based in an alkaline extraction and bleaching of pulp. It is interesting to use a biological alternative capable to degrade lignin without the generation of harmful residues (Kerem et al., 1992, Moreira et al., 1997b; Sinsabaug and Liptak, 1997; Higuchi 2004; Feijoo et al. 2008; Hammel and Cullen, 2008). Selective delignification might also increase the value of wood, straw and bagasse (the residual fiber from sugar cane processing) by conversion of their lignocellulosic material into feed or biofuel (Sinsabaug and Liptak, 1997; Wong, 2008). Moreover, natural phenolic mediator could be obtained as by-product during the industrial process of biomass conversion improving the degradation of lignin by the laccase-mediator system (Cañas and Camarero, 2010).

Degradation of polycyclic aromatic hydrocarbons (PAHs)

These compounds may have toxic effects on microorganisms, plants and animals; and therefore they are classified as compounds with a significant risk to human health. They are difficult to degrade due to its low water solubility and strong lipophilic character; furthermore they tend to accumulate and persist in the environment (Ding et al., 2008). Degradation of anthracene, dibenzothiophene, fluoranthene, chrysene, pyrene, phenanthrene, benz(a)anthracene, perylene, acenaphthene, naphthalene, fluorene and acenaphthylene has been conducted using *Lentinus trigrinus*, *B. adusta*, *I. lacteus*, *P. chrysosporium*, *T. versicolor*, as well as a consortium of several WRF. Evenmore, the enzymes (MnP, LiP and Lac) were also used for this purpose (Johannes et al., 1998; Eibes et al., 2005; Valentin et al., 2006; Valentin et al., 2007; Ding et al., 2008; Mohammadi and Nasernejad, 2009; Chen et al., 2010). On the other hand, the use of the laccase-mediator system using natural mediators has shown the transformation and detoxification of aromatic compounds (Cañas and Camarero, 2010).

Degradation of chlorinated and phenolic compounds

These compounds are among the most abundant recalcitrant waste produced by the pulping and paper industry with a generation of a high biological oxygen demand (BOD), a highly toxicity and a persistent colour (Rubilar et al., 2008). There are studies of the degradation of compounds such as pentachlorophenol (PCP) (Rubilar et al., 2007), hexacychlorohexane (Quintero et al., 2007), phenolic resins (Gusse et al. 2006). In a recent work performed by Xiao et al. (2011), high degradation percentages of two organochlorine pesticides (dieldrin and aldrin) were achieved by several fungal species of the genus *Phlebia*.

Removal of dyes

Dyes are widely used in food, cosmetics, pharmaceuticals and textil industries, generating a large amount of highly coloured effluents. This colouration affects the penetration of sunlight, slowing the photosynthesis with a consequently inhibition of aquatic biota growth and interfering with the solubility of gas in the water. In addition, many of these dyes are toxic and may be carcinogenic (Rodriguez-Couto, 2009). Sewage treatment is unable to remove dyes completely (Chander and Arora, 2007) and therefore, an alternative to physicochemical and some biological treatments for the degradation of dyes present in textile effluents might be the use of WRF and their LMEs. Several *in vitro* studies have been conducted to analyze the degradation of dyes, for example using MnP enzyme produced by *Bjerkandera* sp. BOS55 (Lopez et al. 2004), LiP and MnP produced by *P. chrysosporium* (Moldes et al., 2003; Yu et al., 2006), Lac (Camarero et al., 2005; Mishra et al., 2011; Rodríguez-Couto, 2011). *In vivo* studies to perform the degradation of Orange G and the remazol brilliant blue R dye (RBBR) with 30 fungal strains reported the best results with *B. adusta, P. chrysosporium* and *P. ostreatus* (Eichlerová et al., 2007). Other studies confirm the potential of *Grammothele subargentea, P. chrysosporium* and *T. versicolor* (LPSC strain 436) to degrade dyes in cultures with immobilized and free mycelia, as well as in solid-state fermentation (SSF) (Boehmer et al., 2006; Chander and Arora, 2007; Saparrat et al., 2008).

Degradation of endocrine disrupting chemicals (EDCs) and reduction of their estrogenic activity

EDCs are a group of compounds defined as any substance or mixture of exogenous substances that cause alteration of the functions of the endocrine system and consequently cause adverse health effects of an organism (Esplugas et al., 2007). The presence of EDCs in industrial and STPs effluents are of great interest because they are classified as recalcitrant compounds that may have negative effects on living organisms since they are able to regulate the estrogenic activity (Tsutsumi et al., 2001). These compounds are not easily removed by conventional activated sludge process due to its resistance to biodegradation and several physicochemical treatments (ozonation, photocatalytic degradation and adsorption with activated carbon) (Sei et al., 2008). In the recent years removal of compounds with estrogenic activity such as estrone and genistein (Tamagawa et al., 2005 and 2006a), bisphenol A (Hirano et al., 2000, Tsutsumi et al. 2001; Soo-min et al., 2005), nonylphenol (Tsutsumi et al., 2001; Soares et al., 2005; Soares et al., 2006) 4-tert-octylphenol (Tamagawa et al., 2006b) have been performed using WRF and their enzymes with satisfactory results.

Degradation of pharmaceutical compounds

One of the latest applications of ligninolytic fungi and their enzymes is the degradation of pharmaceutical compounds. Removal of several compounds such as DCF, IBP, NPX and CBZ has been performed during *in vivo* assays by several strains of WRF (*Ganoderma lucidum, Phanerochaete sordida, T. versicolor, I. lacteus, P. chrysosporium,*) achieving satisfactory degradations (Marco-Urrea et al., 2009; Marco-Urrea et al., 2010a; Rodríguez-Rodríguez et al., 2010a, 2010b; Hata et al., 2010a). Also, the use of LiP, MnP, VP and the so-called Laccase-mediator system has been studied for the degradation of several pharmaceuticals (Zhang et al., 2008; Hata et al., 2010b; Lloret et al., 2010; Marco-Urrea et al., 2010b; Rodríguez-Rodríguez et al., 2010a; Zhang and Geiβen, 2010; Eibes et al., 2011).

1.3. Objectives

The general objective of this thesis is the evaluation of the potential ability of three white-rot fungi (WFR) (an anamorph of *Bjerkandera* sp. R1, *Bjerkandera adusta* and *Phanerochaete chrysosporium*) for the removal of eight pharmaceutical compounds belonging to several therapeutic groups: anti-depressants (citalopram and fluoxetine), antibiotic (sulfamethoxazole), anti-inflammatories (diclofenac, ibuprofen, naproxen), antiepileptics (carbamazepine) and tranquilizers (diazepam).

The specific objectives to achieve this general goal are described below:

i) Determination of the possible inhibitory effect of pharmaceuticals and their mixtures on the growth of the three WRF in plates.

ii) Comparison of different cultivation techniques (static, free pellets and immobilized fungus) to determine the optimal conditions that favour the degradation of pharmaceuticals and also avoid any possible adsorption onto the biomass or immobilization support.

iii) Determination of the hydraulic residence time (HRT) required for the removal of these compounds by WRF during batch (24 h time-course) and fed-batch degradation experiments.

iv) Scale-up of the process to a stirred tank reactor (STR) of 0.75 L operated in batch to evaluate if the selected WRF (the fungal strain which presents the highest removal rates) is able to maintain the removal efficiency under these conditions.

v) Study of the degradation of the considered pharmaceuticals in STRs (2 L) using two different aeration conditions (continuous air flow and oxygen pulses) in a fed-batch reactor with pellets of the selected WRF. In addition, an operation of a STR

from fed-batch to continuous will be conducted to determine if the fungus is able to maintain degradation of the target compounds.

vi) A fixed-bed reactor (FBR) with immobilized fungus in polyurethane foam will be operated for the removal of the selected pharmaceuticals, evaluating the influence of gas supply as a continuous air flow or as oxygen pulses. Moreover, a STR with immobilized fungus will be operated to analyze if it is possible to maintain the process efficiency and stability.

vii) The GC-MS chromatograms of the different experiments will be analyzed in order to find the major metabolites of the three anti-inflammatory drugs (DCF, IBP and NPX).

Chapter 2

Materials and methods

Summary

In this chapter the materials and methods required in the development of this doctoral thesis are described. Three white-rot fungi were used and their culture media and incubation conditions specified. Different culture techniques for the inoculums, static, pellet and immobilized cultures are detailed. A list of the pharmaceutical compounds evaluated are presented with comprehensive information about the methodology and operational conditions used for their determination and quantification, such as High Performance Liquid Chromatography with a Diode Array Detector (HPLC-DAD) and Gas Chromatography-Mass Spectrometry (GC-MS) analysis. In addition, the methodology to evaluate the adsorption of the target compounds on the fungal biomass is included. Furthermore, this chapter includes the description of the configuration of the bioreactors applied for the removal of the target compounds: stirred tank reactors (STRs) and fixed-bed reactors, including the formulation of the feed media and the selection of the operational conditions. Under certain bioreactor configurations, a pulsing device was used for the supply of oxygen pulses. The hydraulics behaviour of the bioreactors was determined by means of residence time distribution (RTD) experiments. Finally, several analytical techniques such as pH, reducing sugars, total organic carbon (TOC), total nitrogen content (NT), enzymatic activity of manganese and versatile peroxidases, peroxide concentration, biomass concentration and morphology and size of pellets by stereomicroscopy are described at the end of the chapter.

Outline

2.1 Microorganisms

In this study three fungal strains were considered: an anamorph species of *Bjerkandera*, *Bjerkandera adusta* (ATTC 90940) and *Phanerochaete chrysosporium* (ATTC 24725). The former was isolated in South Chile and provided by the Laboratory of Environmental Biotechnology of Universidad de la Frontera (Temuco, Chile), being previously classified as *Anthracophyllum discolor*. It comprehensive characterization indicated that this strain was an anamorph of *Bjerkandera*, being then classified as *Bjerkandera* sp. R1 (Taboada-Puig et al., 2010). The other species were obtained from the culture collection of the University of Santiago de Compostela (Spain). The strains were grown in maintenance slants with yeast extract agar at 30°C for 7 days and then transferred to plates with malt extract agar and incubated at 30°C for 5 -7 days before the start of the experiments. In Table 2-1 the compositions of the media used are shown.

Table 2-1 Chemical formulation of slants and malt extract agar

Maintenance slants		Malt extract agar	
Composition	(g/L)	Composition	(g/L)
Glucose	20	Glucose	10
Agar	15	Agar	15
Peptone	5	Malt extract	3.5
Yeast extract	2		
KH_2PO_4	1		
$MgSO_4 \cdot 5H_2O$	0.5		

2.2. Pharmaceutical compounds and chemicals

The pharmaceuticals used in this work were antidepressants: citalopram (CTL) and fluoxetine (FLX); antibiotics: sulfamethoxazole (SMX); anti-inflammatories: diclofenac (DCF), ibuprofen (IBP) and naproxen (NPX); antiepileptic: carbamazepine (CBZ), all of them purchased from Sigma-Aldrich as pure grade. Another pharmaceutical compound used was a tranquilizer: diazepam (DZP) from Roche Pharma, (pure grade). A range of solvents were used for the extractions and for the preparation of the pharmaceutical mixtures: acetone (J.T. Baker, 99.5%), ethyl acetate (J.T. Baker, 99.5%), acetonitrile (J.T. Baker, 99.8%), methanol (J.T. Baker, HPLC grade, 99.8% and Panreac, 99.5%) and n-hexane (J.T. Baker, 95%).

2.3. Culture conditions

2.3.1. Pre-inoculum

The pre-inoculum for all the cultures was obtained from static submerged cultures using Fernsbach flasks (1 L) with 100 mL of modified Kirk medium (Tien and Kirk, 1988) and 6-8 plugs of malt extract agar with active fungus. In Table 2.2 the composition of the medium used is shown.

Table 2-2 Composition of modified Kirk medium

Composition	(g/L)	*Formulation of the trace element solution	(g/L)
Glucose	10	$MgSO_4$	3
Ammonium tartrate[a]	0.2	$MnSO_4$	0.5
Peptone[b]	5	NaCl	1
KH_2PO_4	2	$FeSO_4 \cdot 7H_2O$	0.1
$MgSO_4 \cdot H_2O$	0.5	$CoCl_2$	0.1
$CaCl_2$	0.1	$ZnSO_4 \cdot 7H_2O$	0.1
Sodium acetate	3.3	$CuSO_4$	0.1
Tween 80 (10%)[c]	5 mL	H_3BO_3	0.01
Trace elements*	10 mL	$Na_2MoO_4 \cdot 2H_2O$	0.01
		$AlK(SO_4)_2 \cdot 12H_2O$	0.01
		Nitrilotriacetate	1.5

[a] Only for *P. chrysosporium* cultures; [b] Only for the *Bjerkandera* cultures; [c] Only for agitated cultures to improve pellet formation.

2.3.2. Static cultures

Static cultures were performed in Erlenmeyer flasks (100 mL) containing 15 mL of modified Kirk medium. Each flask was inoculated with three plugs (6 mm) of malt extract agar with active fungus and statically incubated at 30°C during different incubation times.

2.3.3. Pellets and immobilized cultures

The grown mycelium from the Fernsbach flasks was used as inoculum for Erlenmeyer flasks (250 mL) containing 90 mL of modified Kirk culture medium and then were inoculated with 9 mL of homogenized mycelium. The immobilization support consisted of polyurethane foam cubes (volume, 0.5 cm^3; density, 20 kg/m^3; superficial area, 414±10 m^2/m^3) obtained from Copo Ibérica (Vigo, Spain) (Moreira et al., 1997a). Before use, the support was washed with methanol and rinsed three times with distilled water, and then it was sterilized in autoclave and dried at room temperature. The support/liquid volume ratio used was 0.015 g/mL. The flasks were filled with 90 mL of modified Kirk culture medium and the support (1.35 g) and then inoculated with 9 mL of homogenized

mycelium. The flasks were incubated at 30°C and agitated at 150 rpm in an orbital shaker (C24 Incubator Shaker, New Brunswick Scientific, USA) during 3-5 days until the formation of pellets or the immobilization of the fungus was evident.

2.3.4. Preparation of the pre-inoculum for the stirred tank reactors (STRs) and the fixed-bed reactors (FBRs)

Flasks with pellets were withdrawn by filtration using sterile gauze and used as inoculum for the STRs. The pre-inoculum used for the bioreactors is depending on the volume of the vessel. Accordingly, pellets from 3 flasks were transferred to the STR of 0.75 L; while 5 flasks were used for the STR of 2 L. Moreover, in the case of the fixed-bed reactors, 3 flasks with immobilized fungus were transferred to the reactor.

2.3.5. Mycelium growth inhibition at different pharmaceuticals concentrations

According to the protocol defined by Soo-Min et al. (2005), a plug of agar with active fungus was inoculated on malt extract agar plates, which contained mixtures of several pharmaceutical compounds at different concentrations. Besides, fungal plates with no pharmaceuticals were used as controls. The plates were incubated at 30°C for 6 days, and the hyphal extension of each fungus was measured daily from the center of the colony to the edge of the plate, considering that the maximal growth corresponded to a hyphal extension of 4 cm, covering the whole surface of the plate. The inhibition is negative if the fungus was able to grow as in the control plates while positive when the fungus showed slow or negligible growth.

2.4. Bioreactors configuration

2.4.1. Stirred tank reactor (0.75 L)

A stirred tank reactor (STR) from Biostat Q multiple fermenter (B. Braun Biotech International, Melsungen, Germany) was used to carry out the pharmaceuticals degradation experiments (Figure 2-1a). The fermenter consisted of four autoclavable vessels (0.75 L) with magnetic stirring. All the vessels were connected to a supply unit which comprised gas supply system (air) and a thermostat unit with a 1 kW heater and a circulation-pump supplies heated water (max. 60°C) and a cooling water supply for temperature control (from 20°C up to 50°C) by circulating thermostated water through the jacket of each vessel.

The aeration flow was measured by four rotameters with a central pressure reducer. Also, each vessel had two auxiliary pumps connected to the control module for pH control, when necessary. As shown in Figure 2-1b the culture vessels have an upper

stainless steel lid with three addition ports (for connection to acid, alkaline agent and feed supply), one rising pipe for sample withdrawal and two adapters for connection of pH and/or dissolved oxygen (PO_2) electrodes. On-line pH data was processed by software MFCS/DA 3.0 (Module operator service program, Sartorius Systems, Germany); while dissolved oxygen concentration was measured off-line by means of an electrode (Oxi 340i from Wissenschaftlich Technische Werk, Germany). The operating conditions of this bioreactor will be described in detail in chapter 3.

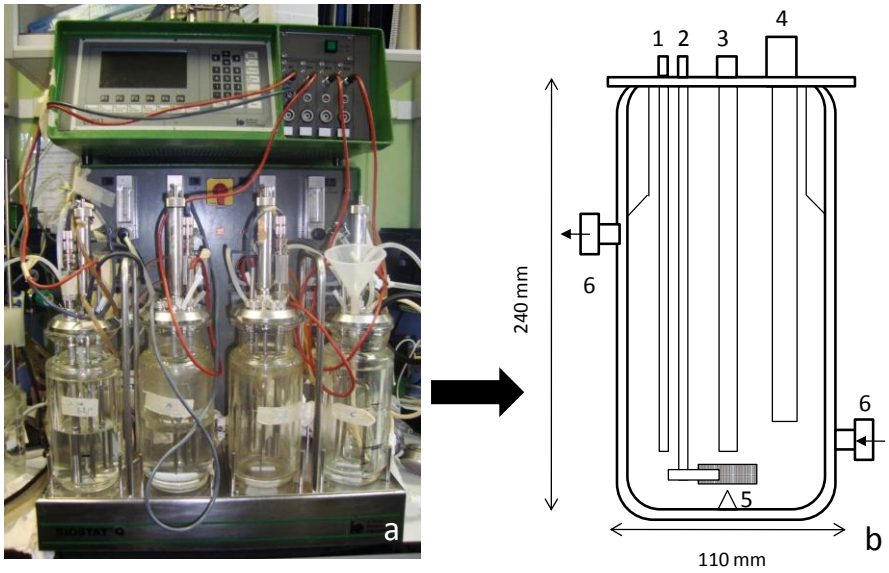


Figure 2-1 Stirred tank reactors (STRs) from a Biostat Q multiple fermenter connected to a control module (a) and main characteristics of each vessel (b). Symbols: (1) sample point; (2) aeration/oxygenation point; (3) PO_2 sensor; (4) pH electrode; (5) magnetic stirre; (6) thermostated water.

2.4.2. Stirred tank reactor (2 L)

Degradation experiments were performed in a 2-L fermenter Biostat B plus (Sartorius, Melsungen, Germany) (Figure 2-2a). The culture vessel consisted of a jacketed glass vessel designed for temperature control via a thermostated system. The configuration includes a stirrer drive and gas supply system. In Figure 2-2b the main characteristics of the culture vessel used are shown. The upper lid of the vessels includes ports for sampling, for connection to acid, alkaline and substrate solutions, for sample withdrawal and for electrodes. Dissolved oxygen concentration and pH were continuously monitored by pO_2 and pH electrodes and data were processed by software MFCS/DA 3.0 (Module operator service program, Sartorius Systems, Germany). The operating conditions of this bioreactor will be described in chapter 4.

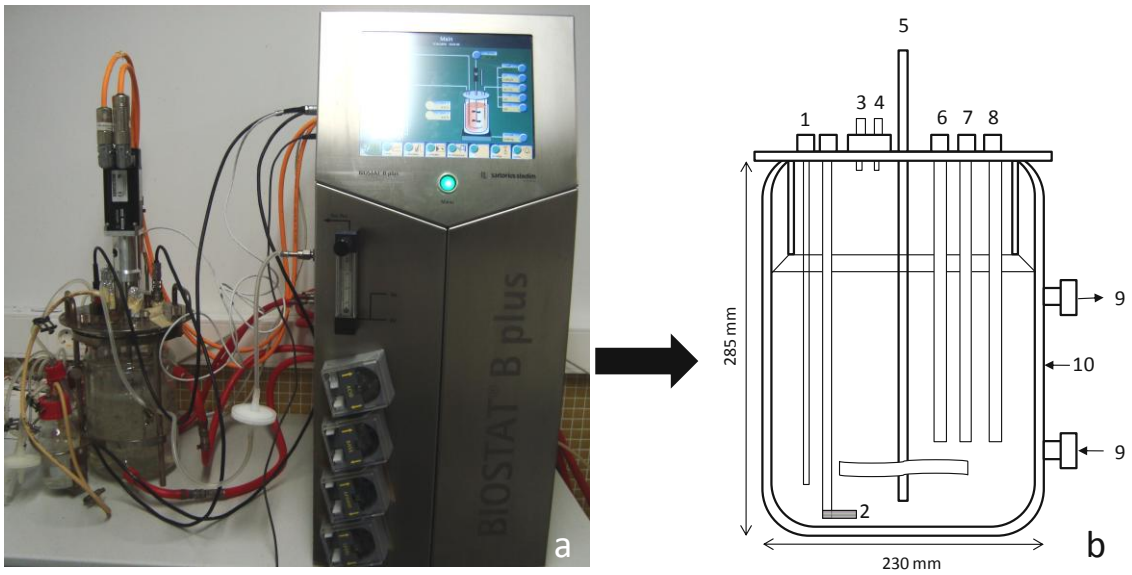


Figure 2-2. Stirred tank reactor (STR) from a Biostat B plus fermenter (a) and main characteristics of the vessel (b). Symbols: (1) sample point; (2) aeration/oxygenation point; (3) feeding pipe; (4) acid pipe; (5) stirrer; (6) PO_2 sensor; (7) temperature sensor; (8) pH electrode; (9) thermostated water.

2.4.3. Fixed-bed reactors (0.167 L)

The bioreactor consisted of a glass column with an internal diameter of 4.5 cm and a height of 20 cm. The bioreactor was designed with a jacket to control the temperature via circulation of thermostated water. Figure 2-3a shows the configuration of fixed-bed reactor (FBR) and Figure 2-3b shows the characteristics of the columns. The immobilization support consisted of polyurethane foam inoculated as described previously. The feeding was added through the bottom of the column and samples were withdrawn from the medium and upper port. Two aeration conditions were used: continuous air flow and oxygen pulses. The operating conditions of this type of bioreactors will be described in chapter 5.

2.4.4. Hydraulics of the bioreactors

Pulsing device

An electrovalve located at the end of a flexible membrane tube (FMT) controlled by a cyclic timer was used to inject oxygen with a pulsing flow (Figure 2-4) (Roca et al., 1994). The pulsing frequency is defined as the inverse of the sum of the opening and shutting times of the electrovalve (Moreira et al., 1996).

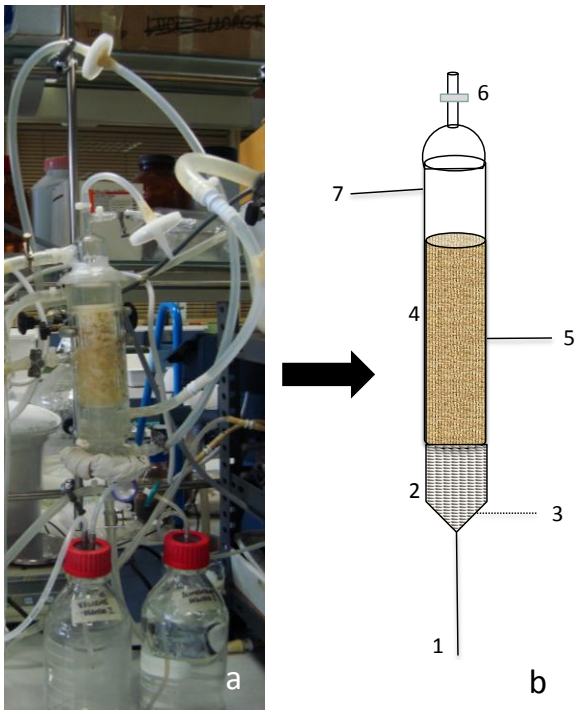


Figure 2-3. Immobilized bioreactor (0.167 L) (a) and main characteristics of the bioreactor (b). Symbols: (1) feeding port; (2) glass spheres; (3) air/oxygen supply port; (4) polyurethane foam support; (5) reactor sampling port; (6) filter; (7) effluent sampling port.

Different shutting times of the electrovalve were assayed (between 15 and 60 min) to maintain the maximum concentration of dissolved oxygen during the degradation assays performed in the 2-L STR and the FBR; therefore, the pulsing frequency used was maintained in a range between 0.0163 min^{-1} and 0.0625 min^{-1}.

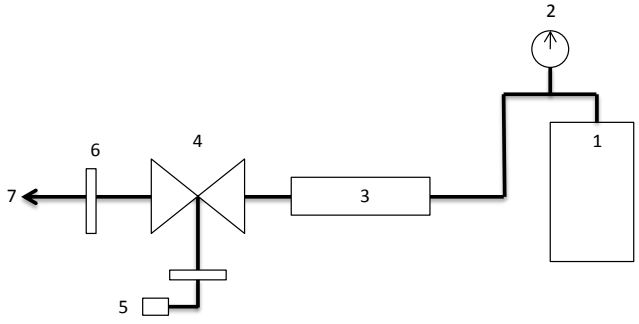


Figure 2-4 Pulsing device. (1) oxygen supply; (2) manometer; (3) flexible membrane tube (FMT); (4) electrovalve; (5) cyclic timer; (6) filter; (7) bioreactor input stream

Determination of the Residence Time Distribution (RTD)

The determination of the RTD curves by stimulus-response experiments was carried out to characterize the hydraulics of the bioreactors. An aliquot of 1 mL of lithium chloride (LiCl, 77 g/L) was injected as a tracer and samples were taken every hour during three residence hydraulic times. Then, the concentration of Li was determined by ionic chromatography in a chromatograph 861 Advanced Compact IC using a Metrosep column C3 (Metrohm). The mobile phase was nitric acid (3.5 mM). The limits of detection were in the range of 0.05–5 mg/L. The experimental data obtained were adjusted to the tank-in-series model to determine the hydraulics of the bioreactors (Levenspiel, 1999). Using the RTD calculated, the number of tanks connected in series (N) can be determined; this expression represents the number of tanks necessary to model the reactor as N ideal tanks in series. If N is small, the characteristics of the reactor will be similar that those from a complete stirred tank reactor (CSTR); while if N is higher, the reactor will have a behavior similar to a plug flow reactor (Fogler, 2006).

2.5 Identification of pharmaceutical compounds

Eight different pharmaceuticals were used in this doctoral thesis: CTL, FLX, SMX, DCF, IBP, NPX, CBZ and DZP. Depending on the available methods to determine the compounds while avoiding interferences between them, three different concentrated mixtures (2000 mg/L each) of pharmaceutical compounds were used (Table 2-3).

Table 2-3. Stock solutions of the pharmaceutical compounds (2000 mg/L)

Mixtures of compounds	Solvent	Determination method
CTL, FLX and SMX	Acetone	HPLC
DCF, IBP and NPX	Methanol	GCMS
CBZ and DZP	Acetone	GCMS

2.5.1. Extraction of pharmaceutical compounds

Before sampling, acetonitrile was added (1:1) for the extraction of the pharmaceutical compounds. The flasks were sealed with Teflon and agitated for 2 h at 180 rpm in a shaker (Ika Labortechnik, HS 501 Digital, Germany). From the supernatant of each flask, samples were withdrawn according to a protocol specific for each analysis.

2.5.2. Quantification of the adsorption extent of pharmaceuticals on fungal biomass

At the end of the operation of the STR, a final extraction was carried out to determine the concentration of pharmaceuticals that could be adsorbed on the biomass. For this purpose, the biomass was taken and blended in order to homogenize the sample. Then,

50 mL of this blended mycelium was placed in an Erlenmeyer flask and 50 mL of acetonitrile were added. The samples were agitated for 2 h at 150 rpm. With the FBR, the immobilized fungus from the column was divided in three parts: the upper, the medium and the bottom of the column. With the upper and bottom parts, an extraction with acetonitrile (1:1) as previously described was carried out in order to determine the residual concentration; meanwhile with the medium part the measurement of the biomass content was conducted. After the agitation of the samples with acetonitrile, samples were analyzed for the determination of the residual concentration of pharmaceuticals by HPLC-DAD and GC-MS.

2.5.3. High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD) analysis

Residual concentrations of CTL, FLX and SMX were measured by High Performance Liquid Chromatography - Diode Array Detection (HPLC-DAD). After extraction with acetonitrile, 2 mL were taken from each flask and centrifuged in an Eppendorf centrifuge for 15 min at 7000 rpm (Alresa, mod Digicen, Spain). Thereafter, 1 mL was withdrawn from the supernatant to determine the concentration of the selected compounds in a HPLC-DAD by using a Lichrosphere column (100 RP-18 5 µ Lichrocart 250-4, Merck). In Table 2-4 the operation conditions of HPLC are shown.

Table 2.4 Operational conditions of HPLC-DAD

	CTL	FLX	SMX
Eluent	Acetonitrile : Buffer phosphate (50 mM, pH 2.2)		
Elution conditions		40:60	
Flow (mL/min)		0.6	
Time (min)	~ 12	~ 25	~ 5
Wavelength (nm)	275	226	205
Volume (µL)		250	

Detection limit (0.1 mg/mL); Retention time (30 min)

The spectrum of each of the three pharmaceutical compounds analyzed by HPLC-DAD as the chromatogram obtained after the analysis of the standard solution is shown in Figures 2-5 and 2-6, respectively.

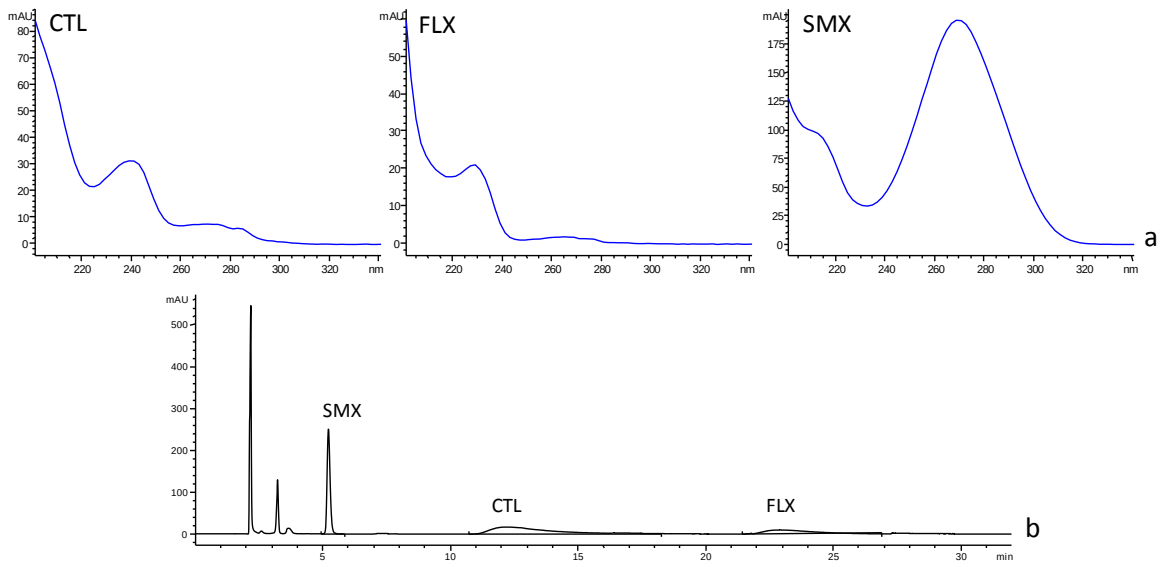


Figure 2-5. HPLC-DAD spectrum of CTL, FLX and SMX (a) and HPLC-DAD chromatogram corresponding to the stock solution of CTL, FLX and SMX (b).

2.5.4. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Residual concentrations of DCF, IBP, NPX, CBZ and DZP were measured by Gas Chromatography-Mass Spectrometry (GC-MS) (Saturn 2100T, Varian, USA). After agitation with acetonitrile, 10 mL from each flask were placed in glass tubes sealed with Teflon and agitated in a shaker at 180 rpm for 2 h (Ika Labortechnik, HS 501 Digital, Germany). From the supernatant of each sample, a volume of 10 mL was withdrawn and placed in glass tubes sealed with Teflon and centrifuged at 7000 rpm for 15 min. An aliquot of 4 mL was taken from each tube, diluted in 100 mL water and then a Solid Phase Extraction (SPE) was carried out with 60 mg OASIS HLB cartridges (Waters closet, Milford, MA, USA) previously supplemented with 3 mL of ethyl acetate, 3 mL of methanol and 3 mL of distilled water adjusted to pH 2 by hydrochloric acid (1 M) (Figure 2-6).

The cartridges were then dried with a nitrogen stream for 45 min and eluted with 3 mL of ethyl acetate. To determine the soluble fraction of DCF, IBP and NPX by GC-MS an aliquot of 0.8 mL of the extract was taken and 200 µL of MTBSTFA (N-Methyl-N-(tert.-butyldimethylsilyl)-trifluoroacetamide) was added for the derivatization of the anti-inflammatory species.

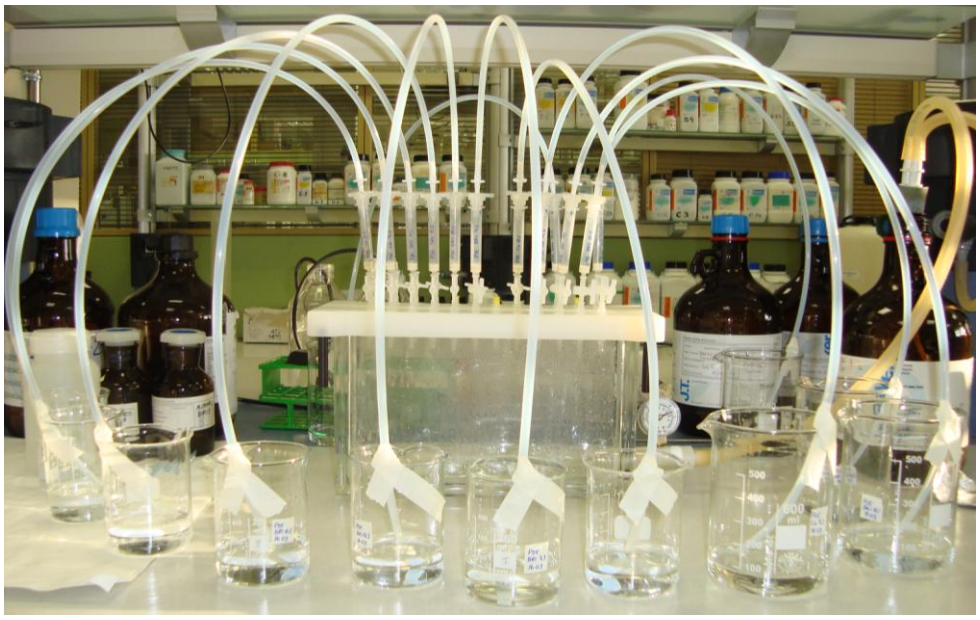


Figure 2-6. Solid phase extraction (SPE) before the GC-MS analysis

The second fraction of the extracted was used for the determination of the concentration of neutral compounds (CBZ and DZP) where an aliquot of 1 mL was withdrawn, supplemented with 10 µL of the internal standard PCB 30 (2,4,6-trichlorophenyl) and thereafter, the samples were analyzed. Table 2-5 shows the GC-MS operational conditions and the detection/quantification limits for each compound (Reddersen and Heberer, 2003; Rodríguez et al., 2003; Reif et al., 2008).

Table 2-5 Operational conditions for the determination of pharmaceuticals by GCMS

Chromatographic parameters	**Acid compounds**	**Neutral compounds**
Split-splitless injector		
Splitless time	1 min	1 min
Injection temperature	280°C	250°C
Gas flow (He)	1 mL/min	1 mL/min
Pressure pulse	No	30 PSI (1 min)
Injector (time/volume)	1 µL	1 µL
Solvent	Ethyl acetate	Ethyl acetate
Temperature program		
Initial temperature	50°C	60°C
Initial time	1 min	2 min
1st ramp	10°C/min	10°C/min
Temperature 1	180°C	250°C
Time 1	7 min	0 min

Table 2-5 (continued). Operational conditions for the determination of pharmaceuticals by GCMS

Chromatographic parameters	**Acid compounds**	**Neutral compounds**
2nd ramp	10°C/min	20°C/min
Temperature 2	230°C	280°C
Time 2	25 min	9.5 min
3rd ramp	-	20°C/min
Temperature 3	-	250°C
Time 3	-	5 min
Mass spectrophotometry		
Ionization mode	Electronic impact	Electronic impact
Filament current	10 µA	20 µA
Ion ramp temperature	220°C	220°C
Transference line temperature	280°C	280°C
Voltage	1700-1750 V	1700-1750 V
Scan velocity	1 s/scan	0.76 s/scan
Mass spectrum	100-330 m/z	45-400 m/z
	(10-25 min)	
	140-420 m/z	
	(25-57 min)	
Quantification ion (m/z)	DCF (352+354+356)	CBZ (193+236)
	IBP (263)	DZP (256+283)
	NPX (287)	
Limits		
Detection limit (mg/L)	DCF (0.1)	CBZ (0.4)
	IBP and NPX (0.025)	DZP (0.2)
Quantification limit (mg/L)	DCF (0.3)	CBZ (1.2)
	IBP and NPX (0.075)	DZP (0.6)

The chromatograms for each of the analyzed compounds (acid and neutral) are shown in Figure 2-7. The mass spectrum of acid (DCF, IBP, NPX) and neutral (CBZ and DZP) compounds can be observed in Figure 2-8.

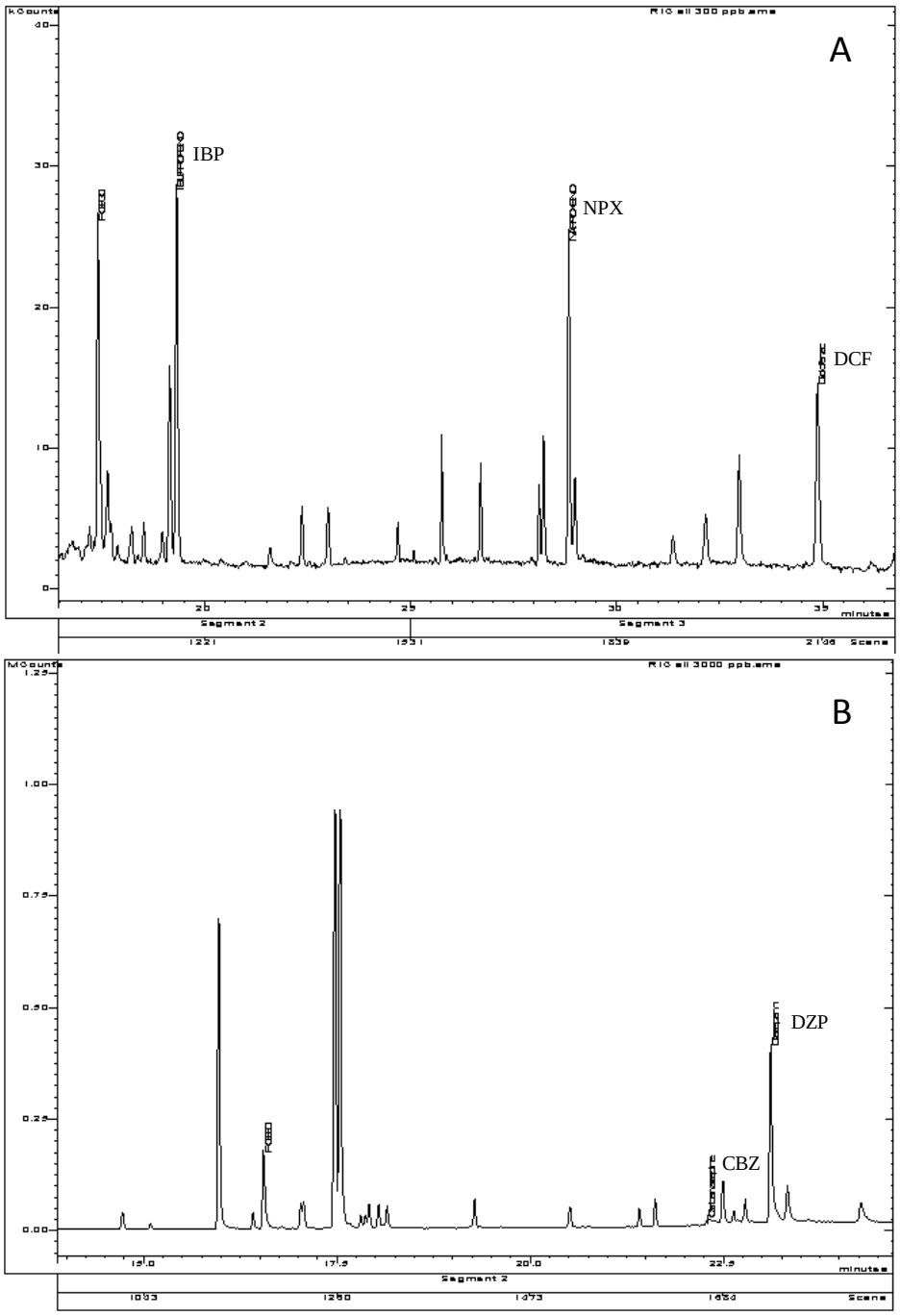


Figure 2-7. GC-MS chromatograms of acid (A) and neutral compounds (B).

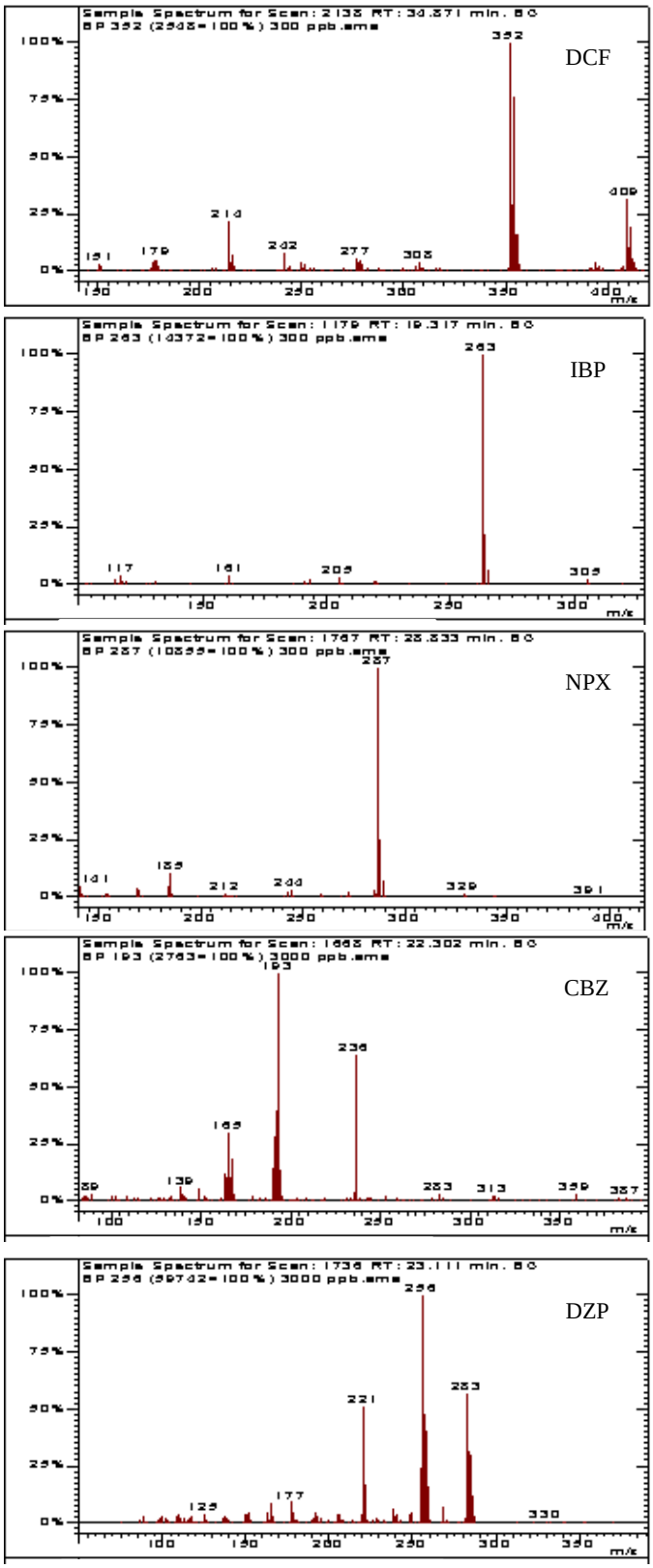


Figure 2-8 Mass spectrum of the pharmaceutical compounds analyzed

2.6. Analytical techniques

2.6.1. Reducing sugars

The concentration of glucose was analyzed with the dinitrosalicylic acid (DNS) method using D-glucose as a standard. An aliquot of 0.5 mL was withdrawn from the sample and placed in a glass tube, then 0.5 mL of DNS was added. The glass tubes were heated in water bath for 5 min. After a rapid cooling with cold water, 5 mL of distilled water were added to each tube. The optical density was measured at 540 nm and a calibration curve was used for the determination of the glucose concentration (Miller, 1959).

2.6.2. Total organic carbon concentration

Total organic carbon (TOC) concentration was measured in a Shimadzu analyzer (TOC 5000) provided with a NDIR detector for the evaluation of carbon dioxide (CO_2) produced during the combustion of the sample at 680°C in presence of platinum as catalyst.

2.6.3. Total soluble nitrogen content

The concentration of total nitrogen (NT) was determined using a Dohrmann nitrogen analyzer at 850°C with vanadium trichloride (VCl_3) as catalyst. The standard solution based in potassium nitrate in a concentration range of 0–20 mg/L. All the chemical compounds were purchased by Sigma-Aldrich.

2.6.4. pH determination

The pH value was measured using a electrode connected to a pH meter GLP 21 from CRISON (Barcelona, Spain). Before the measurement, a calibration of the electrode was carried out at room temperature using two buffer dissolutions at pH 4 and 7.

2.6.5. Peroxide concentration

Peroxide concentration was measured by a colorimetric method with Peroxide-Test strips (0.5-25 mg of H_2O_2 per liter) purchased from Merckoquant (Merck, Darmstadt, Germany).

2.6.6. Biomass determination

Biomass concentration was determined as dry weight with 0.45 µm pore-size filters previously dried until constant weight. The samples were filtered by vacuum and the filters were placed into an oven at 105°C for 2 h. The difference between the weight of the filter before and after drying determines the biomass concentration.

2.6.7. Microscope

Samples of approximately 10 mL were taken every 2-3 days from the bioreactor and then an aliquot was placed in a Petri plate in order to measure the diameter of the pellets. The morphology and size of the pellets were measured by using image analysis with a stereomicroscope (Stemi 2000-C, Zeiss, Germany) with a digital camera (Coolsnap, Roper Scientific Photometrics, Germany). The images obtained were analyzed with the Image Pro Plus software (Media Cybernetics, Inc, USA).

2.6.8. Determination of enzymatic activities

All enzyme activities were determined spectrophotometrically (Shimadzu UV-1603) at 30°C. Enzymatic activity of MnP and VP was measured by the oxidation of dimethoxyphenol (DMP) at 468 nm as described by Field et al. (1992). The reaction mixture contained 200 µL of sodium malonate at pH 4.5 (250mM), 50 µL of DMP (20 mM), 550 µL of distilled water, 50 µL of $MnSO_4 \cdot H_2O$ (20 mM), 50 µL of sample to analyze and 100 µL of H_2O_2 (4 mM). LiP activity was measured by oxidation of veratryl alcohol to veratraldehyde in presence of H_2O_2 at 310 nm of wavelength as described by Tien and Kirk, 1988. The reaction mixtures contained 2 mM of veratryl alcohol, 0.4 mM of H_2O_2 and 50 mM of tartaric acid. Laccase activity was determined by measuring the oxidation of 5mM ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) at 436nm in 100mM sodium acetate buffer, pH 5 ($\varepsilon 436$ = 29,300M^{-1} cm^{-1}) (Muñoz et al., 1997). All the enzymatic activities were expressed as international units (U), defined as the amount of enzyme per 1 µmol substrate/min.

Chapter 3

Removal of pharmaceutical compounds by three white-rot fungi in batch experiments (static, free pellets and immobilized cultures)*

Summary

The removal of eight pharmaceuticals (CTL, FLX, SMX, DCF, IBP, NPX, CBZ and DZP) was performed by three fungal strains (an anamorph strain of *Bjerkandera* sp. R1, *Bjerkandera adusta* and *Phanerochaete chrysosporium*). To evaluate the feasibility of pharmaceuticals degradation, the toxicity of these compounds on fungal growth was assessed. Once the threshold concentrations of the different compounds were determined, batch experiments using static, free pellets and immobilized cultures were carried out. Thereafter, a constrained set of pharmaceuticals and two fungal strains (the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium*) were selected for further experiments in batch stirred tank reactors (STRs). Finally, a fed-batch assay with three compounds: DCF, IBP and NPX, was carried out with the objective of establishing the approximate time required for their degradation and the hydraulic residence time (HRT) to perform future experiments in continuous reactors. All the fungi were able to grow at pharmaceuticals concentrations below 2 mg/L. All the strains removed DCF, IBP and NPX under the different types of culture. The time-course degradation curve showed that the anamorph fungus totally removed IBP in less than 4 h, while DCF and NPX were eliminated up to 85% after 24 h. Moreover, this fungal strain attained to degrade the three compounds, even when daily pulses were added. Concerning to recalcitrant compounds such as CBZ and DZP, the former was totally removed in static and free pellets cultures, whereas partial degradations were observed in immobilized cultures (28%) and in the STR (55%); for the

latter, only partial removals up to 57% were observed during all the assays. The results obtained will be useful for future experiments in bioreactors operating as fed-batch and continuous, to investigate if the fungi are able to degrade these drugs and also to study the effect of the nutrients addition on degradation of pollutants.

* Part of this chapter has been published as:

Rodarte-Morales AI, Moreira MT, Feijoo G, Lema JM. (2010). **Evaluation of two fungal strains for the degradation of pharmaceutical and personal care products (PPCPs).** Chemical Engineering Transactions 20:31-36 (Eds. Bardone E, Viglia A) ISBN 978-88-95608; ISSN 1974-9791.

Rodarte-Morales AI, Feijoo G, Moreira MT, Lema JM. (2011). **Degradation of selected pharmaceutical and personal care products (PPCPs) by white-rot fungi.** World Journal of Microbiology and Biotechnology 27:1839-1846.

Outline

3.1. Introduction

The use of ligninolytic fungi for removal of recalcitrant compounds has generated interest due to their high oxidative potential associated to the secretion of oxidative enzymes (Field et al. 1992; Wesenberg et al., 2003; Ikehata et al. 2006; Ding et al., 2008). In recent years, several studies have been published related to the removal of pharmaceutical compounds by white-rot fungi (WRF) and their ligninolytic enzymes (Marco-Urrea et al., 2009; Hata et al., 2010a; Lloret et al. 2010; Marco-Urrea et al., 2010a; Rodríguez-Rodríguez et al., 2010b; Zhang and Geiβen, 2010). There are several environmental factors that may affect or promote the production of enzymes such as formulation of the culture medium, culture method, agitation, air or oxygen supply, among others (Moreira et al., 1998, Xiong et al., 2007). Therefore, the physiology and morphology of the fungus are factors of major importance, which will depend on the incubation conditions used.

This type of microorganisms typically grow as a biofilm in static cultures, as pellets in shaken flasks or immobilized over a wide range of supports (Moreira et al. 1997a; Rogalski et al., 2006; Xiong et al., 2008, Huang et al., 2010). The fungal biofilm is formed in those cultures with no agitation, where agar plugs with active fungus are used as inoculum and the fungus tends to grow forming a biofilm on the surface of the liquid medium or the solid support. Fernsbach flasks are typically used for this type of culture as they provide a large surface area that favors passive aeration for fungus to grow. Potential disadvantages of this type of culture are related to the fact that lag phase of microbial growth may be longer and consumption of nutrients slower. Some reports indicate that agitation and/or aeration favor the ligninolytic system of these microorganisms (Rogalski et al., 2006).

Pellet formation is considered as a natural immobilization process of the fungal mycelium which occurs by aggregation of free spores to spore clusters with the subsequent germination and formation of small aggregates surrounded by a loose hyphal network; then, aggregation of the primary fungal cores to the final full-size pellets. The main disadvantages are related to the problems encountered by the fact that fungal pellets tend to grow uncontrollably, forming hyphal branches and aggregates of pellets, with their subsequent breakdown into fragments that may cause clogging of lines and probes, preferential paths and increase of viscosity.

The use of porous support enables physical entrapment into the reticulated matrix and provides a large surface area that improves oxygen and nutrient diffusion (Moreira et al., 1998). Moreover, the fungal growth is restricted to the support, which avoids problems derived from the aggregation of mycelia. Certain compounds to be degraded have to assess the potential adsorption of these compounds onto the support surface,

which may render into limited biodegradation and the need of an extraction stage to desorb them.

The aim of this chapter was to study the potential ability of three WRF strains: an anamorph of *Bjerkandera* sp. R1, *Bjerkandera adusta* and *Phanerochaete chrysosporium* to degrade a wide range of pharmaceutical compounds belonging to different therapeutic groups: anti-depressants (citalopram and fluoxetine), antibiotics (sulfamethoxazole), anti-inflammatory drugs (diclofenac, ibuprofen and naproxen), anti-epileptics (carbamazepine) and tranquilizers (diazepam). These compounds have been selected based on their different physicochemical properties that influence their degradability. The experiments were carried out in static, free pellets and immobilized cultures using the three fungal strains selected. An additional objective of the batch assays was the quantification of the potential adsorption of the pharmaceutical compounds onto the biomass or the immobilization support.

The screening procedure will allow selecting those fungal strains with the highest ability to degrade the pharmaceuticals in a batch stirred tank reactor (STR). Finally, a fed-batch assay with three compounds: DCF, IBP and NPX, will be carried out with the objective of determining the approximate time required for their degradation and accordingly, the hydraulic residence time (HRT) to perform future experiments in continuous reactors.

3.2. Materials and methods

3.2.1. Microorganisms

The white-rot fungi used in this chapter were the anamorph strain of *Bjerkandera* sp. R1, *Bjerkandera adusta* and *Phanerochaete chrysosporium*. Different types of cultures were considered: growth in plates, static cultures, free pellets and immobilized mycelia in polyurethane foam. A description of each of these cultures is included in chapter 2.

3.2.2. Pharmaceutical compounds and chemicals

The pharmaceutical compounds used in this chapter were CTL, FLX, SMX, DCF, IBP, NPX, CBZ and DZP in two concentrated stock solution prepared as indicated in chapter 2. The decision of preparing two separated stocks of pharmaceutical compounds depends on the analytical methods considered to determine its residual concentration (See chapter 2).

3.2.3. Mycelium growth inhibition at different pharmaceuticals concentrations

Fungal tolerance to different concentrations of pharmaceutical compounds was studied in agar plates according to the protocol defined in chapter 2.

3.2.4. Removal of pharmaceutical compounds in batch experiments (static, pellets and immobilized cultures)

Static, free pellets and immobilized fungi cultures were prepared individually for the three fungal strains as described in chapter 2. Pharmaceutical compounds were added from two mixtures to provide the desired concentration (~1 mg/L) immediately after inoculation of the static cultures or once pellets were formed or fungus was immobilized (at day 4) in shaken flasks. Experiments were performed in triplicate. In addition, abiotic controls were used to verify any possible adsorption or evaporation. All the cultures were incubated for two weeks and samples from all the assays were taken after 2 h and then after 4, 7 and 14 days to carry out the extraction and analytical determination of the compounds.

3.2.5. Removal of pharmaceutical compounds in a stirred tank reactor

Batch experiments were performed in a 0.75 L stirred tank reactor (STRs) fermenters (Figures 2-1 and 2-2 in chapter 2). Three vessels were filled with 0.7 L of modified Kirk medium (pH 4.5) (Tien and Kirk, 1988) and a single pulse of a mixture of DCF, IBP, NPX, CBZ and DZP (~ 1 mg/L) was added at the beginning of the assay to each vessel. One vessel was used as an abiotic control (culture medium) meanwhile other two vessels were inoculated with pellets previously formed corresponding to three flasks with anamorph fungus and *P. chrysosporium*, respectively. Stirring was fixed at 150 rpm and the temperature was set at 30°C throughout the assay. Samples of 10 mL were taken daily in order to follow the profile fermentation and to estimate the residual concentration of the pharmaceuticals. The dissolved oxygen content was measured to each withdrawn sample with an electrode WTW Oxi 3401 (Weilheim, Germany).

3.2.6. Extraction of pharmaceuticals and determination of residual concentrations

The extraction of compounds with acetonitrile was carried out and the determination of the residual concentrations of pharmaceuticals was carried out by HPLC-DAD or GCMS depending on the type of compound, as described in chapter 2.

3.2.7. Adsorption of pharmaceuticals in the different fungal cultures

Assays were carried out to discard any possible adsorption or volatilization process of the pharmaceutical compounds. A detailed description of the procedure applied for each type of fungal culture is included in chapter 2.

3.2.8. Time-course degradation of anti-inflammatory drugs by pellets of anamorph of *Bjerkandera* sp. R1

A time-course degradation experiment was carried out in pellet cultures as indicated in chapter 2. Samples were withdrawn just after the addition of anti-inflammatories and then after 2, 6, 10 and 24 h in order to follow the degradation of the three compounds and to determine their residual concentration by GC-MS as described in chapter 2.

3.2.9. Degradation of anti-inflammatory drugs by pellets of the anamorph of *Bjerkandera* sp. R1. in a fed-batch experiment

A fed-batch experiment was carried out in pellet cultures as indicated in chapter 2. Once the pellets were formed, a daily pulse of the three anti-inflammatory compounds: DCF, IBP and NPX was added in the period between days 4 and 9. Each day a sample was taken to determine the residual concentration of pharmaceuticals by GC-MS.

3.2.10. Analytical techniques

A description of the analytical techniques used in this study was previously detailed in the Materials and methods chapter.

3.3. Results

3.3.1. Mycelium growth inhibition at different pharmaceuticals concentrations

Initial experiments were performed to determine the threshold concentration of different pharmaceutical compounds, from which the growth of the three fungi was affected and inhibitory effects were evident. In the experiments with CTL, FLX and SMX, no significant differences were observed between the control and experimental plates at concentrations below 2 mg/L (Figure 3-1, left column). However, the mixture of five pharmaceuticals (DCF, IBP, NPX, CBZ and DZP) at 2 mg/L had a significant effect on the growth of the anamorph of *Bjerkandera* sp. R1 and *B. adusta* (Figure 3-1, right column) which showed slower extension. Once checked that the concentration of 1 mg/L of the considered pharmaceuticals did not significantly affect fungal growth, the degradation experiments were performed in flasks, considering this as the concentration to be assayed.

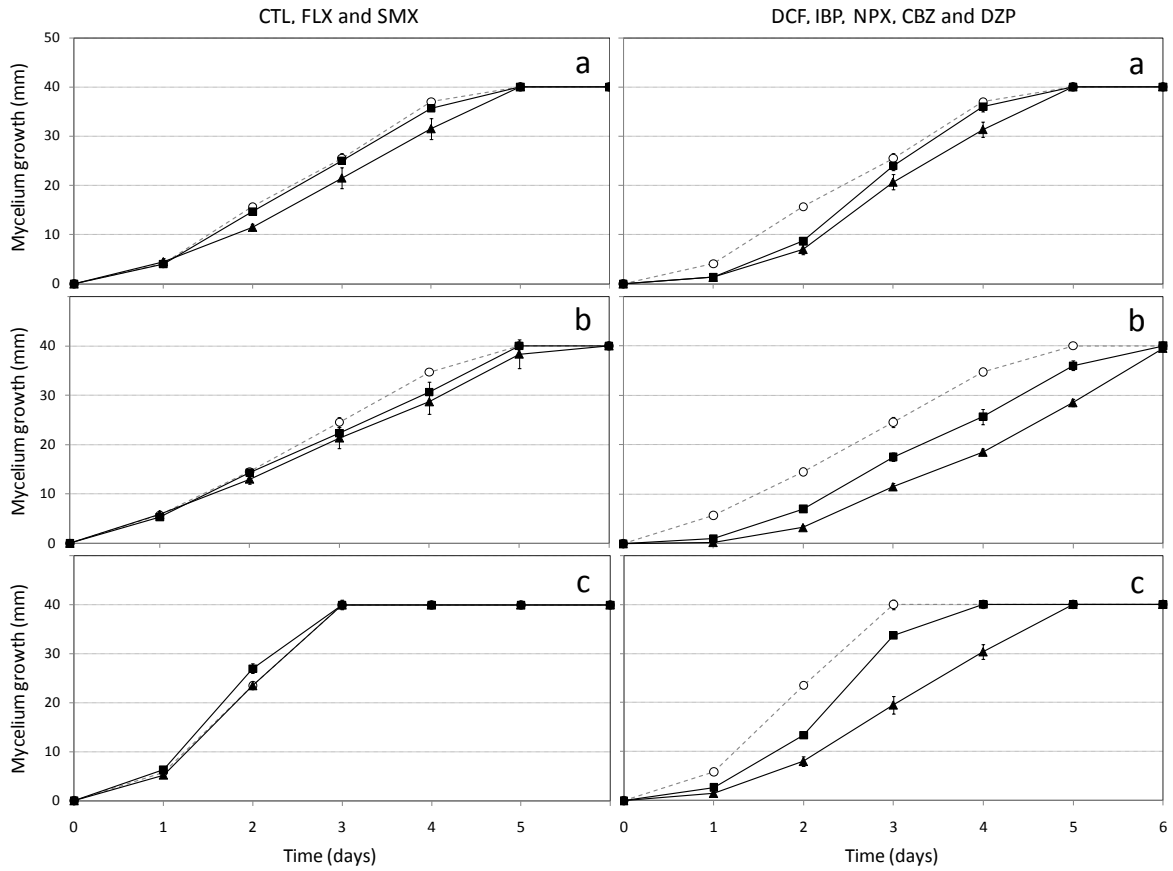


Figure 3-1. Inhibition of the fungal growth of the anamorph of *Bjerkandera* sp. R1 (a)*, B. adusta* (b) and *P. chrysosporium* (c) at different concentrations of two pharmaceuticals mixtures. Symbols: control 0 mg/L (○), 1 mg/L (■), 2 mg/L (▲).

3.3.2. Removal of pharmaceutical compounds in static cultures

The evolution of the fermentation obtained during the static experiments is shown in Figure 3-2. The profile of *B. adusta* is not shown because the results were similar to those obtained for the anamorph of *Bjerkandera* sp. R1, except for the VP activity which it was similar to the MnP activity detected after 2 weeks with *P. chrysosporium* (~20 U/L). Contrarily, after 11 days of static incubation, VP activities up to 900 U/L were detected for the anamorph of *Bjerkandera* sp. R1. LiP nor Laccase activities were detected in any of the analyzed samples. Glucose consumption rate by the anamorph of *Bjerkandera* sp. R1 remained constant during the first week of the experiment (0.24 g/L·d), increasing during the second week (1.54 g/L·d). In the case of *P. chrysosporium*, the consumption rate was similar to the one obtained by the anamorph (0.29 g/L·d); however, at day 5 a slight increase in this consumption rate was observed (0.75 g/L·d). The pH values were detected in the range of 4.5 – 5.6 and the peroxide content was in the range of 0.5 – 2.0 mg H_2O_2/L for the three fungi throughout the experiment (data not shown). The content of biomass

was determined by dry weight and after two weeks, the biomass obtained from the anamorph of *Bjerkandera* sp. R1 was 2.8 g/L, for *B. adusta* was 2.1 g/L and finally, for *P. chrysosporium* was 1.8 g/L.

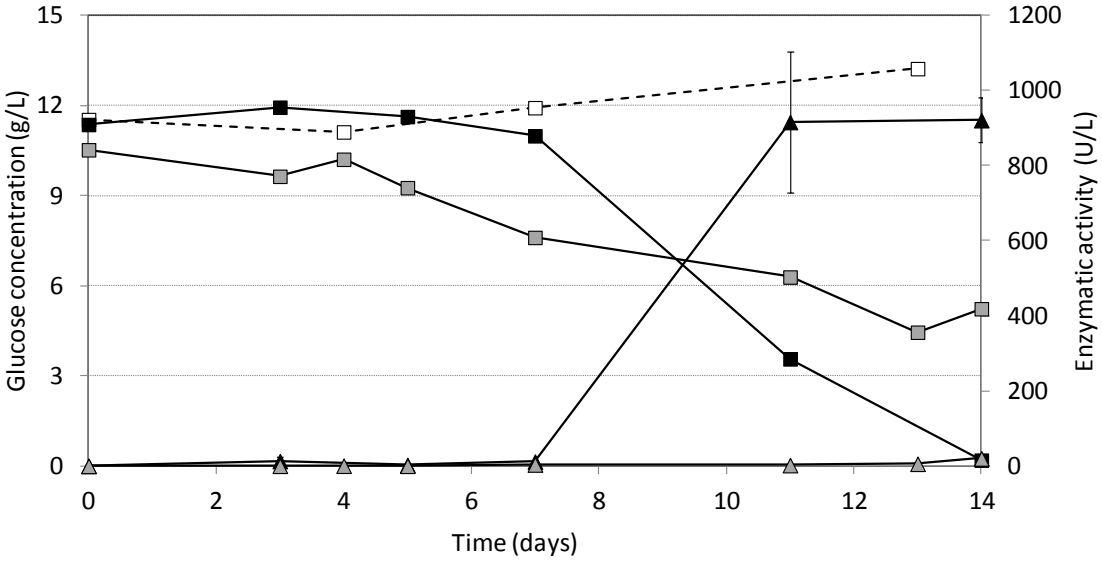


Figure 3-2. Profile fermentation during static experiments. Symbols: glucose control (□), glucose - *Bj.* sp. R1 (■), VP activity - *Bj.* sp. R1 (▲), glucose - *P. chrysosporium* (□), MnP activity - *P. chrysosporium* (△).

In Figure 3-3 the morphology of the fungal cultures after 14 days in static incubation is shown. It can be observed that the anamorph fungus showed higher growth when compared with the other strains. Also, another difference was that the anamorph fungus and *B. adusta* presented brown mycelia, while *P. chrysosporium* showed white mycelia.

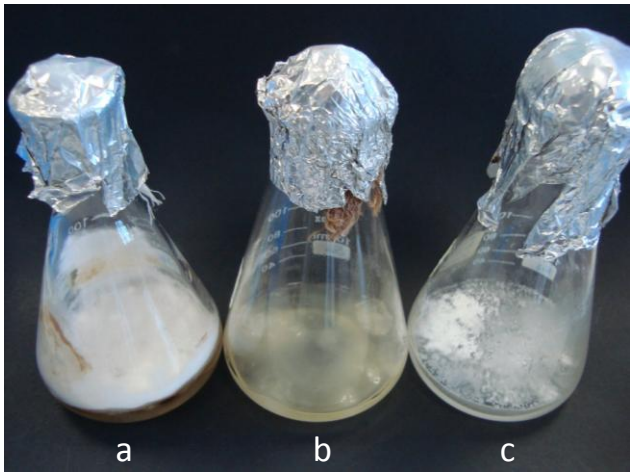


Figure 3-3. Appearance of anamorph of *Bjerkandera* sp. R1 (a), *B. adusta* (b) and *P. chrysosporium* (c) after 14 days of incubation in static cultures.

During the degradation experiments, abiotic controls were used to detect any possible adsorption, volatiliztion and/or photodegradation of the compounds. It is important to note that the abiotic controls for the three fungi were the same and the recovery percentage after 14 days is shown in Table 3-1.

Table 3-1. Recovery of pharmaceuticals from the abiotic control after 14 days in static cultures

Compound	Residual percentage (%)	Compound	Residual percentage (%)
CTL	95	IBP	85
FLX	87	NPX	93
SMX	76	CBZ	96
DCF	85	DZP	69

The degradation extent of the pharmaceutical compounds, the results obtained by the anamorph of *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium* are shown in Figure 3-4. It is important to note that the three WRF were able to remove all the compounds, except FLX and DZP, where partial eliminations were achieved. In the case of the anamorph fungus (Figure 3-4a), compounds such as CTL, SMX and CBZ were partially removed at day 7 (80%, 53% and 91%). Anti-inflammatory compounds such as DCF and IBP were eliminated easily at day 4; meanwhile a high concentration of NPX was detected at this time (57%) reaching total removal at the end of the experiment. Finally, recalcitrant compounds such as FLX and DZP were partially removed at the end of the experiment (46% and 57%, respectively).

Similar results were obtained by *B. adusta* when statically cultured (Figure 3-4b) where FLX and DZP were partially degraded (23% and 39%, respectively) while the other compounds were totally eliminated. Higher percentages of FLX were found at day 7 and 14 (88% and 77%, respectively). After one week of experiment, compounds such as CTL and CBZ were partially degraded (44% and 31%, respectively); however, after 14 days a total elimination was observed. The three anti-inflammatory drugs (DCF, IBP and NPX) and the antibiotic (SMX) were removed even after 7 days (up to 88%), achieving a total elimination at the end of the experiment.

The results obtained with the fungus *P. chrysosporium* (Figure 3-4c) indicate that this fungus was not able to remove totally FLX and DZP, since only partial eliminations were reached (21% for the former and 52% for the latter compound). Anti-inflammatory drugs were highly degraded at day 4 (70% up to 97%). On the contrary, although a total degradation of CTL, SMX and CBZ was achieved at the end of the experiment, more than 75% of the initial concentration of these compounds was detected at day 7.

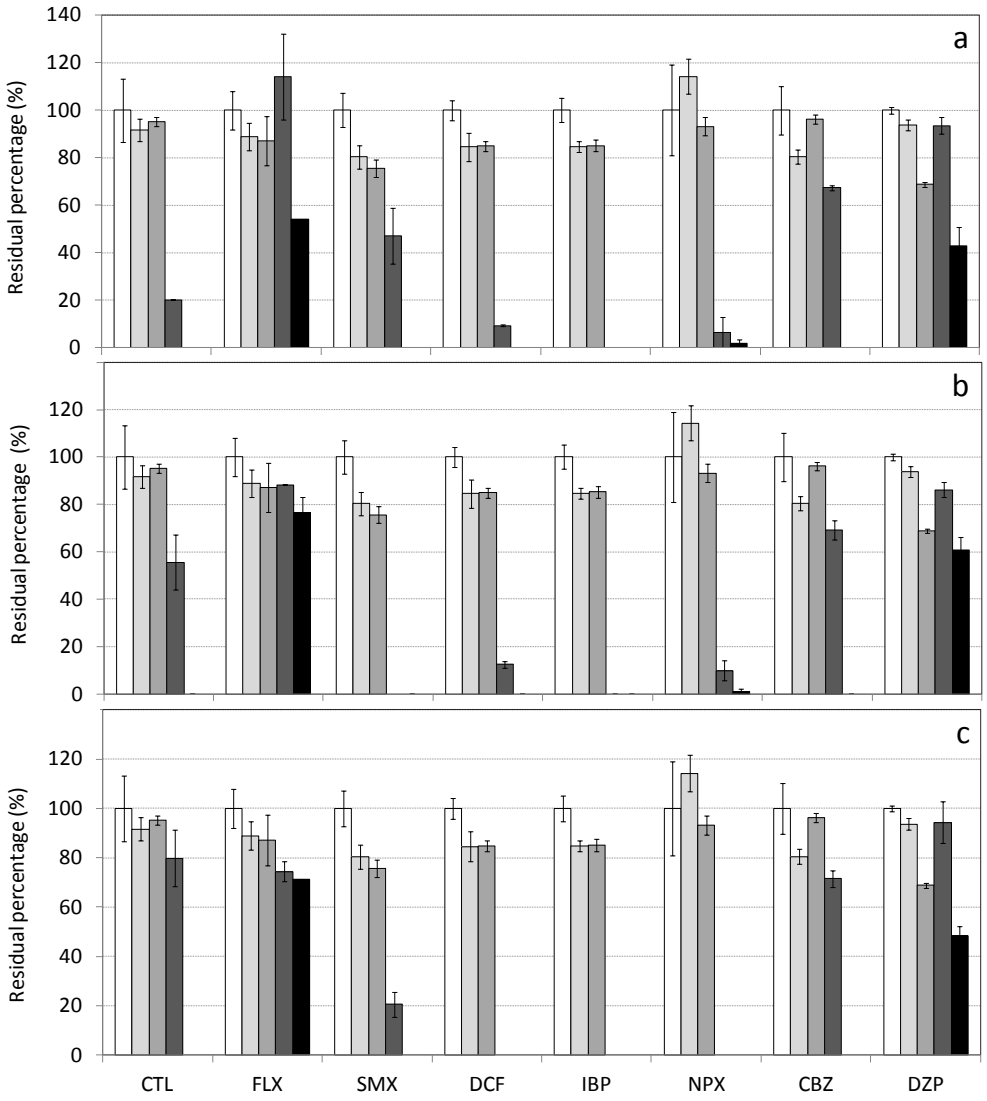


Figure 3-4. Degradation of pharmaceuticals by anamorph of *Bjerkandera* sp. R1 (a), *B. adusta (*b) and *P. chrysosporium* (c) during static cultures. Symbols: abiotic control (day 0) (□), abiotic control (day 7) (□), abiotic control (day 14) (▩), fungal culture (day 7) (■), fungal culture (day 14) (■).

Additionally, adsorption experiments were carried out during the static cultures (Table 3-2). The results showed similar range of concentrations in the flasks with inactivated biomass and in the abiotic controls (referred as 100%). Also it can be observed partial degradations with active biomass of anamorph of *Bjerkandera* sp. R1 (68% of SMX), in the case of *B. adusta* (19% of SMX) and *P. chrysosporium* (9% - 65% for DCF, IBP, NPX and CBZ). Thus, adsorption of the pharmaceutical compounds on the fungal mycelium was excluded.

Table 3-2 Recovery percentage of pharmaceuticals during adsorption experiments

	Anamorph of *Bjerkandera* sp. R1		*B. adusta*		*P. chrysosporium*	
	Active biomass (residual %)	Inactivated biomass (residual %)	Active biomass (residual %)	Inactivated biomass (residual %)	Active biomass (residual %)	Inactivated biomass (residual %)
SMX	32	>100	81	>100	>100	88
DCF	>100	>100	>100	>100	35	100
IBP	>100	>100	>100	>100	89	96
NPX	>100	>100	>100	>100	59	99
CBZ	>100	>100	>100	>100	91	>100
DZP	>100	>100	>100	>100	>100	>100

3.3.3. Removal of pharmaceutical compounds in batch free pellets cultures

The profile fermentation corresponding to the free pellet cultures is shown in Figure 3-5. During this experiment only two fungi were used (the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium*) since during static cultures the fungal strain *B. adusta* showed the lowest degradation percentages. Only DCF, IBP, NPX, CBZ and DZP were considered in this experiment.

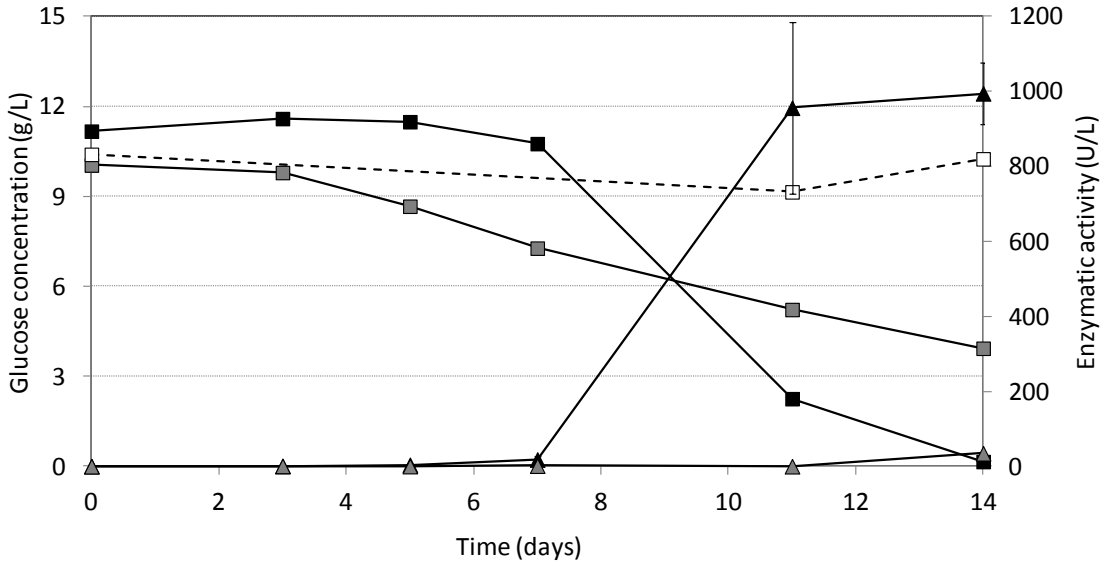


Figure 3-5. Profile fermentation during agitated experiments. Symbols: glucose - control (□), glucose - *Bj.* sp. R1 (■), VP activity - *Bj.* sp. R1 (▲), glucose - *P. chrysosporium* (□), MnP activity - *P. chrysosporium* (△).

The enzymatic activity was measured throughout the experiment and the results showed only VP activities up to 900 U/L with the anamorph fungus while in the case of *P. chrysosporium* only 35 U/L of MnP were detected after 14 days of experiment. Glucose

consumption during the first days of the experiment was similar for both fungi (0.05 – 0.08 g/L·d). In the case of the anamorph of *Bjerkandera* sp. R1, an increase up to 2.13 g/L·d at day 11 was observed; meanwhile, this rate was maintained around 0.55 g/L·d with *P. chrysosporium* throughout the rest of the experiment. The pH values were maintained between 4.3 and 5.7 for both fungi and the peroxide content was similar to that detected during the static experiments (< 2 mg H_2O_2/L) (data not shown).The content of biomass was measured after 14 days obtaining 2.25 g/L for anamorph of *Bjerkandera* sp. R1 and 1.46 g/L of biomass for *P. chrysosporium*. In Figure 3-6 it can be observed that the morphology of the pellets of the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium* was similar, except by the color.

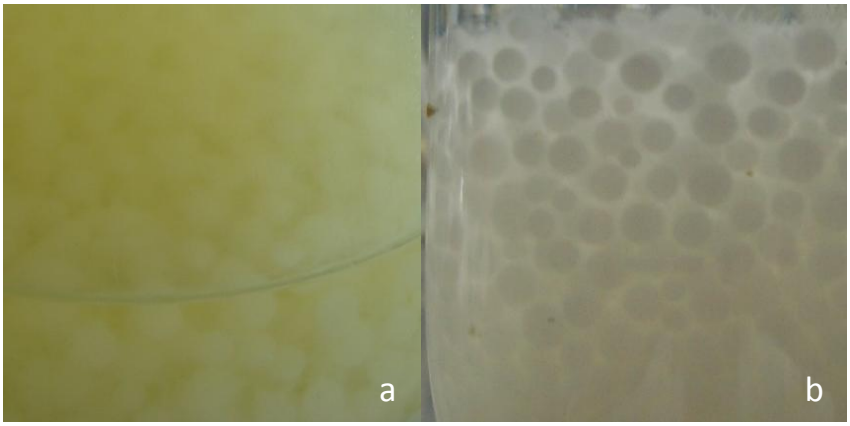


Figure 3-6. Appearance of the pellets of anamorph of *Bjerkandera* sp. R1 (a) and *P. chrysosporium* (b) at day 5 before the pharmaceuticals addition.

During the agitated experiments, abiotic controls were used to discard any possible adsorption, volatilization and/or photodegradation of the compounds. It is important to note that the abiotic controls for the three fungi were the same and the recovery percentage after 14 days is shown in Table 3-3.

Table 3-3. Recovery of pharmaceuticals from the abiotic controls during static experiments

	Residual percentage
Compounds	Abiotic control (day 14th)
DCF	63
IBP	80
NPX	78
CBZ	97
DZP	>100

In Figure 3-7a the results obtained by pellets of the anamorph of *Bjerkandera* sp. R1 are shown. This fungus is able to degrade all the compounds after 7 days of incubation,

some of them even after day 4 (data not shown), such as the anti-inflammatory drugs (DCF, IBP and NPX). In the case of CBZ, a partial degradation of 33% was achieved after only 4 days reaching a total elimination at day 7. Diazepam was partially degraded (27%) at the end of the experiment.

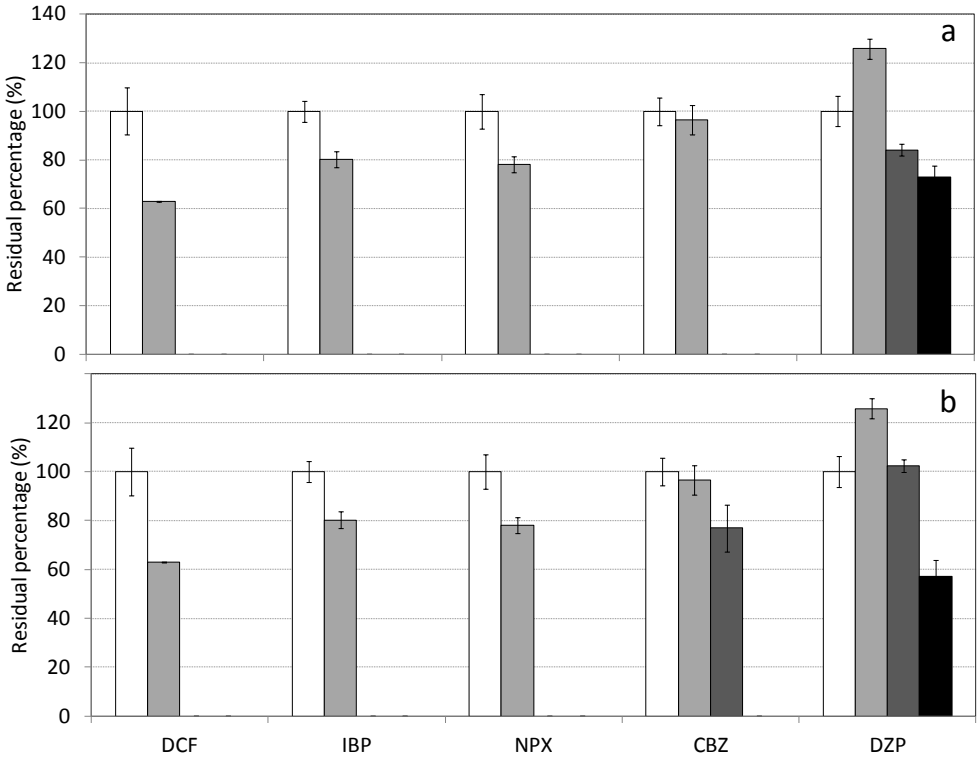


Figure 3-7. Degradation of pharmaceuticals by pellets of anamorph of *Bjerkandera* sp. R1 (a) and *P. chrysosporium* (b) during agitated cultures. Symbols: control (day 0) (□), control (day 14) (▩), fungal culture (day 7) (■), fungal culture (day 14) (■).

According to the degradation of pharmaceutical compounds by pellets of *P. chrysosporium*, a similar behavior to that of the anamorph fungus was observed (Figure 3-7b). A total removal of the anti-inflammatory drugs was observed at day 4; however, CBZ maintained the initial concentration at this time of the experiment. This compound was partially eliminated (33%) at day 7 and a total removal after 14 days was observed. In the case of DZP, this compound was hardly degraded during the first week of experiment (<2%), although an elimination of the 43% of the initial concentration was observed after two weeks of experiment.

The determination of potential adsorption on the flask glass surface and on the fungal biomass was carried out in samples taken before and after the extraction with acetonitrile. The results showed that IBP and NPX were not detected in the samples with

active biomass before the extraction, suggesting total elimination; however, these compounds were detected in the samples after extraction (66% and 4%, respectively). Also, higher concentrations were detected in the abiotic controls if an extraction was carried out, except in the case of DCF (Table 3-4).

Table 3-4. Recovery percentage of anti-inflammatories during agitated experiments.

	Before extraction		After extraction	
	Abiotic control	Active biomass	Abiotic control	Active biomass
DCF	52	9	56	n.d.
IBP	95	n.d.	>100	66
NPX	91	n.d.	99	4

(n.d.) not detected

3.3.4. Removal of pharmaceutical compounds in batch immobilized cultures

In Figure 3-8, the fermentation profiles corresponding to the immobilized cultures of the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium* are shown. The time course of the fermentation was carried out until the 12th day in order to maintain a high volume of the culture medium, since the flasks containing polyurethane foam presented lower volume of free culture medium due to its absorption by the foam.

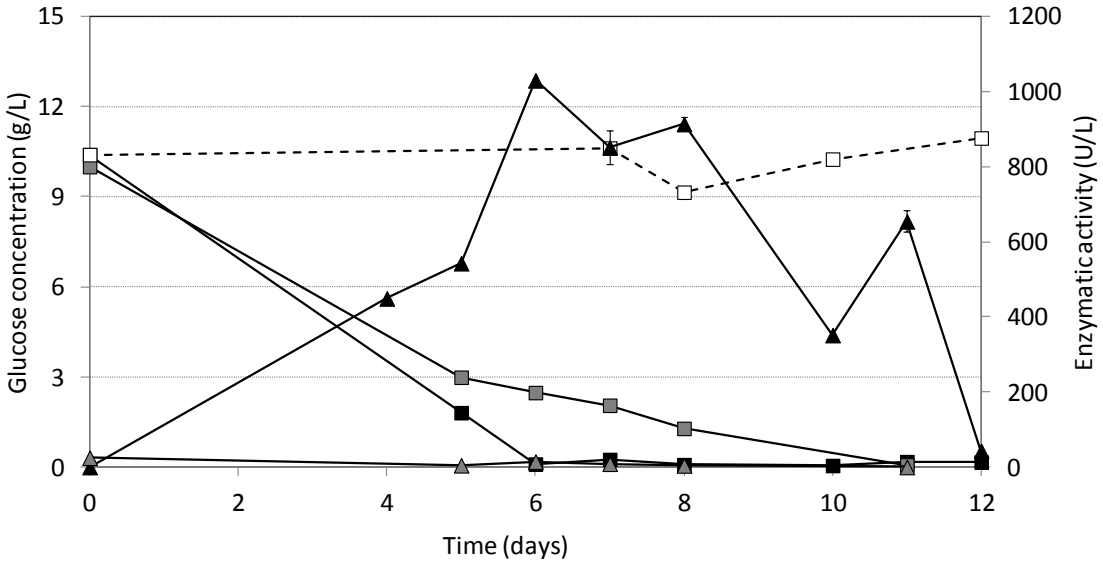


Figure 3-8. Profile fermentation during immobilized experiments. Symbols: glucose - control (□), glucose - *Bj.* sp. R1 (■), VP activity - *Bj.* sp. R1 (▲), glucose - *P. chrysosporium* (▣), MnP activity - *P. chrysosporium* (△).

Concerning the cultures with the anamorph fungus, enzymatic activities of VP where maintained throughout the experiment in the range of 351 – 1029 U/L, reaching the maximum value at day 6. In the case of *P. chrysosporium* low activities of MnP were

detected (24 U/L). Enzymatic activities of LiP or Lac were not detected. The glucose consumption rate for both fungi detected at day 5 was in the range of 1.4 – 1.7 g/L·d, decreasing to values of 0.2 g/L·d for the anamorph fungus at day 6, while these rates were near to zero for the rest of the experiment. Contrarily, in the case of *P. chrysosporium* a constant glucose consumption rate were detected between days 5 and 11 (0.53 g/L·d). The pH was detected in a range between 4.3 and 5.6 for both fungi and the peroxide concentration was similar to static and pellet experiments. A large biomass growth was evidenced during the immobilized experiments (Figure 3-9). The determination of biomass content was carried out after 14 days obtaining 2.66 g/L for the anamorph fungus and 1.54 g/L for *P. chrysosporium*.

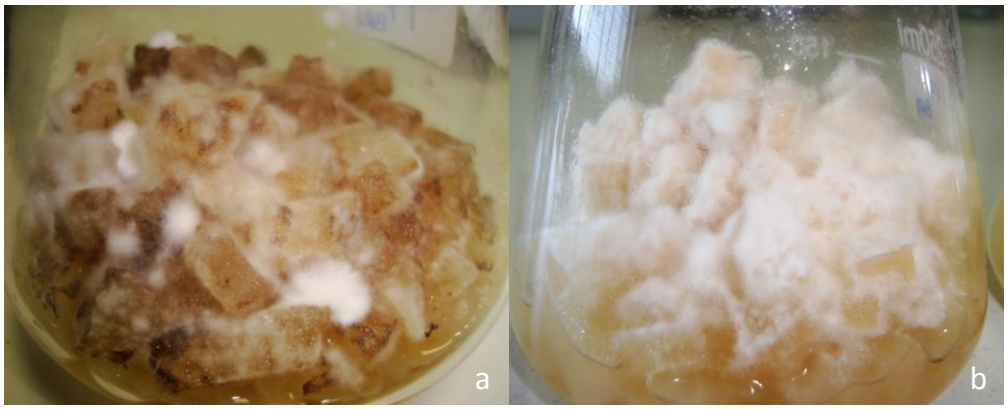


Figure 3-9. Appearance of anamorph of *Bjerkandera* sp. R1 (a) and *P. chrysosporium* (b) after 14 days of incubation in immobilized cultures.

During the experiments, abiotic controls were used to discard any possible adsorption, volatilization and/or photodegradation of the compounds. It is important to note that the abiotic controls for the two fungi were the same and the recovery percentage after 14 days is shown in Table 3-5. Contrarily to static and agitated cultures, higher recovery percentages for all the compounds were detected after 14 days of experiment, except in the case of CBZ, where a recovery of 80% was achieved.

Table 3-5 Recovery of pharmaceuticals from abiotic controls

	Residual percentage		
Compounds	Abiotic control (day 14th)	Compounds	Abiotic control (day 14th)
DCF	>100	DZP	>100
IBP	>100	CBZ	80
NPX	>100		

The immobilized anamorph of *Bjerkandera* sp. R1 was able to remove up to 93% of the initial concentration of IBP and NPX (Figure 3-10a) while only partial elimination of DCF was observed (77%). In the case of CBZ less than 18% of degradation was achieved; meanwhile DZP was not degraded. *P. chrysosporium* was able to eliminate completely DCF and IBP even before 7 days of incubation, although only 50% of elimination was achieved for NPX (Figure 3-10b). In the case of CBZ and DZP, this microorganism showed higher percentages of removal than the anamorph fungus (28% and 21%, respectively).

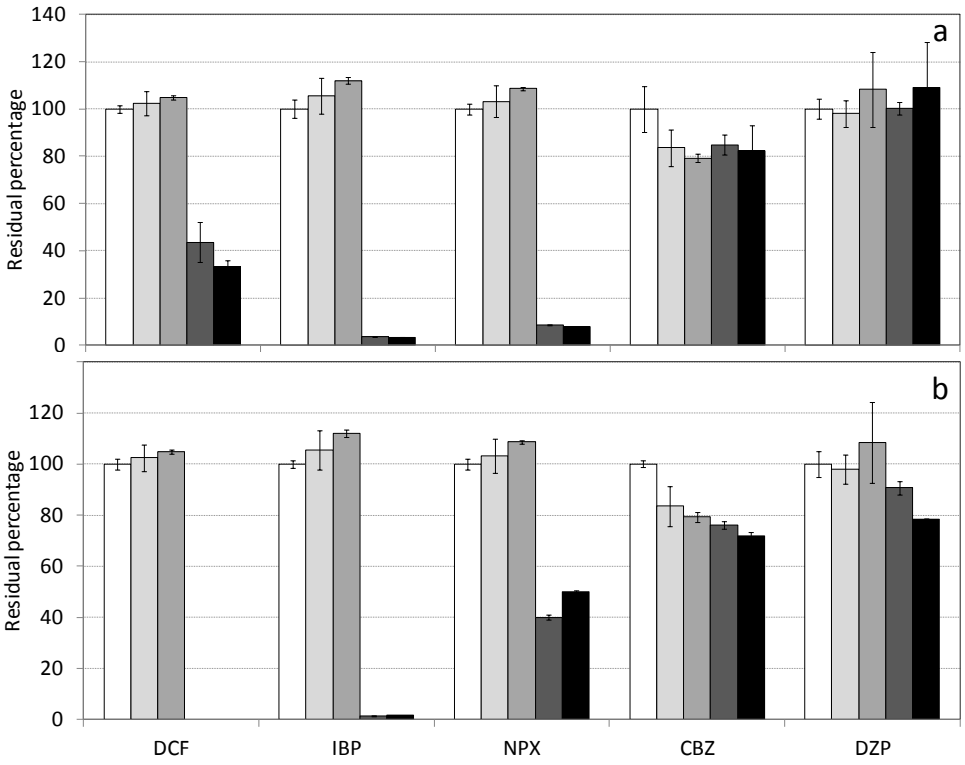


Figure 3-10. Degradation of pharmaceutical compounds by anamorph of *Bjerkandera* sp. R1 (a) and *P. chrysosporium* (b) immobilized in polyurethane foam. Symbols: control (day 0) (□), control (day 7) (□), control (day 14) (■), fungal culture (day 7) (■), fungal culture (day 14) (■).

Adsorption experiments were carried out from samples taken before and after the extraction with acetonitrile. In Table 3-6, the results showed that DCF, IBP and NPX were not detected in the abiotic control either in active biomass before extraction; although after extraction they were detected up to 100% in the case of the abiotic controls and variable percentages in the case of the fungal samples (3% - 110%). CBZ and DZP were detected in high percentages in all the cases.

Table 3-6 Recovery percentage of pharmaceuticals during immobilized experiments.

	Before extraction		After extraction	
	Abiotic control	Active biomass	Abiotic control	Active biomass
DCF	n.d.	n.d.	>100	33
IBP	88	n.d.	>100	3
NPX	87	n.d.	>100	8
CBZ	80	99	79	82
DZP	92	95	>100	>100

(n.d.) not detected

3.3.5. Removal of pharmaceutical compounds in a stirred tank reactor

Treatment of five different pharmaceuticals was carried out during 13 days in a STR using pellets of the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium*. In Figures 3-11 and 3-12 the fermentation profiles obtained during the experiment with both fungal strains are shown. It is important to highlight that the feeding medium used was different for each strain, since it was prepared according to the conditions to maximize the activation of the ligninolytic system of each fungus (see chapter 2). Regarding the anamorph fungus, complete glucose depletion after only 4 days can be observed in Figure 3-11. A single peak of VP activity (17 U/L) was detected at day 9. The pH was not controlled during the experiment and it was maintained in a range of 4.4 - 5.8.

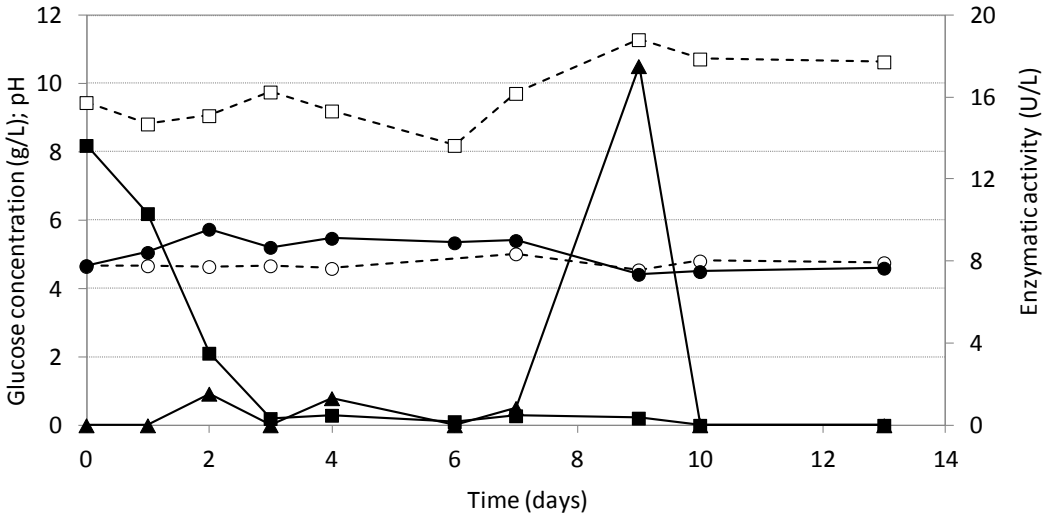


Figure 3-11. Profile fermentation of anamorph of *Bjerkandera* sp. R1 during batch assays in a STR. Symbols: glucose – abiotic control (□), glucose – *Bj.* sp. R1 (■), pH – abiotic control (○), pH – *Bj.* sp. R1 (●), VP activity – *Bj.* sp. R1 (▲).

In Figure 3-12 it can be observed the fermentation profile obtained by pellets of *P. chrysosporium* in a STR. The glucose consumption by these pellets was slower than the fungal pellets of the anamorph, since *P. chrysosporium* consumed only 4.4 g of glucose

throughout the assay. Despite this, a higher enzymatic activity of MnP (32 U/L) was detected after 11 days of assay. As in the case of the anamorph fungus, pH was not controlled during the experiment and it was maintained in a range between 3.6 and 5.2.

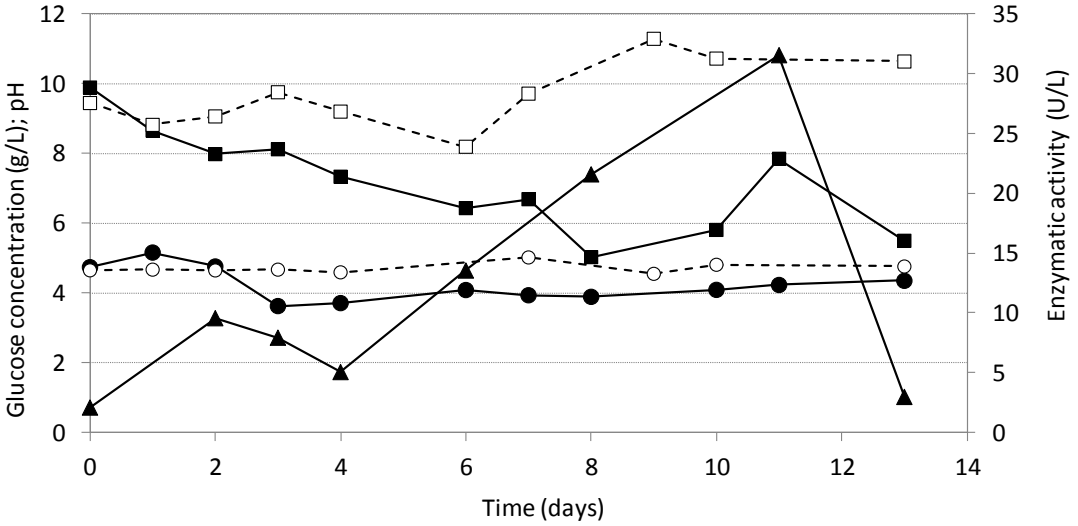


Figure 3-12. Profile fermentation of *P. chrysosporium* during batch assays in a STR. Symbols: glucose – abiotic control (□), glucose - *P. chrysosporium* (■), pH – abiotic control (○); pH – *P. chrysosporium* (●); MnP activity – *P. chrysosporium* (▲).

Concerning to the content of dissolved oxygen in the reactor, the gas flow was maintained at 1 L/min throughout the assay. In Figure 3-13 it can be observed that the oxygen in the reactor with pellets from both strains decreases from 7.8 mg/L until 1.6 mg/L at day 4, maintaining the dissolved oxygen below this concentration until the end of the assay.

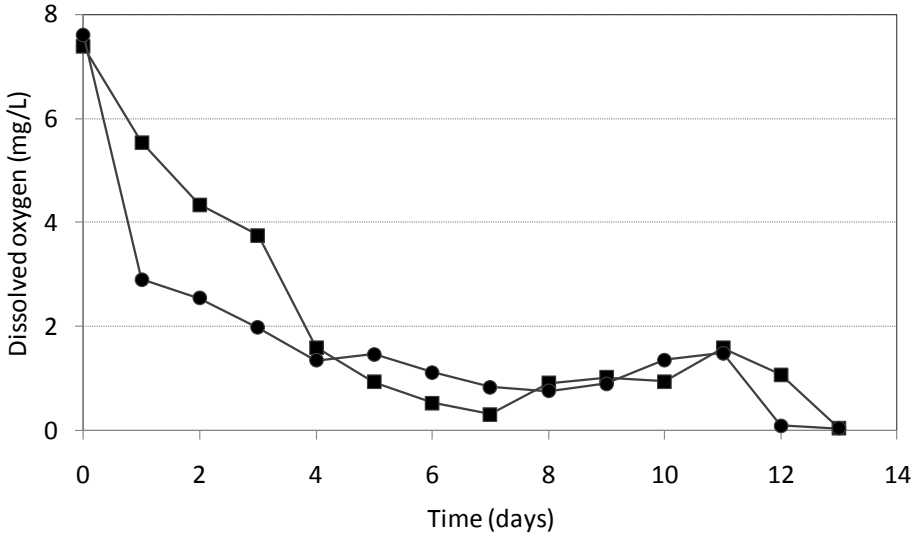


Figure 3-13. Dissolved oxygen concentration during batch assays in a STR. Symbols: *Bj.* sp. R1 (■); *P. chrysosporium* (●).

Figure 3-14 shows a picture of both reactors after 10 days and it can be observed that the pellets from both fungal strains showed a similar size and present hyphal branches; despite this, there were no clogging problems throughout the assay and no growth was observed on the sensors or probes. The estimated concentration of biomass at the end of the assay was of 3.1 g/L for the anamorph of *Bjerkandera* while for *P. chrysosporium* only 1.9 g/L were measured.

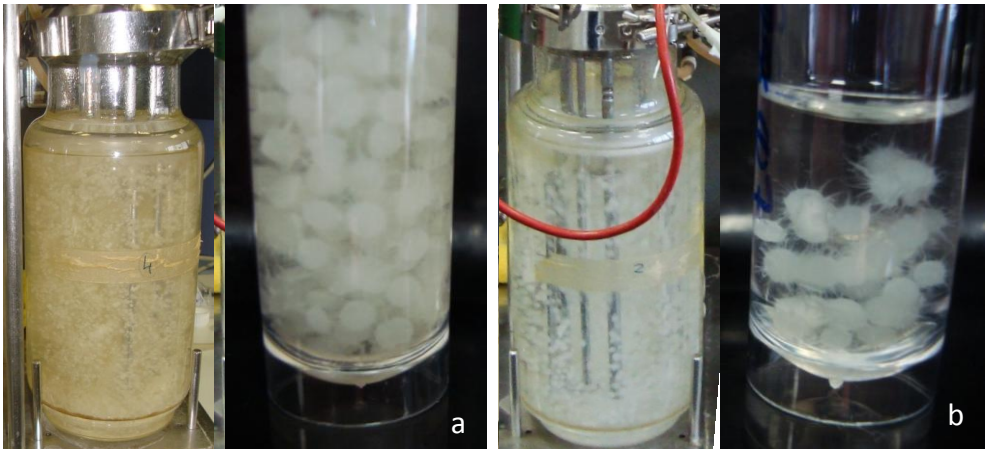


Figure 3-14. Pellets of anamorph of *Bjerkandera* sp. R1 (a) and *P. chrysosporium* (b) in a STR (0.75 L).

Results corresponding to the degradation of anti-inflammatory compounds are shown in Figure 3-15. In the case of the abiotic controls, more than 75% of the initial concentration of these compounds was detected at the end of the assay; although before the 10th day, this residual percentage was higher (>85%) in the case of IBP and NPX; while for DCF this decrease was observed since day 5.

Complete degradation of IBP after only 24 hours was observed in both fungal reactors. Meanwhile DCF and NPX were totally degraded by the anamorph fungus after 4 days. On the contrary, it took more than 10 days to attain total removal by *P. chrysosporium*. The maximum degradation rates for the three compounds were achieved in a range between (0.24 – 1.56 mg/L·d) by both fungal strains during the first day of assay. The results corresponding to CBZ and DZP are shown in Figure 3-16. The residual percentage of these compounds detected in the abiotic controls at the end of the experiment was above 95% of its initial concentration. In the case of fungal cultures, partial removal of CBZ (55%) was achieved by both strains. This behaviour was also observed for DZP in the bioreactor with pellets of the anamorph fungus.

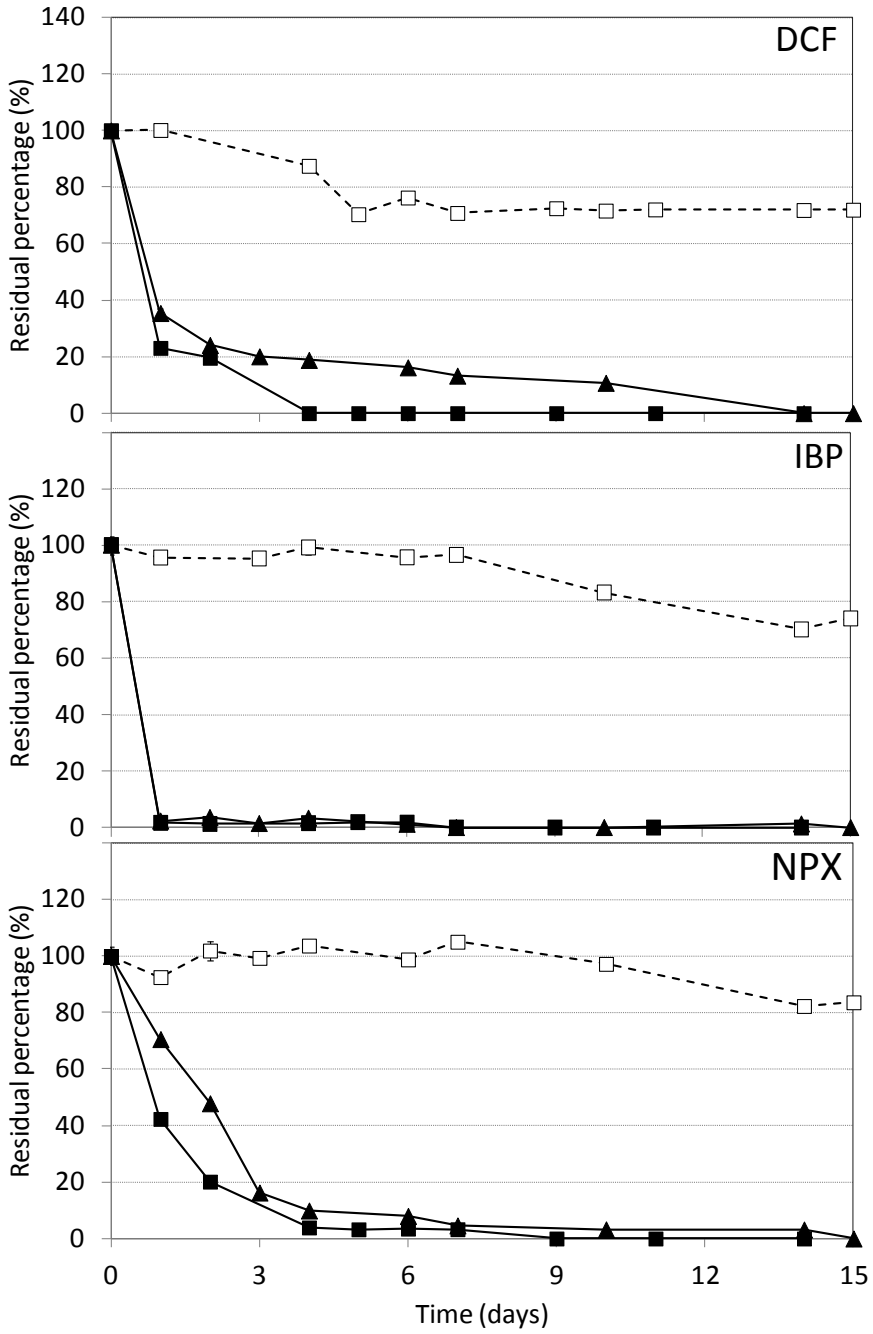


Figure 3-15. Degradation of DCF, IBP and NPX by the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium* in a STR. Symbols: abiotic control (□), *Bj.* sp. R1 (■), *P. chrysosporium* (▲).

Finally, less than 15% of degradation of DZP was detected in the bioreactor vessel with pellets of *P. chrysosporium.* Between days 2 and 4, the degradation rates of CBZ and DZP by the anamorph fungus were in a range of 0.19–0.67 mg/L·d, the maximum corresponding to the antiepileptic compound (CBZ). Contrarily, since day 1 to day 4,

higher degradation rates were observed for both compounds by *P. chrysosporium* (0.16–0.90 mg/L·d).

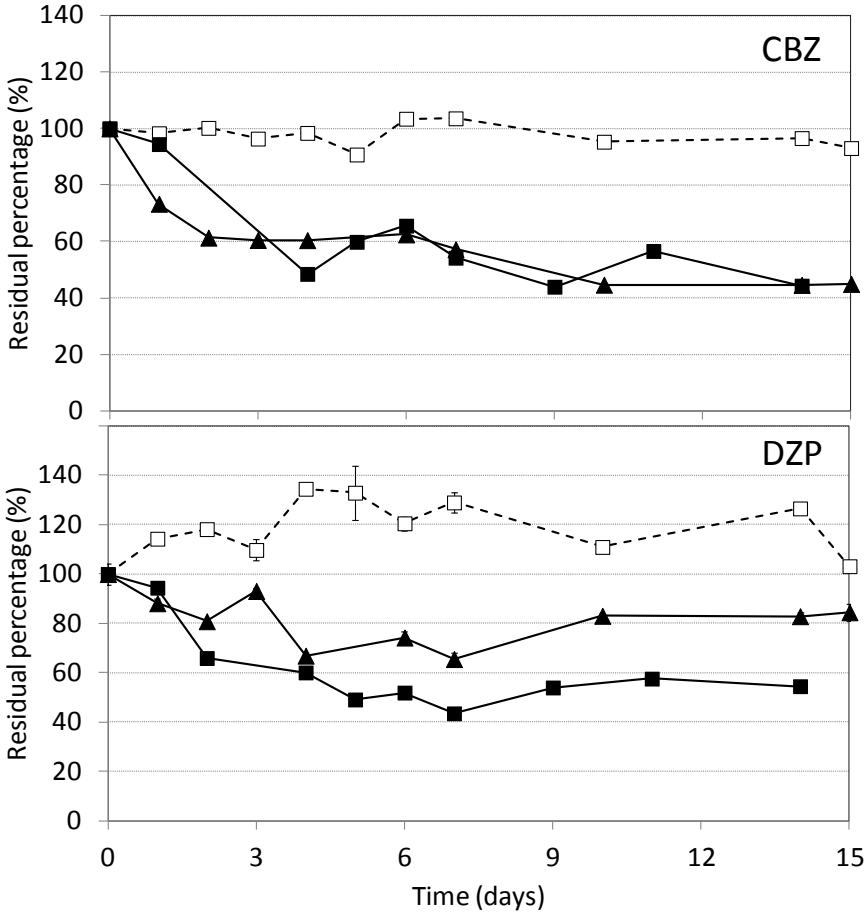


Figure 3-16. Degradation of CBZ and DZP by the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium* in a STR. Symbols: abiotic control (□), *Bj.* sp. R1 (■), *P. chrysosporium* (▲).

In Table 3-7 the results of the final extraction of the biomass with acetonitrile are shown. None of the three anti-inflammatory drugs were detected at the end of the experiment. The biomass of the anamorph of *Bjerkandera* sp. R1 showed high concentrations of the drugs CBZ and DZP (3 and 50 fold, respectively) than the observed onto the biomass of *P. chrysosporium*.

Table 3-7. Residual concentration of pharmaceuticals onto biomass

	Pharmaceuticals concentration (mg drug/g biomass)	
	Anamorph of *Bjerkandera* sp. R1	*P. chrysosporium*
CBZ	0.176	0.056
DZP	0.426	0.008

n.d. (not detected)

3.3.6 Time-course degradation of DCF, IBP and NPX by pellets of the anamorph of *Bjerkandera* sp. R1.

Once the anti-inflammatory drugs are demonstrated to be easily degraded by the three fungal strains, a time-course experiment was carried out with pellets of the anamorph of *Bjerkandera* sp. R1. Figure 3-17 depicts the fermentation profiles during the 10 days of the experiment. Glucose concentration remained constant throughout the experiment in the abiotic controls (Figure 3-17a); meanwhile in the flasks with fungal pellets a slow consumption was observed during the first stage since the fungus depleted 2.4 g of glucose in five days. Complete glucose depletion was observed in the following 24 hours after the addition of the anti-inflammatory drugs.

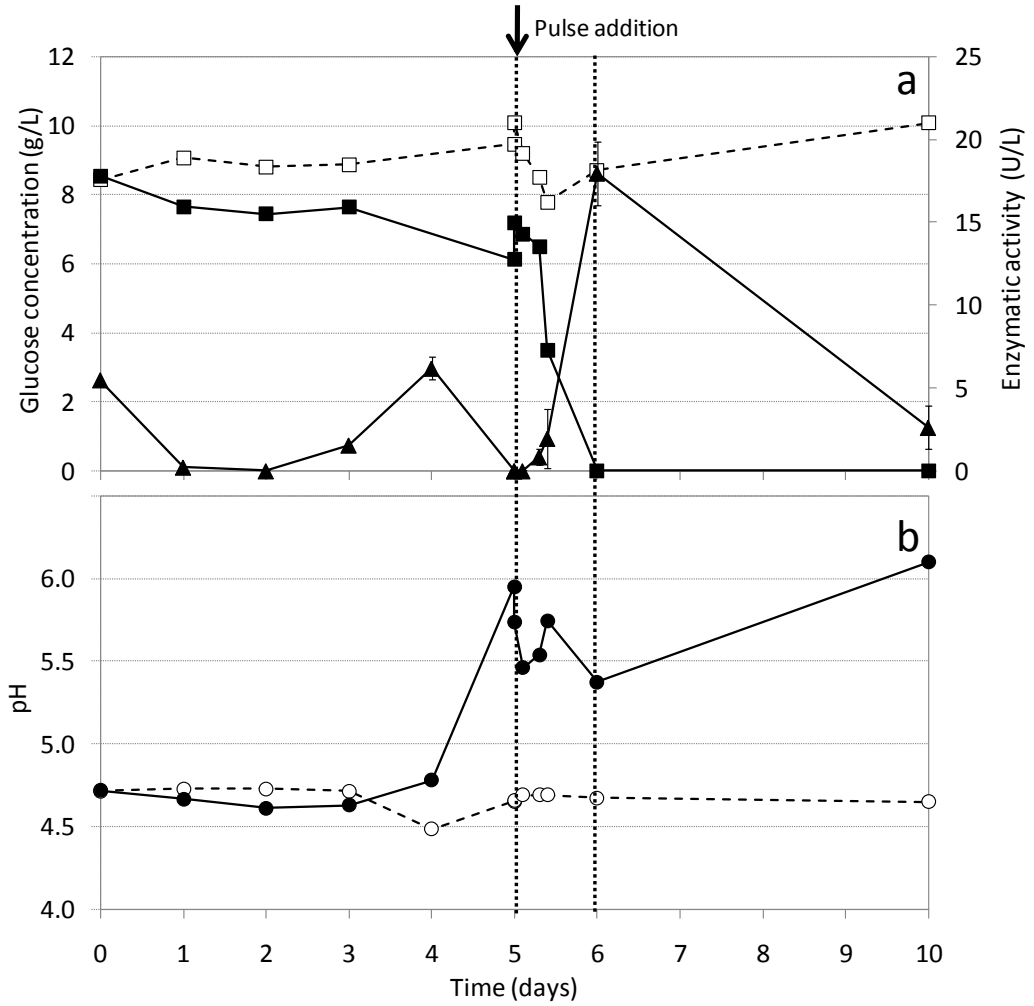


Figure 3-17. Profile fermentation of the anamorph of *Bjerkandera* sp. R1 pellets during 10 days. Symbols: pH – control (○), pH – *Bj.* sp. R1 (●), glucose - control (□), glucose – B*j.* sp. R1 (■), VP activity – *Bj.* sp. R1 (▲).

The enzymatic activity was almost negligible during the first stage of this assay and a single peak was detected after the addition of the anti-inflammatory drugs (18 U/L) (Figure 3-17a). The value of pH was maintained in a range of 4.5 - 4.7 in the abiotic controls. In the flasks with pellets of the anamorph fungus the pH range was between 4.7 and 5.7 until day 6, which correspond to the end of the time-course degradation of 24 hours; while at the end of the fermentation a high pH value was detected (>6) (Figure 3-17b).

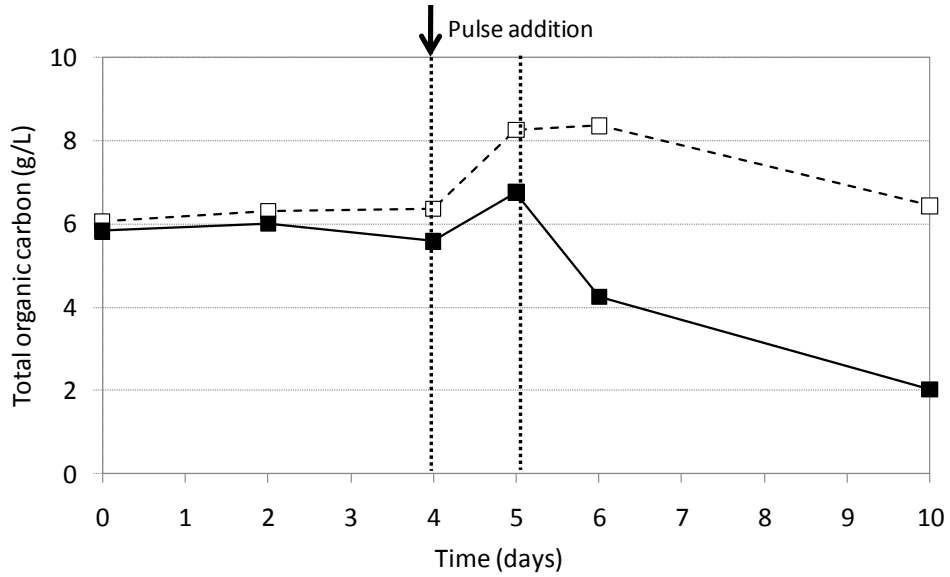


Figure 3-18. Total organic carbon (TOC) content throughout 10 days. Symbols: TOC control (□), TOC – *Bj.* sp. R1 (■).

The total organic carbon content decreased down to 2 g/L in fungal cultures (Figure 3-18) where biomass concentration in the 24 h time-course was quantified in a range from 0.9 g/L at the beginning and 2.2 g/L at the end of the experiment (data not shown).

Concerning to the degradation of three anti-inflammatory drugs (DCF, IBP and NPX) by pellets of anamorph of *Bjerkandera* sp. R1, a time-course assay was carried out after 5 days of incubation and the results are shown in Figure 3-19. The concentration of the three compounds was maintained constant in the abiotic controls; meanwhile it is noteworthy that the anti-inflammatory IBP was completely eliminated after only four hours by the fungal pellets and degradation above 85% for DCF and NPX were achieved after 24 hours.

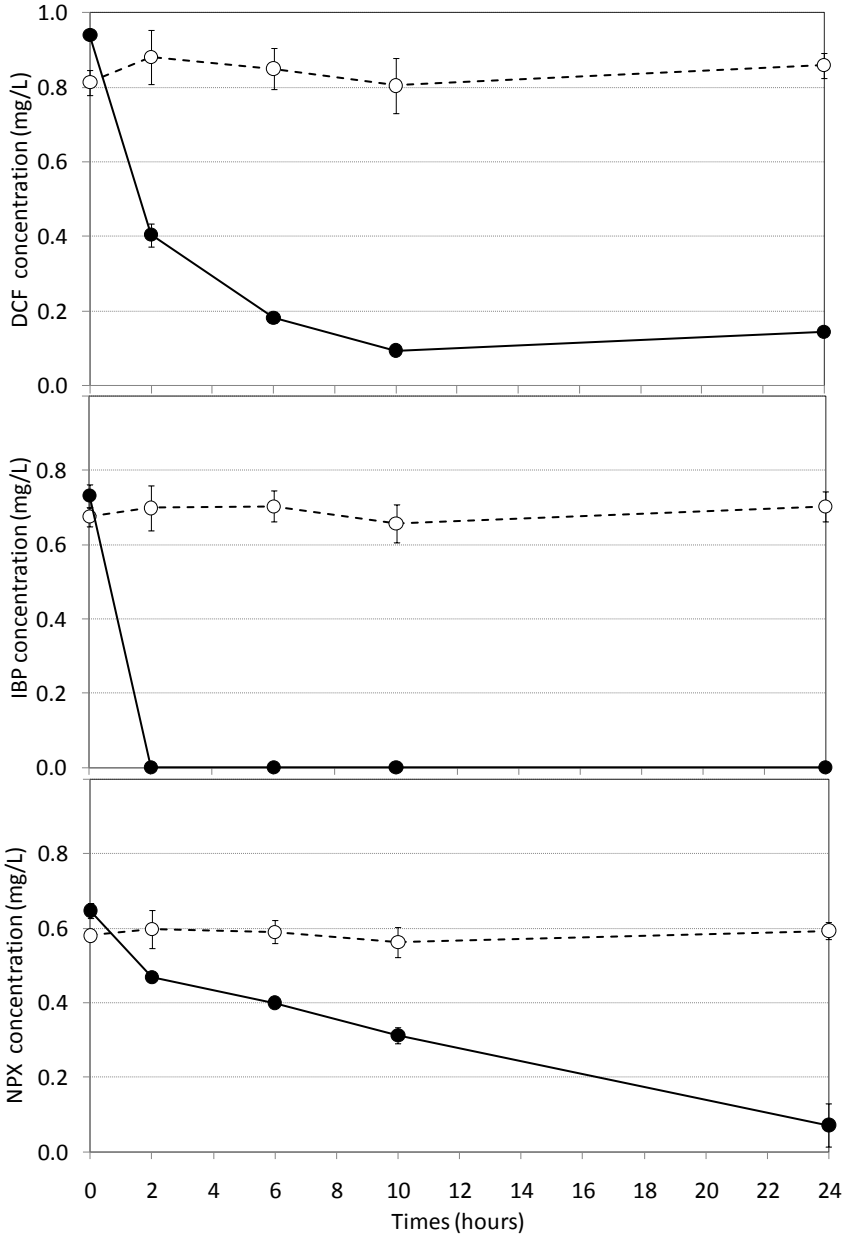


Figure 3-19. Degradation of DCF, IBP and NPX during batch assays with pellets of anamorph of *Bjerkandera* sp. R1. Symbols: abiotic control (○), *Bj.* sp. R1 (●).

3.3.7. Fed-batch assay for the degradation of DCF, IBP and NPX by pellets of the anamorph of *Bjerkandera* sp. R1.

A fed-batch assay was carried out for the degradation of the anti-inflammatory drugs by *Bjerkandera* sp. R1. After 5 days of incubation, a pulse of DCF, IBP and NPX was added daily for the next 4 days (Figure 3-20). As expected, glucose concentration was maintained in concentrations near to 9 g/L in the abiotic control while in the fungal cultures, 3.4 g of

glucose were depleted during the first stage (until day 5). In the fed-batch experiment, complete glucose consumption was observed between days 5 and 7. The enzymatic activity was measured and a peak was observed at day 3 (200 U/L). After glucose depletion, the enzymatic activity of VP increased until values of 350 U/L and this activity was maintained at the end of the experiment.

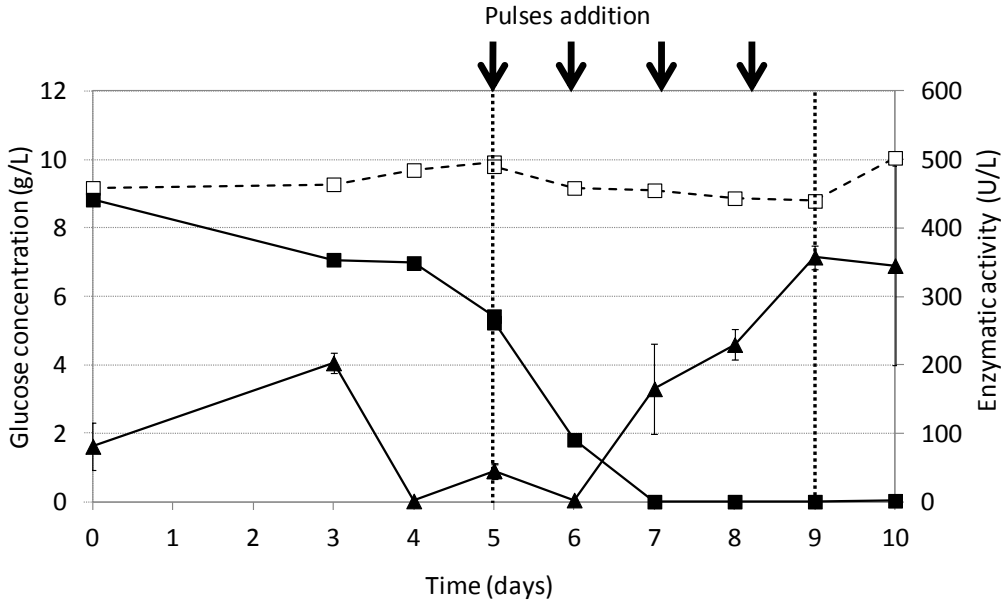


Figure 3-20. Profile fermentation during fed-batch assay with pellets of the anamorph of *Bjerkandera* sp. R1. Symbols: glucose - control (□), glucose – *Bj.* sp. R1 (■), VP activity – *Bj.* sp. R1 (▲).

During the first stage of this assay (until day 3) the content of organic carbon was invariable (6 g/L) in both, abiotic control and fungal pellets flasks; after the addition of the first anti-inflammatory drugs pulse at day 5, the TOC was detected in a range between 6.9 - 10.1 g/L, showing an increase until 14 g/L in the abiotic controls and a slight increase in the fungal pellets flasks (9.9 g/L) after the addition of four drugs pulses. At the end of the assay, a decrease of the concentration of TOC was observed in the abiotic controls and flasks with pellets (6.5 g/L and 1.9 g/L, respectively) (Figure 3-21a). Fungal biomass was measured throughout the experiment (Figure 3-21b). When the fed-batch strategy started, 1 g/L of biomass was detected in the flasks with fungal pellets. This concentration increased until values near to 2 g/L after the addition of the four drugs pulses, detecting 2.1 g/L of biomass at the end of the experiment.

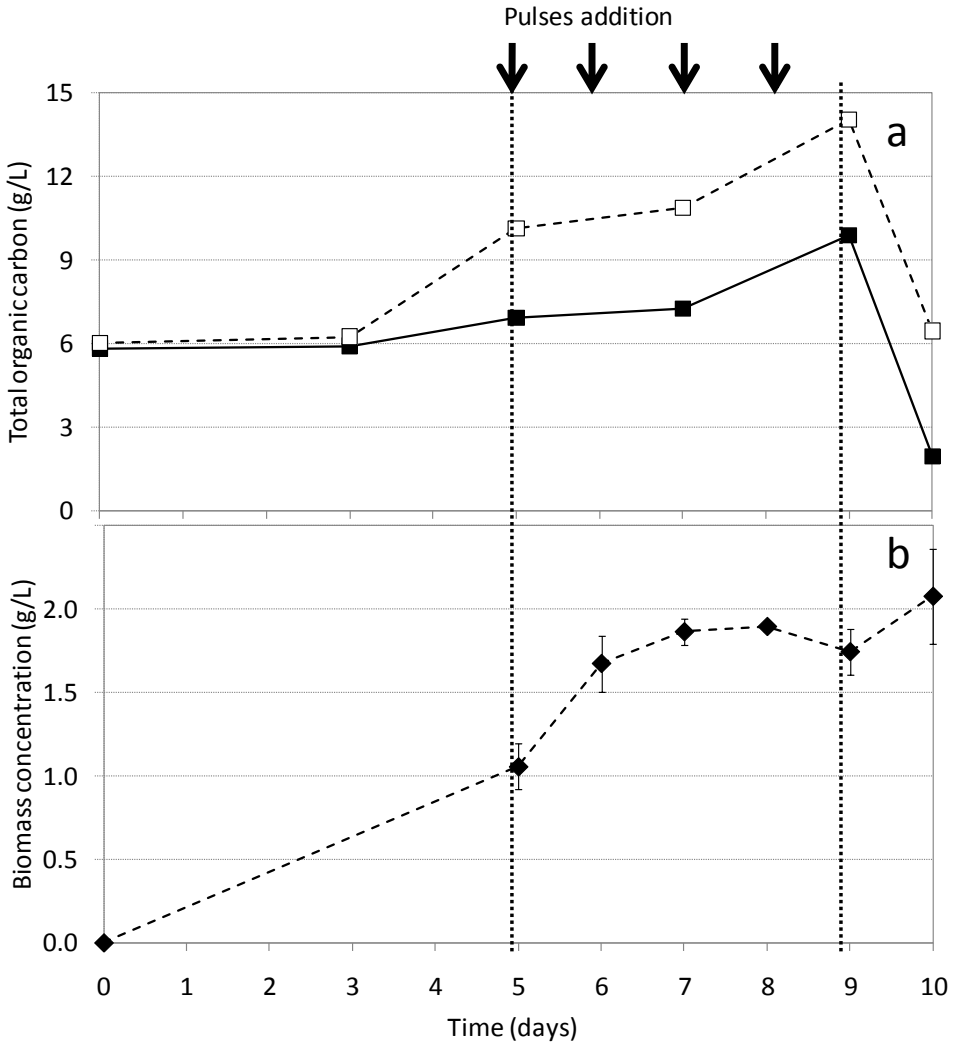


Figure 3-21. Total organic carbon (TOC) content (a) and biomass concentration (b) measured throughout 10 days batch assay. Symbols: TOC control (□), TOC – *Bj.* sp. R1 (■), biomass – *Bj.* sp. R1 (♦).

Figure 3-22 shows the degradation of DCF, IBP and NPX during a fed-batch experiment. Accumulation of the anti-inflammatories concentrations in the abiotic controls was observed; also, after the last pulse addition (day 3) the residual concentration of these compounds was maintained stable for 24 hours. In the case of the flasks with fungal pellets, the concentration of these compounds in the fed-batch operation was similar to that of the abiotic controls, but in lower concentrations; after four days a total degradation up to 93% for the three compounds was observed.

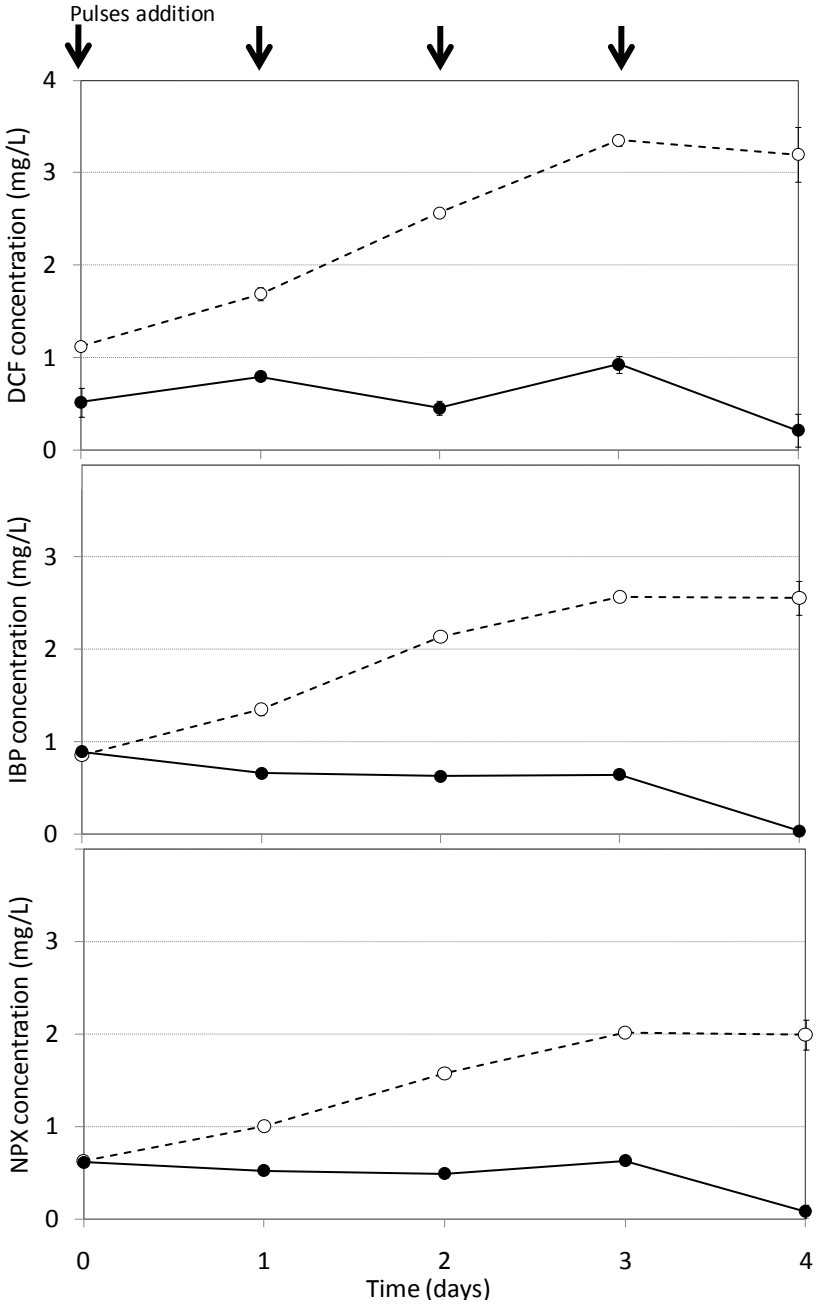


Figure 3-22. Degradation of DCF, IBP and NPX by anamorph of *Bjerkandera* sp. R1 during batch assays with daily pulse addition. Symbols: abiotic control (○), *Bj.* sp. R1 (●).

3.4. Discussion

In this chapter, three white rot fungi (anamorph of *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium*) were evaluated for their capacity to degrade eight pharmaceutical compounds. Previously to the degradation experiments, the possible inhibition of the

fungal growth in malt agar plates at different concentrations of pharmaceuticals was evaluated and the results showed that concentrations below 2 mg/L did not affect mycelium growth. It is important to mention that not only the pharmaceuticals concentration could affect the fungal growth, but also the mixtures and the type of compounds; this means that a concentration up to 2 mg/L of a mixture of three or less pharmaceutical compounds does not affect fungal growth. Furthermore, compounds such as SMX could have a greater inhibitory effect over the mycelium than the rest of the compounds, since this compound is an antibiotic. The effect of the five pharmaceuticals at 1 mg/L was quite low and accordingly this was the concentration selected for all experiments.

Three types of fungal culture were investigated: static, free pellet and immobilized cultures. The results of these batch assays showed that *B. adusta* was the fungal strain with the lowest degradation percentages; for this reason, this WRF was discarded in the following experiments. With the selected strains (the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium*) the degradation of five pharmaceutical compounds in a STR was carried out using free pellets, since this type of culture showed the best results concerning to the total elimination of all the compounds and partial removal for recalcitrant compounds such as CBZ and DZP. In addition, since during the experiments in the STRs it was demonstrated a rapid elimination of anti-inflammatory drugs, the degradation of these compounds was analyzed during a 24 h time-course degradation experiment and a fed-batch experiment using pellets of the anamorph fungus to determine the HRT required for further assays and to investigate the feasibility of further treatment after daily pulses addition.

High enzymatic activities of VP were observed using the three types of cultures (static, free pellets and immobilized cultures) for the anamorph fungus; while *P. chrysosporium* achieved low MnP activities. In the case of the STR and the time course degradation experiment, low enzymatic activities (MnP and VP) by both strains were detected throughout the assay; however, during the fed-batch assay higher activities were observed by the anamorph fungus. These differences between the anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium* might be due to the glucose consumption rate during the experiments was higher for the former. This could affect the enzymatic production since nutrient deprivation enhances the production of ligninolytic enzymes (Wesenberg et al., 2003). Also, each fungus showed a different growth; in this chapter it was observed that the anamorph of *Bjerkandera* showed a noticeable growth, while *P. chrysosporium* presented a limited and controlled growth. Another explanation of this difference could be the nitrogen sources used for each fungus: peptone for the anamorph

fungus and ammonium tartrate for *P. chrysosporium*; these sources has been selected to produce a positive effect on the ligninolytic system of each fungus.

Regarding the degradation of antidepressants and antibiotic drugs, the elimination of these compounds was carried out only during static assay. Citalopram and fluoxetine are very persistent and significant concentrations have been detected in effluents of STPs (Kwon and Armbrust, 2005; 2006). In this chapter, the three fungal strains degraded completely CTL whereas lower removals percentages were observed for FLX. At the present time, there is no research on the degradation of these compounds by action of WRF or their ligninolytic enzymes. In fact, there are a few investigations about the elimination of anti-depressants using different technologies. In example, Kwon and Armburst (2005) reported the photodegradation of CTL in alkaline conditions (pH 9) after more than 60 days of treatment, while the degradation was negligible at pH 5-7. Other authors have reported only limited removal percentages of FLX, lower than 10% after 60 days of treatment (Redshaw et al. 2008). This compound is highly recalcitrant to hydrolysis, photolysis and microbial degradation, and its total degradation is still a challenge to be attained (Kwon and Armbrust, 2006). Another compound that was totally removed during static cultures was SMX, a pharmaceutical drug that belongs to the therapeutic class of the antibiotics and for this reason it could be a problem to investigate its degradation using microorganisms. Previous studies reported degradations of 60% after treatment in STPs and 52% in a membrane bioreactor (MBR) (Carballa et al. 2004; Reif et al. 2008). In the case of advanced oxidation processes (AOPs), Dantas et al. (2008) reported that the nearly complete abatement of SMX by means of an ozonation process at a dose of 0.4 g ozone/L.

Anti-inflammatory drugs degradation was investigated using the three fungal strains incubated in three types of cultures. Also, the degradation of these compounds was analyzed in a STR by pellets of anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium*. In addition, a time-course assay and a fed-batch assay were carried out using pellets of the anamorph fungus. These compounds are one of the major classes of pharmaceuticals used worldwide, and residues of these drugs have been found in treated wastewater at concentrations ranging from 42 to 2556 ng/L (Gagnon et al. 2008). In this chapter, a total elimination of DCF, IBP and NPX was observed using the three fungal strains, regardless of the type of culture. Also, it was demonstrated that they can be degraded when the process was scaled up to a STR. These results led into a more accurate investigation of the degradation during a 24 h time-course experiment and a fed-batch experiment demonstrating that the anamorph fungus has the ability to eliminate these compounds in shorter periods of time, even after only 4 hours; also it was observed that this strain can

eliminate these compounds even when daily pulse additions were added into the medium. The removal of these compounds has been studied using different technologies such as MBRs finding that DCF was not removed, while the rest of the anti-inflammatory drugs was highly eliminated (Clara et al., 2005; Reif et al., 2008); activated sludge process in STPs demonstrated a partial removal for NPX and IBP (up to 70%) (Carballa et al., 2004); coagulation-flocculation and flotation processes achieved a limited removal of these compounds (Suárez et al., 2009); on the other hand, the use of AOPs may improve the removal efficiencies of the three compounds (Ikehata et al., 2006; Gagnon et al., 2008). As it can be observed, the results of the degradation of DCF, IBP and NPX using the previously mentioned technologies is strongly depending on the selected compound and the characteristics of the used technology. Although the results obtained in this chapter were better than those reported above, the removal efficiency will mainly depend on the HRT, the physicochemical properties and the initial concentration of the pharmaceuticals used. In the recent years, the use of WRF and its ligninolytic enzymes has demonstrated that pellets of the fungi *Trametes versicolor* and *P. chrysosporium* were able to achieve a complete degradation of high concentrations of IBP and NPX (10 mg/L) (Marco-Urrea et al., 2009; Marco-Urrea et al., 2010b). Also, Rodríguez-Rodríguez et al., (2010a) using pellets of the same fungus showed a partial removal of NPX (47%) after 24 hours in bioslurry cultures and complete depletion in solid-phase systems after three days. Another WRF used in a recent study was *Phanerochaete sordida,* a fungus that showed a complete removal of DCF (Hata et al., 2010a). These results were similar to those obtained in this chapter with respect to fungal pellets, where a complete degradation of three anti-inflammatory compounds was achieved even in short periods of time. In the case of ligninolytic enzymes, *in vitro* assays were carried out using purified Laccase finding a complete removal of DCF after less than 5 h (Marco-Urrea et al., 2010a). These results are similar to those obtained by Lloret et al., (2010) who found that to achieve a complete removal of the drug DCF it was necessary 1 hour under the action of Laccase using several mediators, while only a partial removal of NPX was observed after 8 h using the mediators 1-hydrozybenzotriazole (HBT) and violuric acid (VA). Other enzymes such as versatile peroxidase (VP) and lignin peroxidase (LiP) have achieved total depletion of DCF (Zhang and Geiβen, 2010; Eibes et al., 2011). The results reported above demonstrated that when concentrated enzymes were used, a total removal was observed in shorter periods of time (hours) than those obtained in our results (days) using fungal pellets. Despite this, it is important to highlight that apparently there is not a strict dependence on a given level of peroxidase production to carry out the degradation, since a complete degradation of these compounds was achieved in this chapter even at low MnP activities using *P. chrysosporium*.

Two of the most persistent pharmaceuticals in the environment are carbamazepine (CBZ) and diazepam (DZP). The former is a carboxamide-type anti-convulsant and has been found ubiquitously in the aquatic environment at 1-2 µg/L; meanwhile the latter is a benzodiazepine-type anti-anxiety agent used for the treatment of many other neurological and psychiatric disorders and motion sickness (Ikehata et al. 2006). Carbamazepine was totally eliminated during static and free pellets cultures, meanwhile low degradations were detected in immobilized cultures. In the case of DZP, none of the fungi or cultures (static, pellets and immobilized) were able to completely degrade this compound. The removal of these compounds by an anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium* was analyzed when the process was scaled up into a STR; however, fungal pellets were only able to achieve partial eliminations. Degradation percentages in the range of 9% up to 50% of CBZ and DZP have been reported using several technologies such as coagulation-flocculation and flotation processes, nitrifying-denitrifying plants and MBR (Carballa et al., 2005; Clara et al., 2005; Reif et al., 2008; Suárez et al., 2005). Comparing our results with those mentioned above, higher removals were achieved in this chapter; however, as it was mentioned previously these removal efficiencies will depend on several factors such as the type of process and the HRT, among others. The use of WRF or its ligninolytic enzymes for the degradation of CBZ have attained partial removal of this compound in fungal cultures (Marco-Urrea et al., 2009; Rodríguez-Rodríguez et al., 2010a). In the case of *in vitro* experiments, when the VP and LiP enzymes were used no significant removal was observed (Zhang and Geiβen, 2010; Eibes et al., 2011). Contrary to these results, using the so-called Laccase-mediator system a partial removal of CBZ (26%) was observed after 24 h enhancing this removal up to 66% when a fed-batch assay was carried out for a period of 48 h (Hata et al., 2010b). It has not been found further investigations about the degradation of DZP by fungal pellets or fungal enzymes action. Comparing the results mentioned above with those obtained in this chapter, it can be seen that the removals were similar using pellets or enzymes; however in the case of the latter, these eliminations were achieved in a shorter period of time (hours).

Concerning to adsorption processes, it was demonstrated that all compounds, maintain their initial concentration in the abiotic controls; also it was proved that the use of acetonitrile to extract these compounds from the biomass or support was successful. Since an extraction was carried out to each withdrawn sample, adsorption of pharmaceuticals on fungal mycelium was excluded. At the end of the STR operation an extraction of the final biomass content is always necessary in order to determine the residual concentration of the pharmaceutical compounds. In this chapter it was demonstrated that the anti-inflammatory drugs were not adsorbed onto the biomass of

both fungal strains and great difference was observed between the biomass corresponding to the anamorph fungus and *P. chrysosporium* in the case of CBZ and DZP. Anti-inflammatory drugs were eliminated rapidly comparing with CBZ and DZP during all the experiments. This could be due to the differences in their chemical structure and their physicochemical properties. A deeper study should be necessary to understand the different biodegradability of each compound. The difference between these compounds might be due to the fact that the biodegradability of the anti-inflammatory drugs can be influenced by different factors as the chemical structure and physicochemical properties of each compound as their liphophilic character (K_{ow}), the acid dissociation constant (pKa) and the pseudo first-order degradation constant (K_{biol}) (Suárez et al., 2008). The K_{ow} and the pKa are related to a sorption process into the solids; however, in this chapter these compounds were not detected onto the biomass. In addition, CBZ and DZP are classified as hardly biodegradable compounds due to their K_{biol} constant (Joss et al., 2006).

Accordingly to the operation of the STR, this bioreactor was operated as batch culture in order to analyze if the degrading ability showed by both fungal strains during batch experiments in flasks could be maintained or even improved when the process was scaled-up to a STR. The results obtained after almost two weeks of operation as batch culture showed that the performance of the bioreactor was stable throughout the assay, although worst elimination than those obtained during batch assays in flasks were attained for CBZ and DZP. A further investigation is necessary for future assays in STR with a greater volume and using different operation conditions in order to improve the degradation of these recalcitrant compounds. Biomass content measured at the end of the assay was higher in the case of the anamorph fungus and no clogging problems were observed; however this assay was carried out for only two weeks. In order to find out if these fungal strains could generate clogging problems is necessary to carry out an assay for a longer period of time. The aeration system applied to this bioreactor was a continuous air flow with the aim to favour the enzymatic production, although low MnP activities were detected. It has been reported that the use of oxygen pulses in this type of bioreactors may have a positive influence over pellets morphology and helps to avoid an excessive mycelia growth (Moreira et al., 1996). In future assays, the influence of a continuous air flow or oxygen pulses over the pellets growth and the degradation of the pharmaceuticals will be studied.

It has been demonstrated by comparing the results of degradation of pharmaceutical compounds that the three types of cultures and the three fungal strains used in this chapter were very different. Static cultures tend to present a slow metabolism than those cultures with agitation or aeration supply; this could affect their ability to

degrade the compounds. Despite this, in this chapter it can be observed that the three fungal strains showed glucose consumptions and enzymatic activities similar during static and free pellets cultures; meanwhile during immobilized assays higher glucose consumption was obtained and high enzymatic activities were maintained throughout the assay. Related to free pellets cultures, this type of culture demonstrated to be the optimal culture for the elimination of several compounds; this could be due to the higher aeration in the system that could favour the nutrients consumption rate. Finally the use of immobilized fungus on a support like polyurethane foam helps to control the mycelia growth depending on the strain used; also provide a higher aeration into the system, a higher fungal active surface, the nutrients consume rate is higher, among other; in this chapter two fungal strains were immobilized (anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium*) without clogging or growth problems.

3.5. Conclusions

In this chapter a batch degradation of a mixture of eleven pharmaceuticals was carried out by three different fungal cultures (static, free pellets and immobilized cultures) using three fungal strains: an anamorph of *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium*. The results were different depending on the incubation type and the fungus used. The best culture conditions were using free pellets, since high elimination of the pharmaceutical compounds was achieved in shorter periods of time and also high enzymatic activities were observed. It was demonstrated that an extraction with acetonitrile is always necessary to recover these compounds from the biomass or support. The fungal strain *B. adusta* was excluded for future assays since this WRF obtained the lowest removals. When the process was scaled up to a STR using pellets of the fungal strains selected (anamorph fungus and *P. chrysosporium*), the degradation of the anti-inflammatory drugs was maintained, although worst results were observed for the elimination of CBZ and DZP than the results obtained during batch experiments in flasks. The operation of the reactor was steady throughout the assay even when a high content of biomass was measured in the case of the anamorph fungus. Low enzymatic activities were detected using both fungal strains; however this concentration was enough to carry out the degradation of the considered pharmaceuticals. It was demonstrated that the anti-inflammatory drugs could be degraded in less than 24 hours and that the WRF are able to eliminate these compound even after a daily addition. Since the results obtained in this chapter demonstrated that both fungal strains have the ability to achieve from total to partial degradation of five pharmaceuticals (DCF, IBP, NPX, CBZ and DZP), all these compounds were selected for future experiments. In addition, since it was demonstrated that these compounds were slightly adsorbed onto the fungal pellets of *P. chrysosporium*, this fungus was selected to

continue the experimental investigation. Another advantage to use this fungus instead of the anamorph is the lower mycelia growth that will avoid clogging problems facilitating the operation of the bioreactor for longer periods of time. Furthermore, these findings will be useful to analyze the degradation of these compounds and to optimize the operation and performance of STRs and/or fixed bed reactors (FBRs) operating under different feeding conditions (fed-batch or continuous).

Chapter 4

Development of a free pellets reactor with *Phanerochaete chrysosporium* for removal of DCF, IBP, NPX, CBZ and DZP. From fed-batch to continuous strategy*

Summary

Different operational conditions were sequentially evaluated to maximize the removal of DCF, IBP, NPX, CBZ and DZP in a stirred tank reactor operating with free pellets of *Phanerochaete chrysosporium*. In a first experiment, a fed-batch operation was conducted for 30 days to carry out the degradation of the three anti-inflammatory drugs. Additionally, the influence of gas supply, either by continuous aeration or periodic pulsation of oxygen, on the process efficiency was evaluated. Evolution of pellet morphology was monitored to establish the potential effect that the gas composition and mode of supply may exert on pellet formation and development. The three anti-inflammatory drugs were efficiently removed at high oxidation rates, especially under oxygen pulsation. Among these compounds, NPX presented a slightly lower removal with variable percentages (77% up to 99%) and a slight accumulation between days 12 and 22. Not only the pulsation of oxygen maintained higher levels of dissolved oxygen but it also controlled pellet extension and morphology. In a subsequent experiment, the bioreactor was operated in a fed-batch mode (stage I) for 26 days and thereafter, it was changed to continuous mode until day 70 (stage II). The target compounds considered for this experiment were DCF, IBP, NPX and CBZ. During the fed-batch stage the fungus was able to completely remove DCF, IBP and NPX meanwhile only partial removal was attained for CBZ (30% - 63%). During the continuous stage, the bioreactor was capable to maintain

efficient removal of all the compounds with a slight decline from day 55th. Finally, a continuous experiment was carried out to confirm the degradation of the former four compounds, including a more recalcitrant one: DZP. The bioreactor operation was maintained steady for 50 days and attained high removal efficiencies of the three anti-inflammatories, partial removal of CBZ (<53%) and negligible removal of DZP were achieved.

* Part of this chapter has been published as:

Rodarte-Morales AI, Feijoo G, Moreira MT, Lema JM. (2011) **Biotransformation of three pharmaceutical active compounds by the fungus *Phanerochaete chrysosporium* in a fed batch stirred reactor under air and oxygen supply.** Biodegradation. DOI 10.1007/s10532-011-9494-9.

Rodarte-Morales AI, Feijoo G, Moreira MT, Lema JM. (2011). **Evaluation of two operational regimes: fed-batch and continuous for the removal of pharmaceuticals in a fungal stirred tank reactor**. Chemical Engineering Transactions (Submitted).

Rodarte-Morales AI, Feijoo G, Moreira MT, Lema JM. (2011). **Operation of stirred tank reactors (STRs) and fixed-bed reactors (FBRs) with free and immobilized *Phanerochaete chrysosporium* for the continuous removal of pharmaceutical compounds.** Biochemical Engineering Journal (Submitted).

Outline

4.1. Introduction

On-going initiatives rely on the use of white-rot fungi (WRF) for the oxidation of a wide range of organic pollutants structurally similar to lignin, such as synthetic dyes, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pharmaceutical compounds and endocrine disrupting chemicals (EDCs) (Field et al., 1992; Zeddel et al., 1993; Wesenberg et al., 2003; Ikehata et al., 2006). Specifically for pharmaceuticals, this type of compounds are considered as emerging pollutants and are suspected to present a remarkable impact on the environment because they have been designed to affect biochemical and physiological functions of humans and animals. Moreover, pharmaceuticals compounds may cause increased aquatic toxicity and endocrine disruption (Ikehata et al., 2006; Jjemba, 2006). Among the different classes of pharmaceuticals, some of the most commonly used worldwide are anti-inflammatory drugs, such as diclofenac (DCF), ibuprofen (IBP) and naproxen (NPX) which possess analgesic, antipyretic and anti-inflammatory effects by inhibiting the synthesis of prostaglandin (Ikehata et al., 2006). Belonging to the group of pharmaceuticals, two of the most recalcitrant compounds are carbamazepine (CBZ), a carboxamide-type anti-convulsant as well as diazepam (DZP), a tranquilizer recommended for depression and anxiety; both compounds have been found ubiquitously in the aquatic environment at low concentrations: 1-2 μg/L (Fent et al., 2006; Ikehata et al. 2006).

Different fungi (*Trametes versicolor*, *Phanerochaete sordida, Phanerochaete chrysosporium*) have been used for the removal of anti-inflammatory and antiepileptic drugs, either by whole cultures or by the oxidative action of the enzymes produced (Marco-Urrea et al., 2009; Hata et al., 2010a; Lloret et al. 2010; Rodríguez-Rodríguez et al., 2010). Although the application of oxidative enzymes has been proven efficient, they require the presence of specific cofactors and mediators to activate the catalytic cycle, some of which have been reported to be toxic or scarcely biodegradable (Cañas and Camarero, 2010). Moreover, their use is beset by additional operational barriers: reusability, cost and denaturation of the enzyme (Cabana et al., 2007). In this chapter, the fungal strain selected was *P. chrysosporium,* the best known WRF that produces two extracellular peroxidases: lignin peroxidase (LiP) and manganese peroxidase (MnP) (Glenn et al., 1983; Tien and Kirk, 1983; Kuwahara et al., 1984). These ligninolytic enzymes are produced during the secondary metabolism of the fungus and their synthesis and secretion are induced by nutrient limitation (Wesenberg et al., 2003). The use of this fungal strain has some advantages over other WRF since this fungus has no restrictions due to genetic modifications as it is a readily available strain. In addition, this microorganism is ideal for discontinuous processes since its ligninolytic system is activated

during secondary metabolism. Several environmental conditions can be modified to improve the production of ligninolytic enzymes: culture age, medium composition, culture technique (static, agitated and immobilized), reactor configuration, aeration supply (air or oxygen) and agitation rate, among others (Feijoo et al. 1995; Moreira et al., 2000b; Rogalski et al. 2006). Specifically for *P. chrysosporium,* this fungus has been reported to require high levels of oxygen concentration to promote the production of ligninolytic enzymes (Dosoretz et al., 1990; Moreira et al. 1996; Jiménez-Tobon et al., 1997, Rothschild et al., 1999).

The enzymatic production by ligninolytic fungi has been scaled up to stirred tank reactors (STRs) in the last years (Moreira et al., 2000a; Rogalski et al., 2006). The stability of the bioreactor will depend on the operational conditions and the ability of the fungus to produce enzymes. When fungal pellets are used, excessive fungal growth with the release of mycelial fragments may limit the viability of the bioreactor operation. This event may cause clogging problems inside the reactor with the formation of preferential pathways, which will render in a dead volume of the reactor with low availability of nutrients and oxygen (Ehlers and Rose, 2005). A strategy to avoid the overgrowth of pellets could be based on the way how gas is supplied, either air or oxygen in a continuous or pulsing mode. A continuous gas supply maintains a constant level of dissolved oxygen, which may be beneficial for the enzymatic production; however, this alternative cannot be used for oxygen supply as it would largely increase the cost of operation. On the other hand, the supply of gas by means of a pulsation device could have a favorable effect on the stress generated on the bioparticle and may prevent excessive fungal growth by the shear stress on the pellet surface. In this case, the use of oxygen could be considered as an option because the consumption is limited and controlled during the process and its cost may be affordable (Dosoretz et al., 1990; Moreira et al., 1996; Xiong et al. 2008; Kenealy and Dietrich, 2004). As was evidenced in a previous work, the supply of gas in periodic pulses not only controlled the extension of pellets but also their size. Pellets from the pulsed bioreactor showed a spherical and compact shape (Moreira et al, 1996). On the contrary, when the bioreactor was continuously aerated, the fungal pellets tended to grow and aggregate and mycelial fragments were released with the subsequent clogging of the bioreactor bed.

This research work approaches a degradation system based on the use of a fungal STR operated with free pellets of *P. chrysosporium*. Two different aeration systems (continuous air flow and oxygen pulses) and two nutrient addition strategies (fed-batch and continuous) were considered for the degradation of DCF, IBP, NPX, CBZ and DZP. The operation under fed-batch regime, with the controlled addition of glucose and pharmaceutical compounds, aimed at maintaining the fungal culture active and avoiding

overflow metabolism (formation of side metabolites) and nutrient deprivation. Later on, the reactor was changed to a continuous mode with the objective of assessing if the degradation of these pharmaceutical compounds could be maintained in a continuous operation.

4.2. Materials and methods

4.2.1. Microorganism and inoculum preparation

The white rot fungus used in this chapter was *Phanerochaete chrysosporium*. A detailed description of the preparation of the pre-inoculum for the stirred tank reactor (STR) was included in chapter 2.

4.2.2. Pharmaceutical compounds and chemicals

Five pharmaceutical compounds (DCF, IBP, NPX, CBZ and DZP) were taken from a stock solution prepared as indicated in chapter 2. Different concentrations were used during the three experiments: 1 mg/L (DCF, IBP, NPX), 0.5 mg/L (CBZ) and 0.25 mg/L (DZP).

4.2.3. Features of the stirred tank reactor

In this chapter, all the experiments were conducted using a STR (2 L) using different operation conditions. A detailed description of the main characteristics of the bioreactor used was included in chapter 2.

4.2.4. Influence of the gas supply (in continuous and in pulses) on the operation of a fed-batch stirred tank reactor for the degradation of DCF, IBP and NPX.

The configuration of the STR used during this experiment is shown in Figure 4-1. Agitation was fixed at 200 rpm and temperature maintained at 30°C. The hydraulic retention time (HRT) was 24 h, as concluded from previous batch experiments, which showed efficient degradation of the three anti-inflammatory drugs in less than 24 h (results from chapter 3). The vessel was filled with 1.5 L of modified Kirk medium (pH 4.5) (Tien and Kirk, 1988) and fungal pellets previously formed from 5 flasks were added as inoculum. Pulses of glucose (3 - 6 g/L) and pharmaceuticals (~1 mg/L) were added sequentially every 3 - 4 d when decay in the glucose concentration was observed in the air and oxygen reactors. Daily pulses of anti-inflammatory drugs were added during the third week of the oxygen experiment to analyze if the fungus was able to remove these compounds at this moment of the operation. In the air reactor, the vessel was continuously aerated for 30 d with a variable air flow to maintain the dissolved oxygen concentration as high as possible.

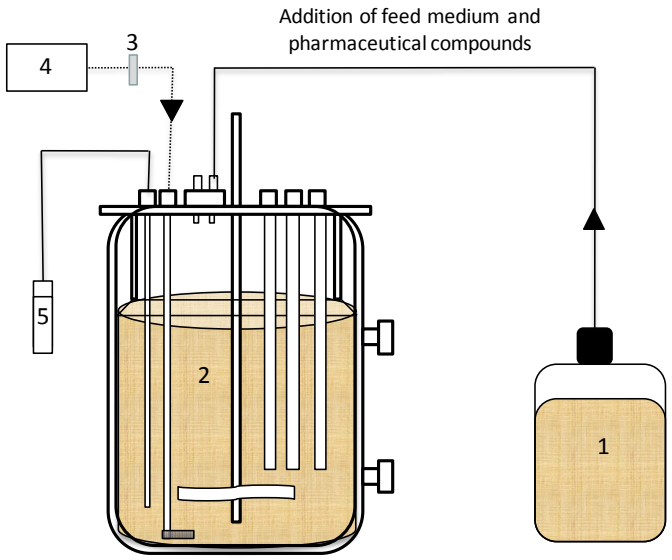


Figure 4-1. STR in fed-batch operation for degradation of DCF, IBP and NPX.

In the oxygen reactor, a pulsing device consisting of an electrovalve located at the end of a flexible membrane tube (FMT) described in chapter 2 was implemented to supply oxygen in pulses. The pulsing frequency is calculated by the inverse sum of the opening and shutting times of the electrovalve. Different pulsing frequencies were assayed to assure high levels of dissolved oxygen (Table 4-1). Samples were withdrawn from the bioreactor every 3-4 days throughout the experiment.

Table 4-1. Pulsation frequencies of the bioreactor operated with oxygen supply.

Days	Opening time (min)	Shutting time (min)	Pulsing frequency (min^{-1})
0-2	1.5	15	0.0606
2-6	1	15	0.0625
7-15	1	60	0.0164
15-20	1.5	60	0.0163
20-21	2	45	0.0213
21-30	1.5	60	0.0163

4.2.5. Influence of the feed regime (fed-batch and continuous) on the operation of a stirred tank reactor for the degradation of DCF, IBP, NPX and CBZ.

Two configurations were used in this experiment: a fed-batch experiment (Figure 4-1) and continuous feeding (Figure 4-2). Fungal pellets previously grown from 5 flasks were transferred into the reactor which was filled with 1.5 L of modified Kirk medium (pH 4.5) (Tien and Kirk, 1988). During stage I (days 0-24), pulses of glucose (3-6 g/L) and

pharmaceuticals were added sequentially at days 0, 4, 7, 14 and 20; meanwhile from day 25, the reactor operated with a continuous feeding of glucose (50-300 mg/L·h) and pharmaceutical compounds (~0.04 mg/L·h). The bioreactor was continuously aerated for 70 d with a variable air flow to maintain the dissolved oxygen concentration close to saturation values. The modification of the glucose feeding rate depends on the substrate consumption rate by the fungus (Table 4-2). The glucose addition rate was modified to maintain a set-point of glucose in the effluent below 2 g/L. The rationale behind this strategy is based on the fact that a low concentration of glucose in the effluent is desirable as it will assure the maintenance of the culture viability while it avoids the secretion of secondary proteases, which will inactivate the produced enzymes.

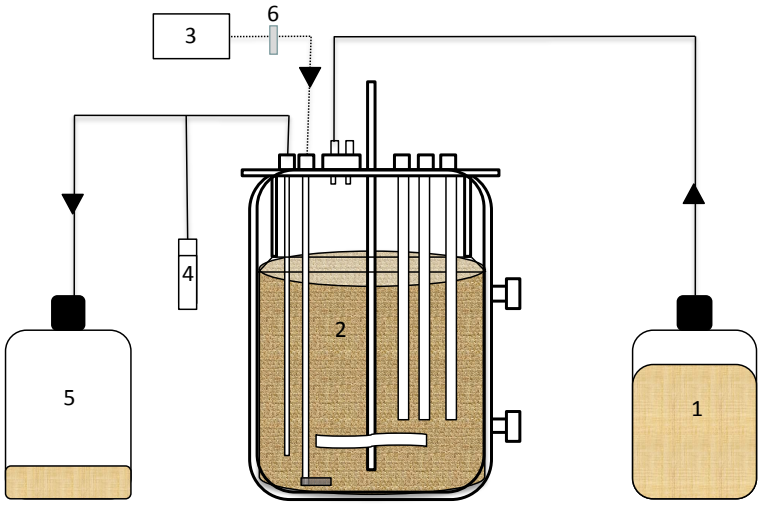


Figure 4-2. Configuration of a continuous operation in a STR using free pellets. Symbols: (1) Feed reservoir, (2) *P. chrysosporium* immobilized in polyurethane foam, (3) air supply, (4) sampling point, (5) effluent reservoir, (6) filter.

During stage I, the monitoring of the main variables were controlled in a time course experiment at days 0, 4, 7, 14 and 20 to analyze the ability of the fungus to remove these compounds at different stages of the fermentation. These experiments were based in samples taken immediately after the moment of the pharmaceutical compounds addition, then every half hour until 2 hours, then every 2 hours until 8 hours and finally after 24 hours. Before the addition of the drugs, a sample was withdrawn from the bioreactor (~60 mL) and filtered; the extracellular liquid was placed in a flask and pharmaceutical compounds pulses were added to assess the capability of the extracellular fluid containing MnP to carry out the degradation of the target compounds under an *in vitro* perspective, that is, by the single action of the enzyme in the absence of the fungal culture. In parallel, abiotic controls were carried out using sterile distilled water to discard

any volatilization and/or photodegradation of the compounds. When a continuous operation was performed (stage II), samples were taken twice per week to monitor the process.

Table 4-2. Glucose feeding rates used during the continuous stage.

Period (days)	Glucose feeding rates (mg/L·h)
27-29	300
31-35	285
35-41	220
42-46	50
48-54	70
55-60	90
61-70	130

4.2.6. Operation of a continuous stirred tank reactor for the degradation of the DCF, IBP, NPX, CBZ and DZP.

Configuration of a continuous operation in a STR was shown in Figure 4-2. As in previous experiments, the bioreactor vessel was filled with 1.5 L of modified Kirk medium (pH 4.5) (Tien and Kirk, 1988) and the inoculum consisted of pellets from 5 flasks. A continuous operation was conducted for 50 days of operation and feeding rates of glucose are shown in Table 4-3. The glucose addition rate was modified to maintain a set-point of glucose in the effluent below 2 g/L.

Table 4-3. Glucose feeding rates used during the continuous experiment.

Period (days)	Glucose feeding rates (mg/L·h)
0-5	150
5-7	175
8-11	260
12-15	300
16-19	220
20-24	260
25-29	170
29-40	270
41-50	225

A continuous air flow was used to maintain significant levels of dissolved oxygen in the culture broth. Pharmaceutical compounds were added in the feed medium at different concentrations: 1 mg/L (DCF, IBP, NPX), 0.5 mg/L (CBZ) and 0.25 mg/L (DZP). Samples were withdrawn daily for monitoring the main operational variables and twice per week to determine the residual concentration of the pharmaceutical compounds.

4.2.7. Extraction of pharmaceuticals and determination of residual concentrations

Samples of 10 mL containing culture medium and fungal pellets were withdrawn during the bioreactor operation at the sampling times indicated above. Then 10 mL of acetonitrile were subsequently added for the extraction of pharmaceuticals. The procedures for the extraction and determination of the residual concentration of the considered pharmaceuticals by gas chromatography-mass spectrometry (GC-MS) were described in chapter 2.

4.2.8. Adsorption of pharmaceutical compounds on the fungal biomass.

Determination of pharmaceuticals adsorption on the mycelia was carried out to discard any possible adsorption, volatilization and/or photodegradation process of the pharmaceutical compounds. The methodology to carry out these experiments was described in chapter 2.

4.2.9. Analytical techniques

A description of the analytical techniques used in this study was indicated in Materials and methods chapter.

4.3. Results

In this chapter three different experiments were performed using pellets of *P. chrysosporium* in a STR for long operational periods (up to 70 days). The first experiment aimed at the degradation of three anti-inflammatory drugs (DCF, IBP and NPX) using a fungal reactor under different aeration conditions (continuous air flow and oxygen pulses). In a subsequent experiment the effect of the feeding regime (fed-batch and continuous feeding) was evaluated for the efficiency of removal of the anti-inflammatory compounds mentioned above as well as an antiepileptic drug (CBZ). Finally, a third experiment was conducted to assess if the fungus was able to maintain a continuous removal of five pharmaceutical compounds (DCF, IBP, NPX, CBZ and DZP). In addition, the evolution of the main variables of the process was monitored in time course experiments at certain operational days to assess the ability of the fungus to remove these compounds at different stages of the fermentation. The evolution of the main parameters of the operation was monitored during the different experiments. The operational strategy has to assure that the viability of the bioreactor operation is maintained both in fed-batch or continuous operation. It is important to remark that the fungal reactor should be operated in conditions close to secondary metabolism when the ligninolytic system is promoted. For this reason, it is necessary to analyze several variables that could affect the

process, such as glucose concentration, total organic carbon (TOC), total nitrogen (TN), pH, dissolved oxygen concentration and enzymatic production. Variables indicative of nutrient concentrations are glucose, TOC and TN. If these parameters are controlled and maintained in optimal concentrations, primary metabolism may be avoided and cell lysis prevented. The optimal pH value to maintain the fungus in secondary metabolism is in a range of 4.5 and 5.0; with restricted variations towards acidic or basic values, depending on the secretion of organic acids or proteases, respectively. A level of dissolved oxygen close to saturation favours the activation of the ligninolytic system. This is also related with the activity of oxidases which are indirectly determined by the level of free peroxide, since they generate peroxide in their cycle and peroxidases corresponding to MnP for this fungus, *P. chrysosporium*. Another important aspect to take into account is the evaluation of the hydrodynamics of the reactor as it may influence the efficiency of the operation and thus the degradation of the selected drugs.

4.3.1. Influence of the gas supply (in continuous and in pulses) on the operation of a fed-batch stirred tank reactor for the degradation of DCF, IBP and NPX

A fed-batch experiment was carried out in a STR operated with pellets of the fungus *P. chrysosporium* for the degradation of three anti-inflammatory drugs (DCF, IBP and NPX). The configuration of the bioreactor is shown in Figure 4-3.

Figure 4-3. Configuration of a STR operated as fed-batch with fungal pellets. Symbols: (1) module operator service program, (2) air supply, (3) vessel with fungal pellets, (4) oxygen supply

Two aeration strategies were considered to compare the effect of the gas supply on the enzymatic production and the degradation of the pharmaceutical compounds. The

operation was steady for 50 days for the bioreactor with air supply and 30 days for the bioreactor with oxygen pulsation; however, for comparison purposes, only the data corresponding to the transformation of the pharmaceuticals until day 30 are presented.

The operation of air and oxygen reactors is presented in Figures 4-4, 4-5 and 4-6. The values of pH showed different behavior depending on the level of oxygen in the medium (Figure 4-4a).

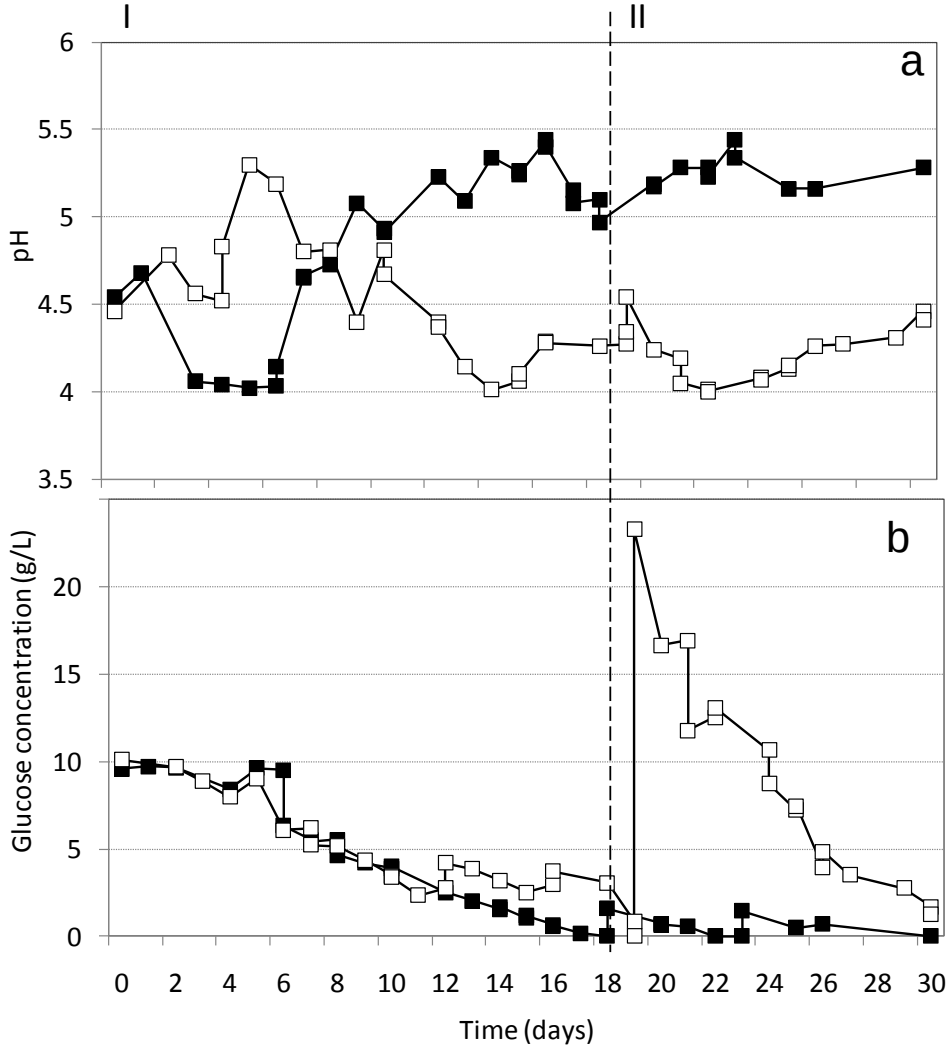


Figure 4-4. pH value (a) and glucose concentration (b) in the air (□) and oxygen (■) bioreactors

At the beginning of the operation of the air reactor, pH increased from 4.5 until 5.3; then a decrease until values near to 4 was observed. When high levels of oxygen were detected, pH decreased during the first 6 days of the experiment and an increase until 5.4 was detected for the rest of the experiment. Regarding glucose depletion, glucose was added at the beginning of both experiments (10 g/L). It was depleted with similar

consumption rates in both reactors (0.5 g/L·d) (Figure 4-4b, stage I). During the second period of the air reactor, a pulse of 20 g/L of glucose was added ensuing into a high consumption rate of glucose: 1.7 g/L·d (Figure 4-4a, stage II).

Similar trends to those obtained for the glucose concentration were found for TOC, starting at a concentration of 4 g/L and decreasing up to values of 1.4 g/L. The addition of glucose in pulses reached maximum values of 10.5 g/L and 2.6 g/L for TOC in the air and oxygen reactor, respectively (Figure 4-5). The concentration of nitrogen was maintained below 0.02 g/L (data not shown).

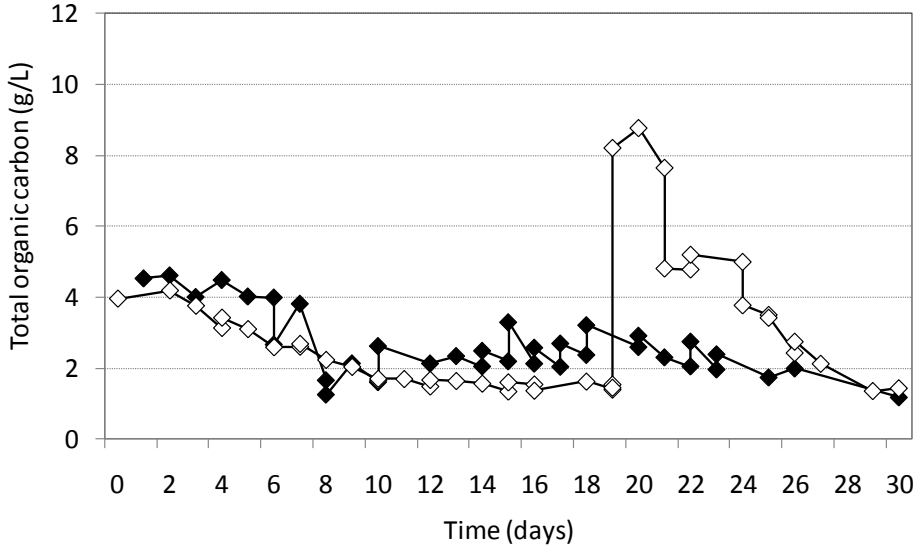


Figure 4-5. Total organic carbon in the air (◊) and oxygen (♦) reactors

Enzymatic activity was detected at low levels in a range of 5 U/L up to 40 U/L during both assays (Figure 4-6a). Peroxide concentration was detected at low levels, between 0.5 and 5 mg/L throughout both experiments. The concentration of dissolved oxygen in the oxygen reactor was maintained between 22 and 29 mg/L; except for the period between days 3 and 6, when this concentration decreased to a range of 7-8 mg/L due to a shortage in the operation and a change to air supply (Figure 4-6b). Low oxygen levels were detected in the experiment with air supply (2-7 mg/L).

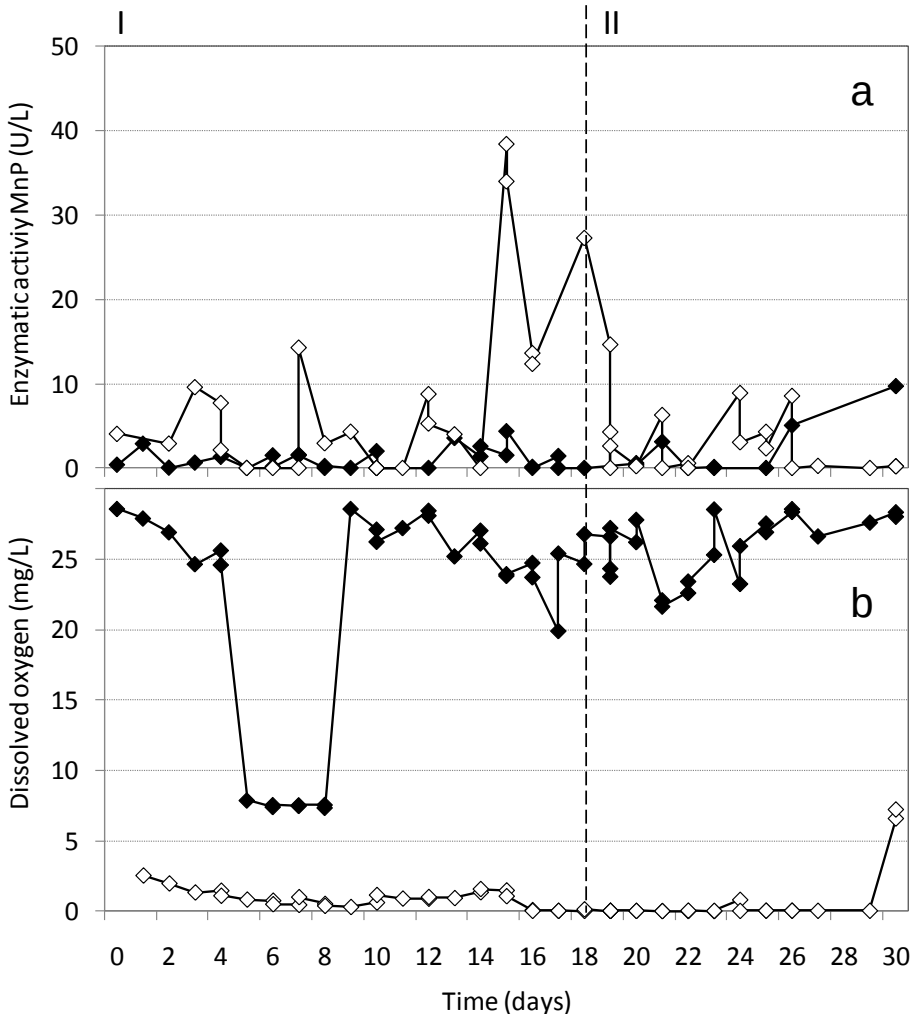


Figure 4-6. Enzymatic activity of MnP (a) and dissolved oxygen content (b) in the air (◊) and oxygen (♦) reactors

During the operation of the reactor with air supply, 8 pulses of pharmaceuticals were added. In the case of DCF, transformation percentages in the range of 65%-99% were obtained (Figure 4-7a). A similar behavior was observed in the reactor with oxygen pulsation, where a considerable reduction of the initial concentration up to 93% was obtained after the addition of 4 pulses of pharmaceuticals during the first 2 weeks of the experiment. During the period of daily pulses of pharmaceuticals, DCF was transformed by 94% after a short period, between 6 and 18 h. Finally, during the last week of experiment, this compound was totally degraded after each pulse (Figure 4-7b).

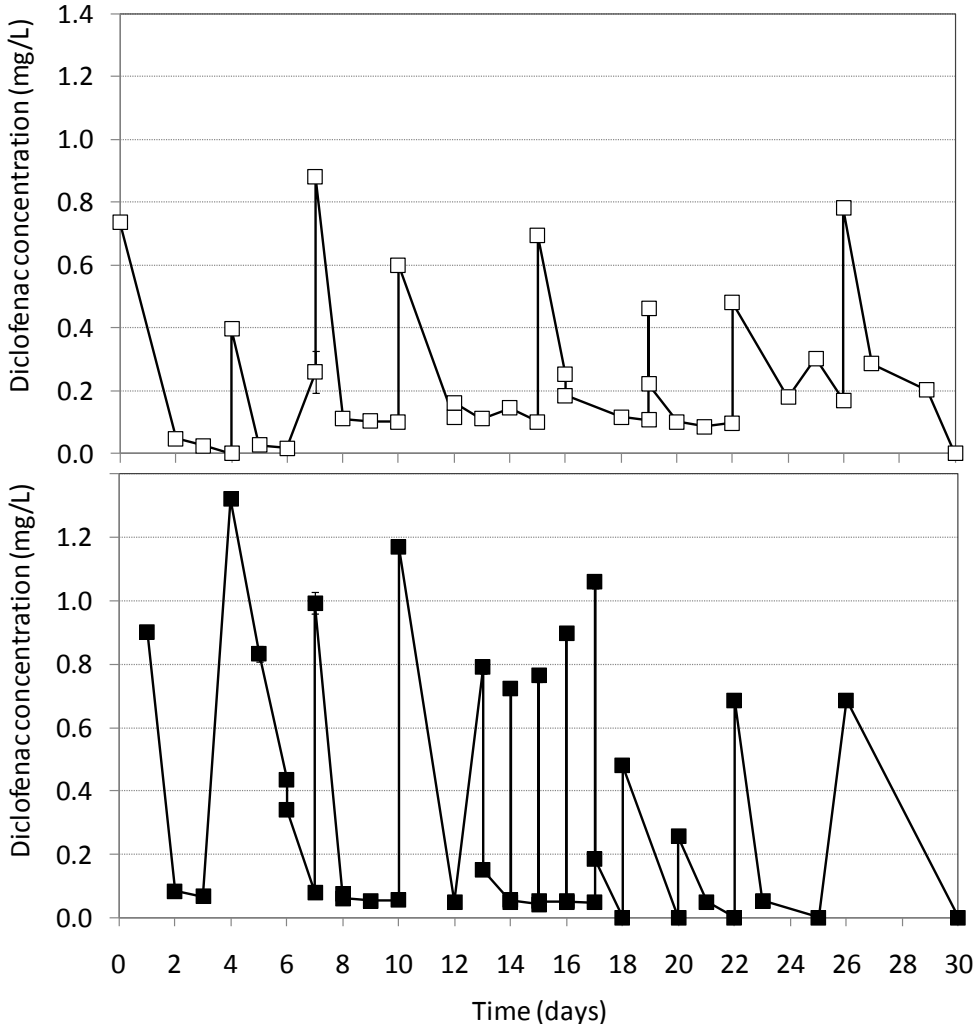


Figure 4-7. Degradation of diclofenac by pellets of *P. chrysosporium* in presence of air and oxygen. Symbols: reactor with air supply (□), reactor with oxygen pulsation (■).

Regarding IBP, complete removal was achieved in both reactors, even when daily pulses were added (Figure 4-8). For the case of NPX, transformation percentages between 78%-99% in the experiment with air supply were obtained (Figure 4-9a). Similar results were obtained in the oxygen reactor, where after the first 3 weeks of experiment, transformations up to 82% were achieved; however, during the last week of incubation, an increase of this percentage was observed (up to 97%) (Figure 4-9b).

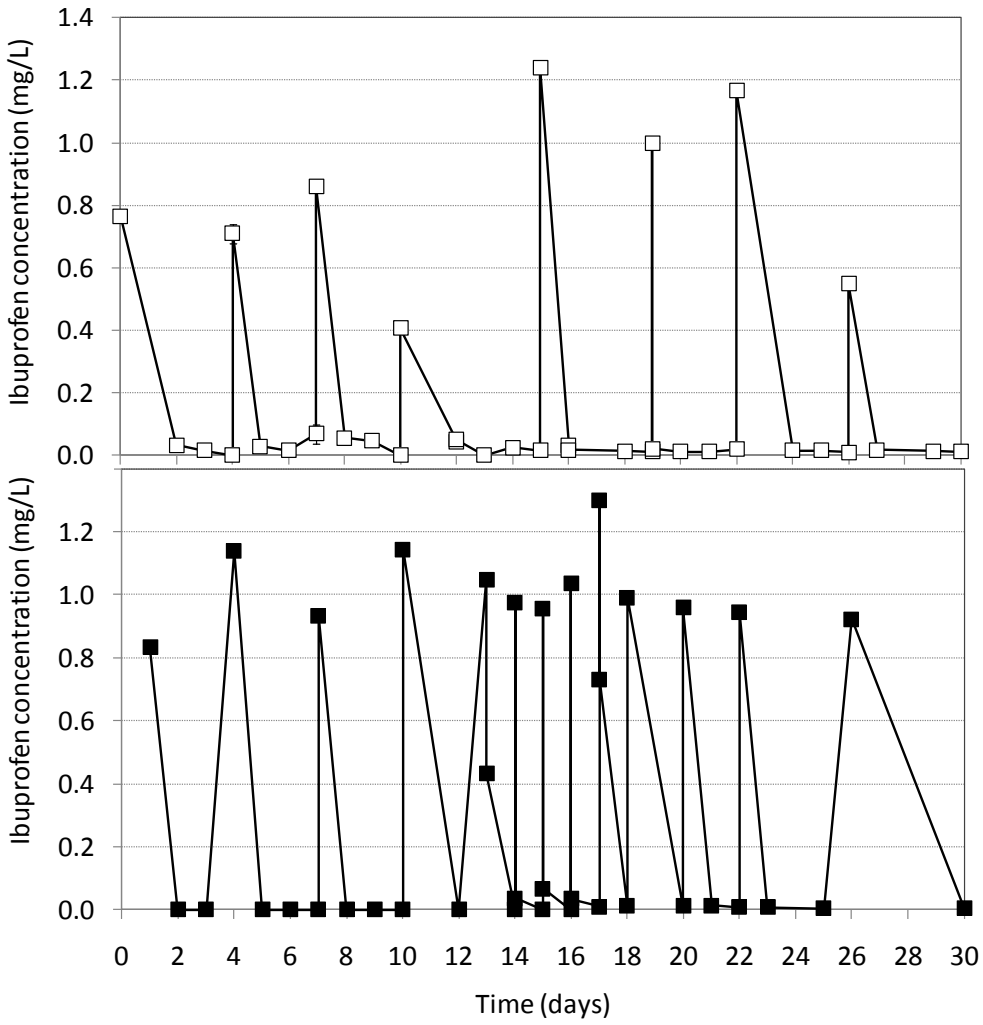


Figure 4-8. Degradation of ibuprofen by pellets of *P. chrysosporium* in presence of air and oxygen. Symbols: air reactor (□), oxygen reactor (■).

In Figure 4-10, a summary of the degradation kinetics obtained during the third week of the experiment with oxygen pulsation can be observed. Diclofenac was easily transformed in the short term, 83% after only 2 h while in the longer term, total removal was observed after 23 h. In the case of IBP, this compound was transformed only by 30% after 2 h and completely transformed after 15 h. Finally, for NPX, transformation percentages of 9% and 83% were achieved after 2 and 23 h, respectively. The degradation rates obtained during this experiment were in the range of 1.03-1.07 mg/L·d for all the analyzed compounds.

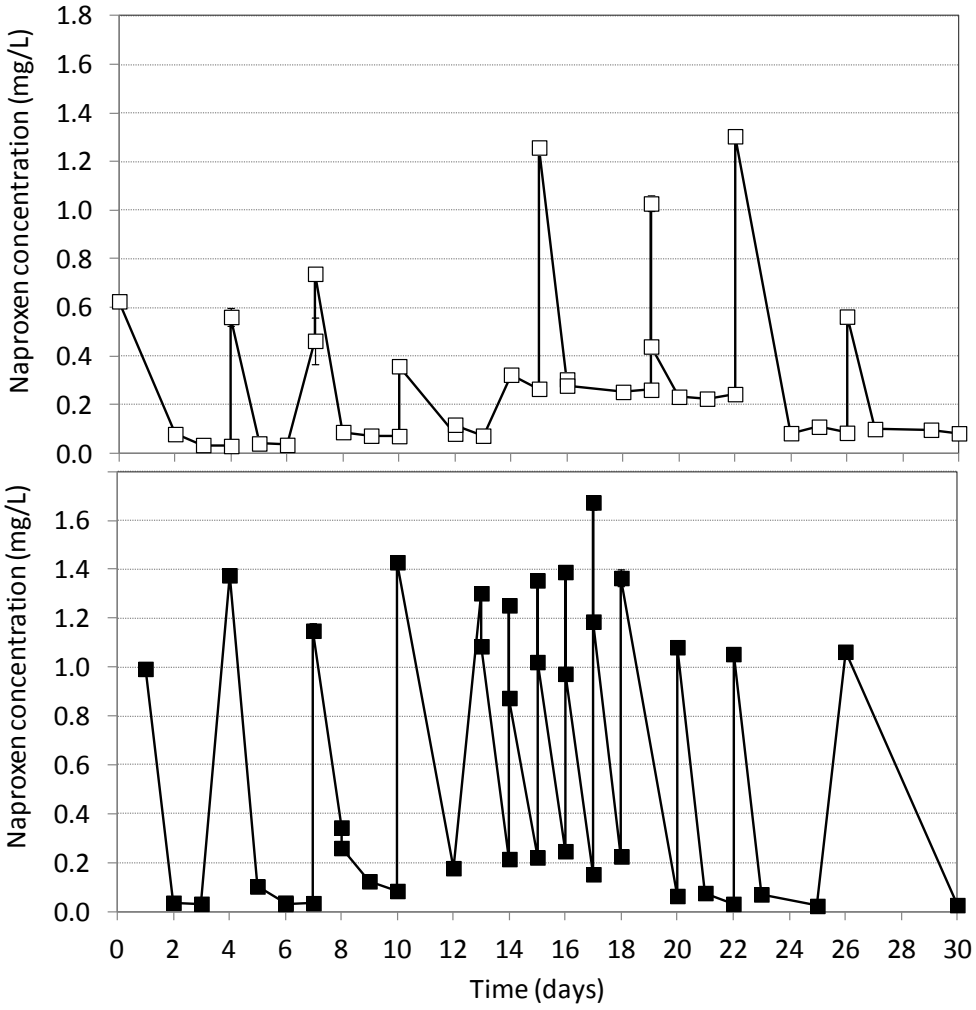


Figure 4-9. Degradation of naproxen by pellets of *P. chrysosporium* in presence of air and oxygen. Symbols: reactor with air supply (□), reactor with oxygen pulsation (■).

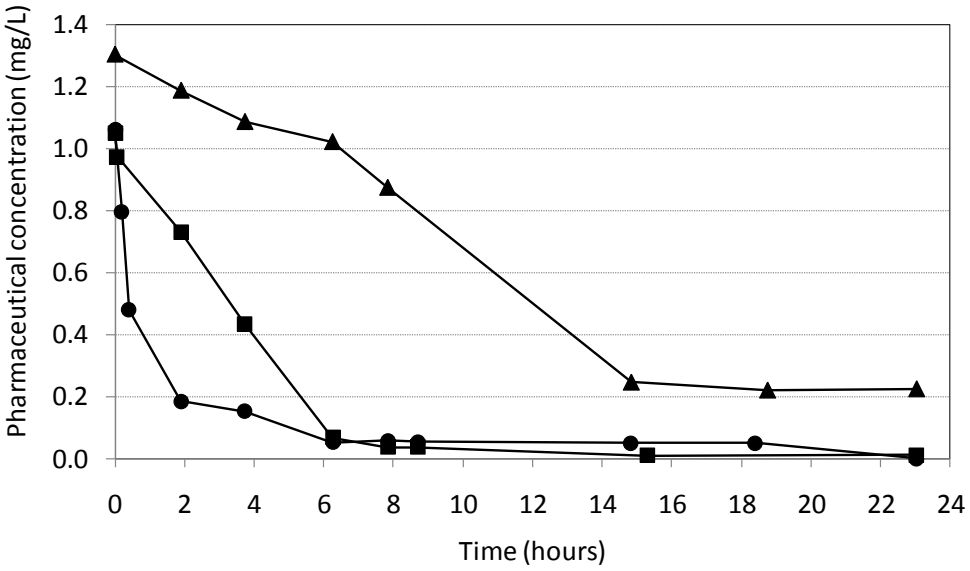


Figure 4-10. Time-course of the degradation of diclofenac, ibuprofen and naproxen during the third week of the oxygen reactor. Symbols: DCF (●), IBP (■), NPX (▲).

Residual concentration of anti-inflammatory drugs adsorbed onto the biomass is shown in Table 4-4. Low quantities of the three anti-inflammatory drugs were detected per gram of biomass corresponding to the reactor with air supply and almost undetectable amounts were found in the reactor vessel (1112.9 cm^2). In the case of the experiment with oxygen pulsation, only NPX was detected in low quantities per gram of biomass and per cm^2 of the reactor vessel.

Table 4-4 Residual concentration of anti-inflammatory drugs onto biomass and reactor vessel

	Anti-inflammatory concentration	
	Biomass (mg drug/g biomass)	Vessel (mg drug/cm^2 vessel)
Air reactor		
DCF	0.011	1.0 E $^{-5}$
IBP	0.001	7.2 E $^{-7}$
NPX	0.005	6.5 E $^{-6}$
Oxygen reactor		
DCF	n.d.	n.d.
IBP	n.d.	n.d.
NPX	0.017	8.8 E $^{-6}$

n.d. (not detected)

A high concentration of biomass was measured at the end of the experiment with air supply (6.5 g/L). This excessive growth of fungal mycelium caused an evident increase of the broth viscosity (Figure 4-11). It is important to highlight that this experiment was carried out until day 50. Nevertheless, for comparison purposes with the oxygen reactor, data were depicted only until day 30. During the second stage of the oxygen reactor, two pulses of glucose (~2 g/L) were added at days 18 and 23, which led to a limited consumption of glucose: 0.3 g/L·d and restricted biomass growth (1.5 g/L) after 30 days of operation (Figure 4-4, stage II). Thus, glucose consumption per gram of biomass was 0.26 g glucose/g biomass per day for the air reactor; while in the oxygen reactor it was 0.20 g glucose/g biomass per day.

Figure 4-11. Appearance of the reactor with air supply (a) and the reactor with oxygen pulsation (b) operated with pellets of *P. chrysosporium*.

Figure 4-12 shows the photographs corresponding to the pellets from the air and oxygen reactors at days 7 and 30. As expected, the pellet diameters from both reactors were similar at the beginning of both experiments: 2.8 mm. However, shortly after day 7, the size of the pellet from the oxygen pulsed reactor was smaller than the one from the continuous supply of air (2.3-fold approx.) and the pellet was dense and hyphae-free while the one of the air reactor presented filamentous growth.

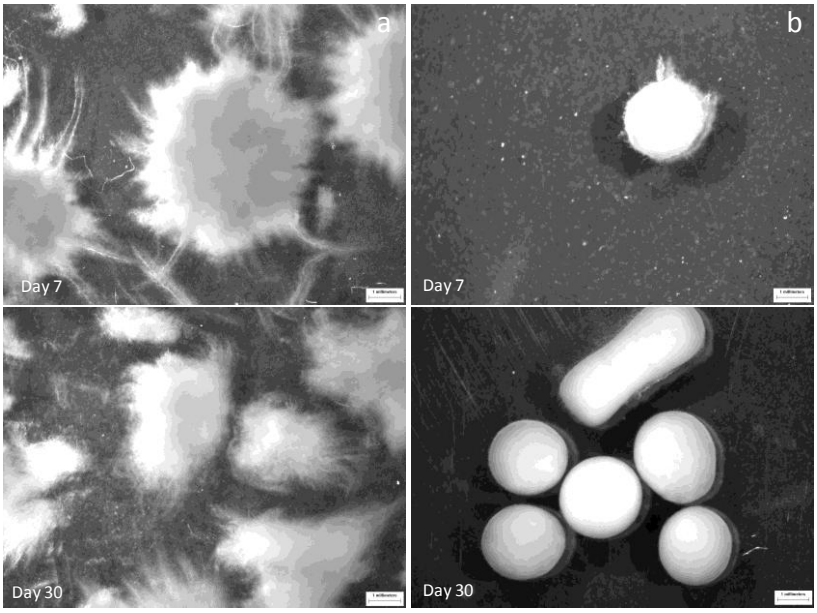


Figure 4-12. Pellet morphology by stereomicroscope from the reactor with air supply (a) and the reactor with oxygen pulsation (b)

4.3.2. Influence of the feed regime (fed-batch and continuous) on the operation of a stirred tank reactor for the degradation of DCF, IBP, NPX and CBZ

Configuration of the STR operated as fed-batch was already showed in Figure 4-3 with a continuous air supply. After 26 days of operation, the feeding regime of the reactor was changed to continuous until day 70 (Figure 4-13).

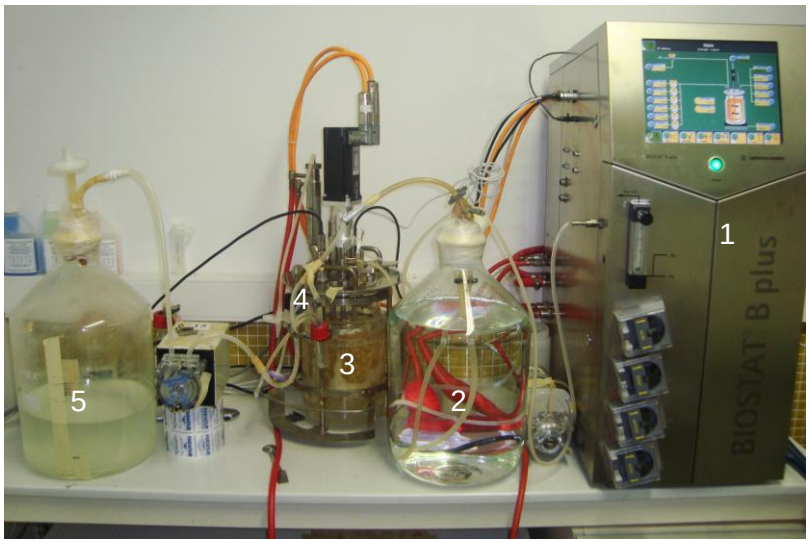


Figure 4-13. Configuration of a STR with fungal pellets during a continuous operation. Symbols: (1) module operator service program, (2) feed medium port, (3) reactor vessel filled with fungal pellets, (4) effluent sampling, (5) effluent reservoir

The evolution of the fermentation during the operation of the reactor with air supply and fed-batch and continuous feeding are presented in Figures 4-14, 4-15 and 4-16.

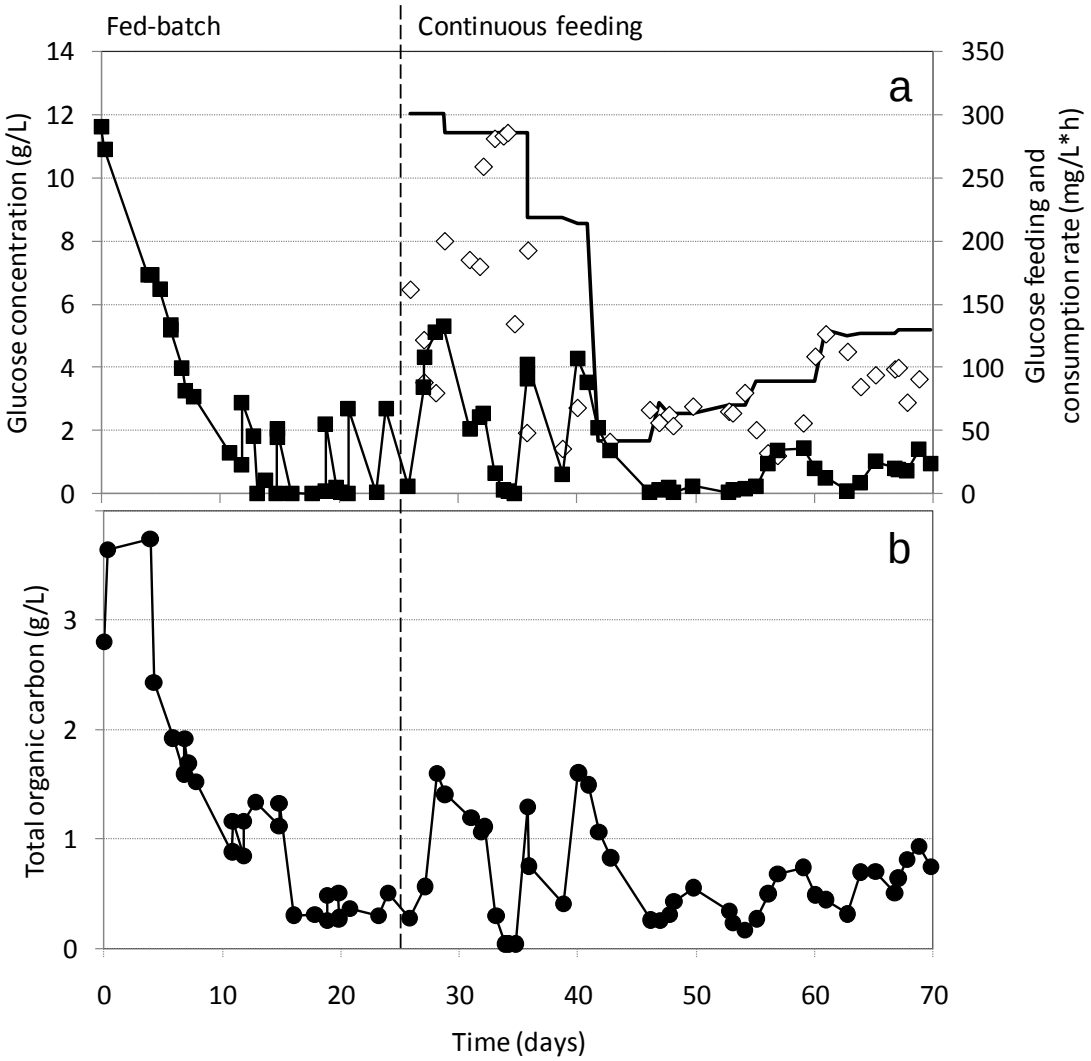


Figure 4-14. Glucose concentration, consumption and feed rate (a) and total organic carbon content (b) in the free pellets reactor. Symbols: glucose concentration in the reactor effluent (■), glucose feeding rate (—), glucose consumption rate (◊), TOC (●)

During the fed-batch stage, the reactor was fed with 10 g/L of glucose that was depleted at day 12; then, five pulses of glucose were added to the reactor (~2.5 g/L) at days 12, 14, 19, 21 and 24. During this stage, the consumption rate of glucose was maintained around 1.3 g/L·d (Figure 4-14a, fed-batch stage). At day 26th, the reactor started the operation with continuous feeding using different glucose feeding rates (50-300 mg/L·h) and substrate concentrations were detected below 5 g/L in the effluent during the first 5 days of this stage. Thereafter, the feeding rate was reduced in order to

maintain the glucose concentration in the effluent below 2 g/L (Figure 4-14a, continuous stage). Besides, TOC was detected during the fed-batch stage at concentrations of 3.7 g/L, decreasing to values of 0.3 g/L (Figure 4-14b, fed-batch stage). When the reactor started to operate with a continuous feeding, TOC was detected at a concentration of 1.5 g/L until day 40, and then decreased to values below 0.5 g/L due to the low glucose addition rate (Figure 4-14b, continuous stage). Total nitrogen content was maintained below 0.03 g/L during the 70 days of the experiment (data not shown).

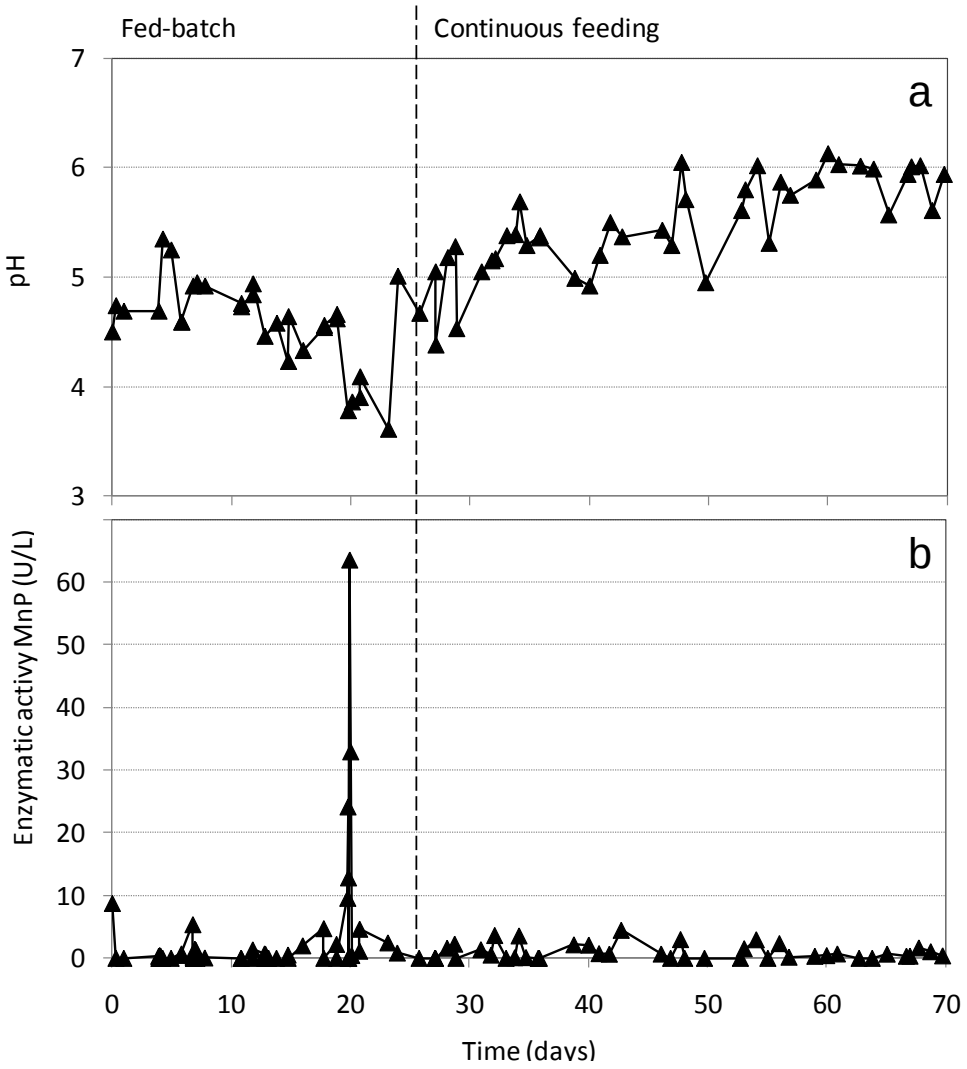


Figure 4-15. pH value (a) and enzymatic activity (b) detected during the fed-batch and continuous experiment in a STR.

The pH value in the reactor was detected in a range of 3.7 up to 5.3 during the fed-batch stage (Figure 4-15a). Then, an increase of pH was observed until values of 6.1 during the continuous stage, although the highest values were measured during the last 10 days

of experiment. Negligible activities of the enzyme MnP were detected throughout the experiment; only a peak of 60 U/L at day 20 was observed (Figure 4-15b). Peroxide content was maintained below 2 mg H_2O_2/L during both stages of the experiment (data not shown).

Gas flow was maintained at 3.0 L/min during the fed-batch stage; meanwhile during the continuous feeding stage, aeration flow was in a range between 1.0-2.5 L/min. Agitation was fixed at 200 rpm throughout the experiment, except during the last week when 250 rpm were used. Dissolved oxygen concentration during the fed-batch stage was maintained below 4 mg/L during the initial 20 days of experiment, decreasing to values near to zero during the last days of this stage (Figure 4-16, fed-batch stage). Contrary to this, when a continuous feeding was added, oxygen was maintained in a range between 2–8 mg/L until day 69, then this value decreased until zero (Figure 4-16, continuous stage).

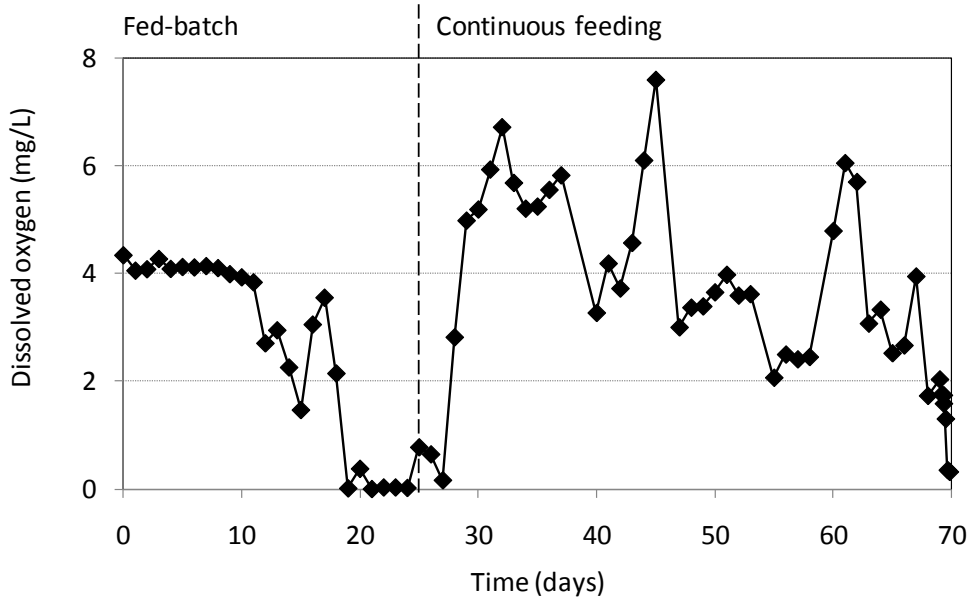


Figure 4-16. Dissolved oxygen (♦) during the experiment with pellets of *P. chrysosporium*

Figure 4-17 shows the results of the time-course degradation experiments that were carried out at days 0, 4, 7, 14 and 20. Diclofenac was slightly removed at the beginning of the bioreactor operation: only 30% at day 1. In the following days, removal of this compound was enhanced with degradation percentages around 80% (days 4, 7 and 14) and a maximum removal of 94% at day 20 (Figure 4-17, DCF). In terms of degradation rate, the lowest rate was obtained at day 0: 0.4 mg/L·d. Average degradation rates between 0.8-1 mg/L·d were observed at days 4, 7 and 14 and finally, the highest removal rate of 1.5 mg/L·d was achieved at day 20.

Ibuprofen shows a similar trend as DCF since the lowest rate corresponded to day 0 where less than 66% were degraded after 8 h. Thereafter, 95% of this compound was efficiently degraded after 24 h. Even more, a degradation percentage of 50% was achieved after a very short period of time: only 30 min at day 20. These results implied that overall degradation rate was maintained around 1.0 mg/L·d during the first 20 days of operation (Figure 4-17, IBP).

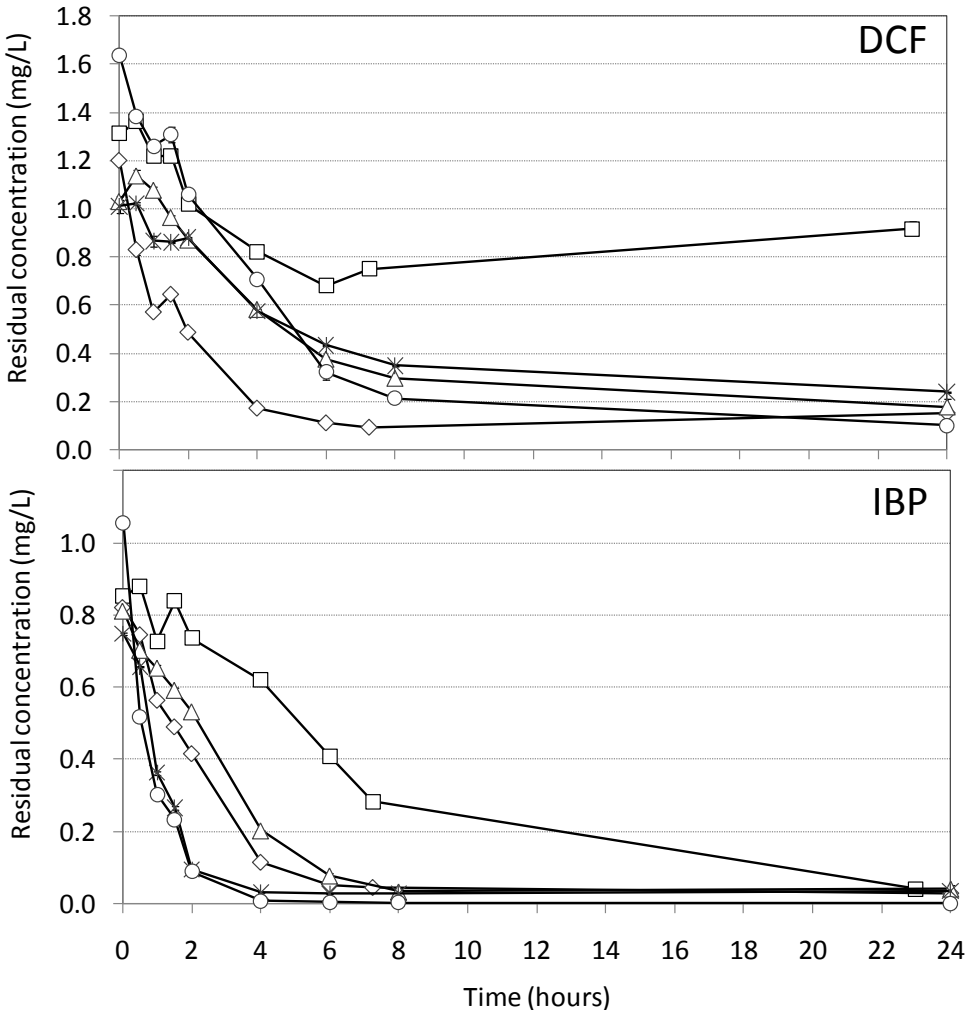


Figure 4-17. Time-course degradation of diclofenac (a) and ibuprofen (b) by pellets of *P. chrysosporium* during the fed-batch stage. Symbols: day 0 (□), day 4(◊), day 7 (Δ), day 14 (*), day 20 (○).

Naproxen was partially degraded (57%) after 24 hours at day 0, higher percentages achieved at days 4, 7 and 14 (~80%) and finally the maximum removal was reached at day 20 (94%) (Figure 4-18, NPX). The corresponding degradation rates were ranged between 0.6-1.3 mg/L·d. Contrary to the results shown above, the antiepileptic CBZ was only partially degraded at day 4 and 7 (64% and 32%, respectively) with degradation rates in the range of 0.5-0.7 mg/L·d (Figure 4-18, CBZ).

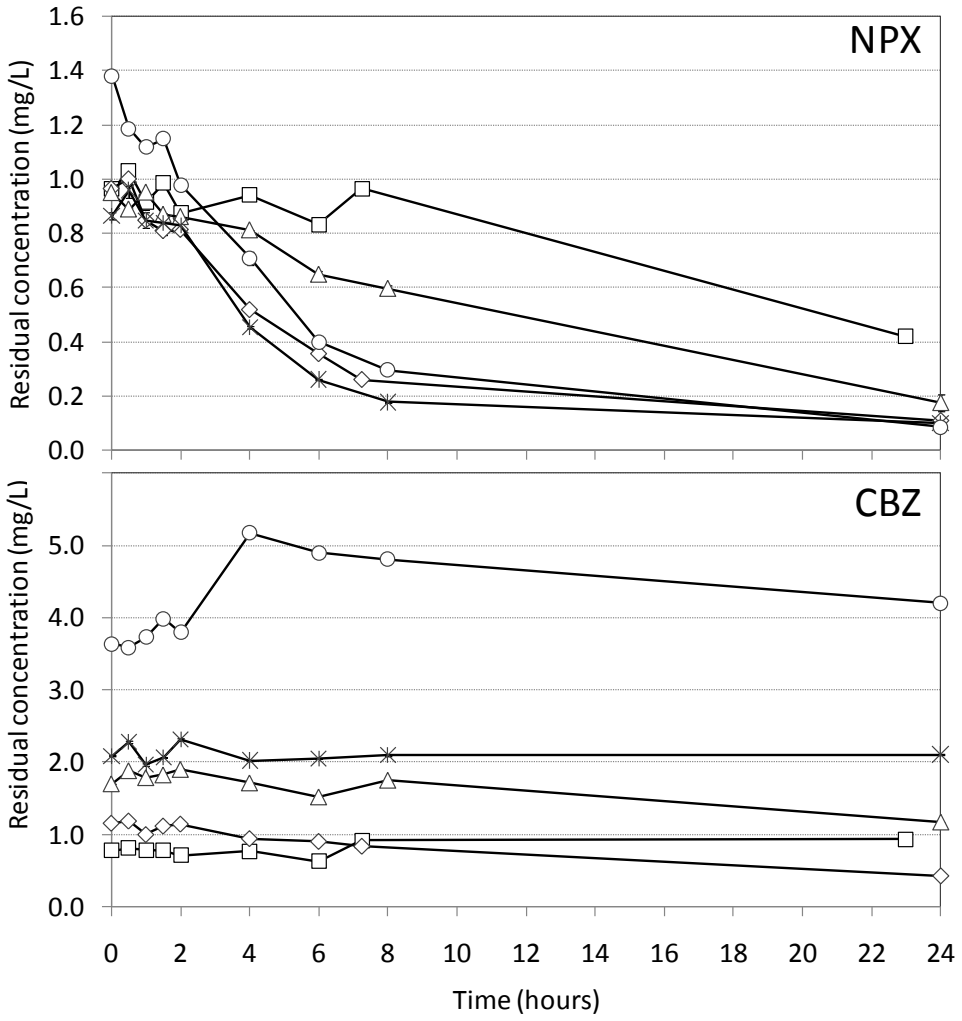


Figure 4-18. Time-course degradation of naproxen (a) and carbamazepine (b) by pellets of *P. chrysosporium* during the fed-batch stage. Symbols: day 0 (□), day 4(◊), day 7 (Δ), day 14 (*), day 20 (○).

Table 4-5 shows the residual percentage of the drugs analyzed in the extracellular liquid and the abiotic control after 24 h. These experiments were performed to assess the capability of the extracellular fluid containing MnP to carry out the degradation of the target compounds under an *in vitro* perspective, that is, by the single action of the enzyme in the absence of the fungal culture. Moreover, the monitoring of abiotic controls would allow quantifying any volatilization process. A slight removal in the extracellular liquid was observed specially in the case of DCF (day 7) as well as IBP and NPX (day 20). In addition, low activities of MnP were detected throughout this stage, except at day 20 where above 60 U/L were detected. In the case of abiotic controls more than 83% of the compounds were detected after 24 hours in all the experiments analyzed.

Table 4-5. Residual percentage in the extracellular liquid and abiotic control after 24 h.

	Residual percentage (%)			
Days	DCF	IBP	NPX	CBZ
Extracellular liquid				
0	93	87	95	>100
4	>100	94	87	98
7	75	93	96	>100
14	96	>100	>100	99
20	80	76	70	>100
Abiotic control				
0	n.d.	n.d.	n.d.	n.d.
4	94	96	97	90
7	95	90	84	83
14	90	99	95	93
20	89	96	92	93

n.d. (not determined)

Figures 4-19 and 4-20 shows the feeding and degradation rates of DCF, IPB, NPX and CBZ obtained during the continuous stage in this experiment. The reactor feeding medium was prepared every 5 or 7 days, as needed, including the pharmaceutical compounds. For this reason, feeding rates of pharmaceutical compounds to the reactor were varied. In the case of DCF feeding rates, these were detected in a range between 0.9 and 1.4 mg/L·d, except between days 40 and 47 when an increase up to 1.7 mg/L·d was applied. Concerning DCF degradation rates, it can be observed that this compound was highly degraded during the period between days 26 and 54 (up to 90%); then, a decrease in this rate was observed between days 55 and 63 (34%-70%). Finally, the degradation rate started to increase from day 66 until the end of the experiment (69%-82%) (Figure 4-19, DCF). Regarding the feeding rates of IBP, a range of 0.8-1.2 mg/L·d was applied with removal percentages above 94% until day 54th. Thereafter, a slight decrease was observed until the end of the experiment, although the percentages were higher than those obtained for DCF (65%-95%) (Figure 4-19, IBP).

Figure 4-20 shows the results for NPX, where it can be observed that the feeding rates of this compound were detected in a range of 0.9-1.3 mg/L·d throughout the experiment, although during the last two days of the experiment this rate was of only 0.7 mg/L·d. This compound was highly degraded in the period between the days 26-54 in a range of 77% up to 94%; from the day 55 until the day 66, the removal rates have suffered a notorious decline, until 21% of removal. Finally, during the last days of experiment, the fungus was not able to eliminate this drug, though a 17% or removal of NPX was detected the last day.

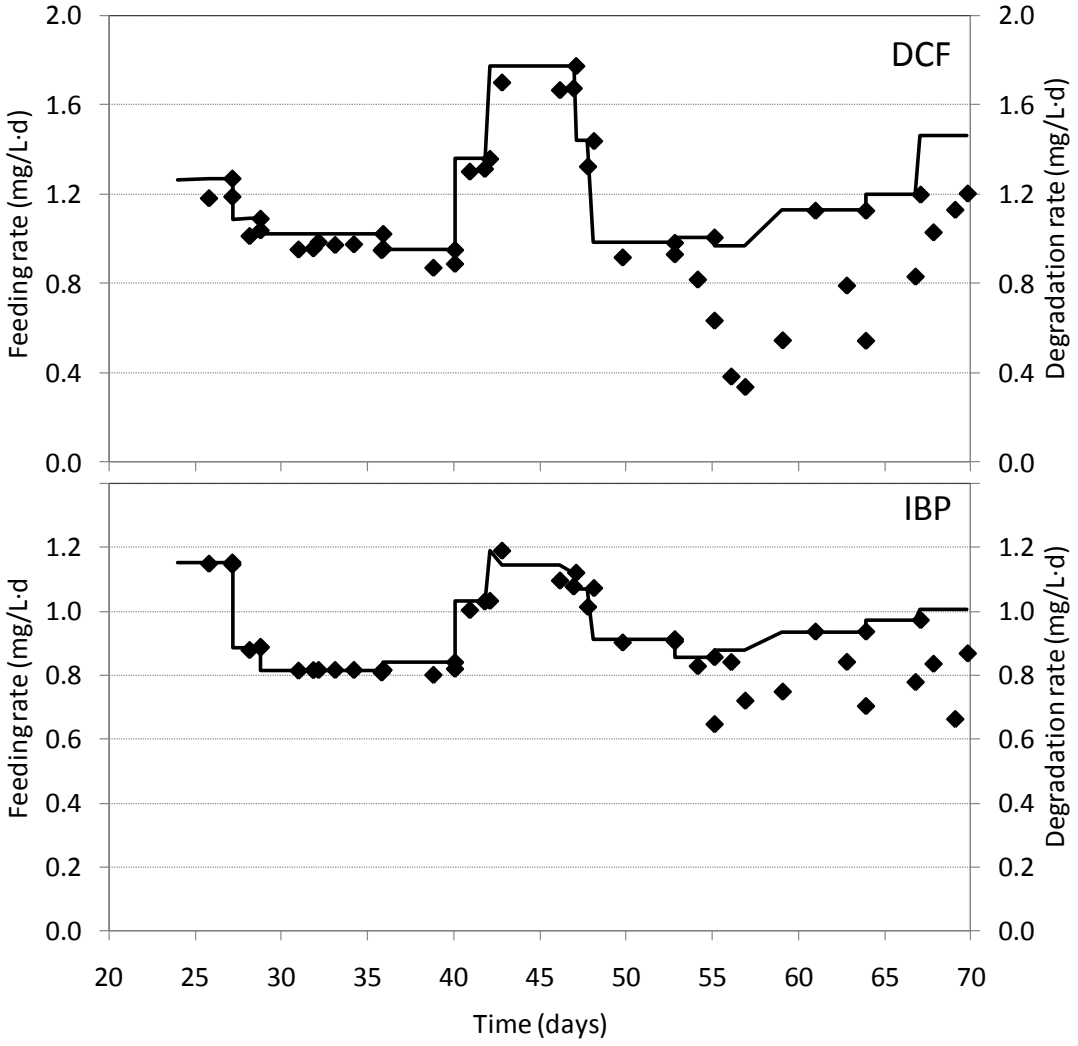


Figure 4-19. Feeding rates (—) and degradation rates (◊) of DCF and IBP during the continuous stage of the bioreactor with pellets of *P. chrysosporium*.

On the other hand, CBZ feeding rates were maintained with a decreasing tendency from 2.2 mg/L·d until 1.0 mg/L·d throughout the experiment; although a slightly increase can be observed during the last days of experiment (1.3 mg/L·d). The results obtained during the degradation of CBZ were different from those obtained for the anti-inflammatory drugs (Figure 4-20, CBZ). Regarding to the degradation of CBZ, during the first days of this experiment (26th until 28th) the fungus was able to remove this compound in a range of 58%-80%. After this day, the reactor was able to maintain high degradation rates (72%-93%) until day 53; however at day 40 a decrease until 38% was observed. From day 54, the removal rates of this compound were decreasing again until only 5%; despite this, an improvement in the degradation rate can be observed during the last week of the experiment achieving removals ranging from 25% to 44%.

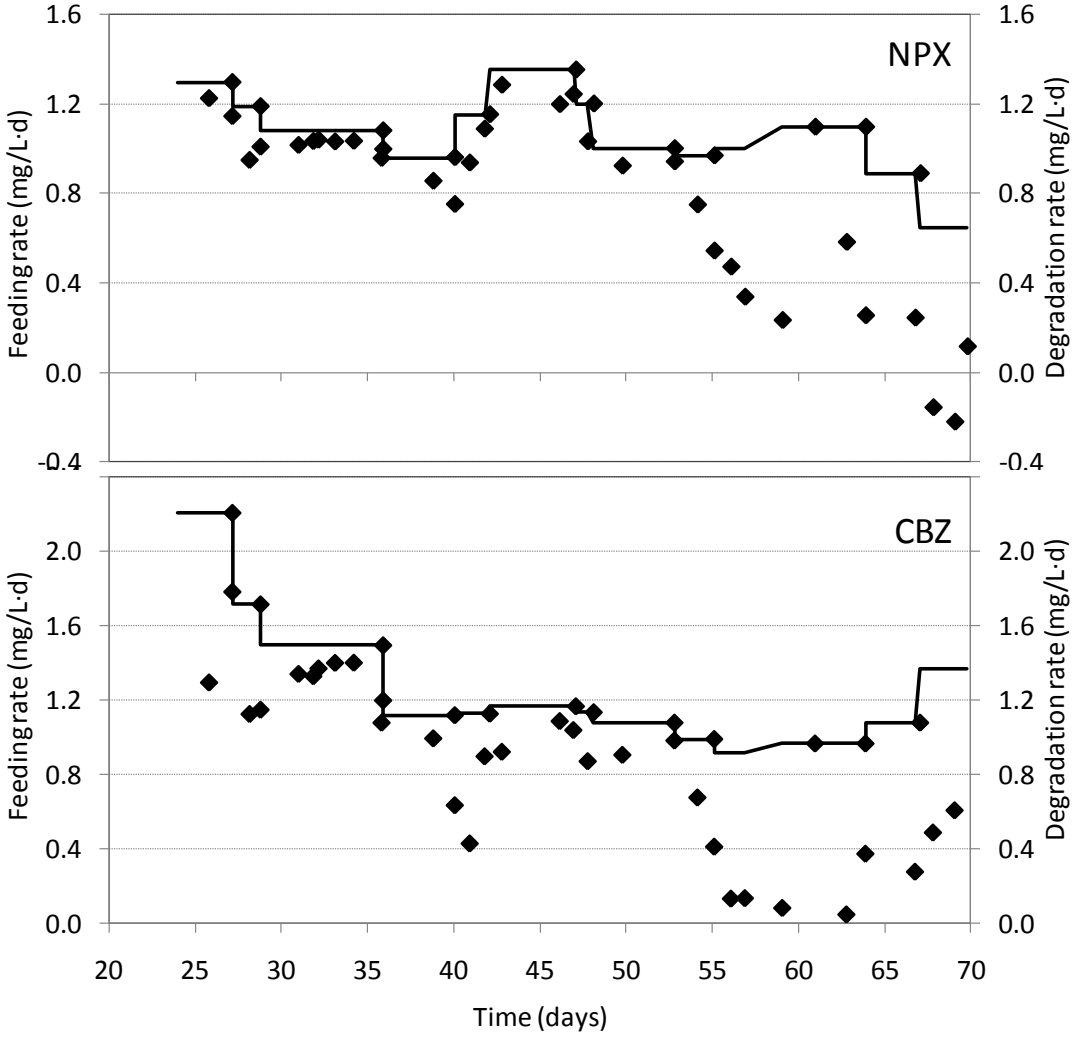


Figure 4-20. Feeding rates (—) and degradation rates (◊) of NPX and CBZ during the continuous stage of the bioreactor with pellets of *P. chrysosporium*.

Table 4-6 shows the residual concentration of three anti-inflammatory drugs and an antiepileptic drug that were absorbed on the fungal biomass and reactor vessel. At the end of this experiment, it was possible to detect all the analyzed compounds in the biomass. High quantities of NPX and CBZ were detected per gram of biomass (up to 0.099 mg of drug). In the case of DCF and IBP, around 0.022 and 0.039 mg of this drugs were detected per gram of biomass. On the other hand, almost undetectable amounts of the three anti-inflammatories were found in the reactor vessel, while CBZ could not be quantified since the amount of this compound was below the minimum detection limit.

Table 4-6 Residual concentration of pharmaceuticals on the fungal biomass and reactor vessel

	Pharmaceuticals concentration	
	Biomass (mg drug/g biomass)	Vessel (mg drug/cm^2 vessel)
DCF	0.039	$5.6\ E^{-6}$
IBP	0.022	$1.3\ E^{-5}$
NPX	0.067	$1.1\ E^{-5}$
CBZ	0.099	BDL

BDL (below detection limit)

The operation of the reactor was steady during the 70 days of the experiment achieving a final biomass concentration of 7.8 g/L. During the fed-batch stage, the appearance of the reactor pellets was maintained as shown in Figure 4-21 (day 8), where the pellets were dense and compact; however a filamentous growth started to show after 3 weeks of experiment (Figure 4-22, day 20). Once the continuous feeding started (Figure 4-21, day 40), excessive growth of the fungus was observed; then hyphal branches and aggregates of pellets started to breakdown.

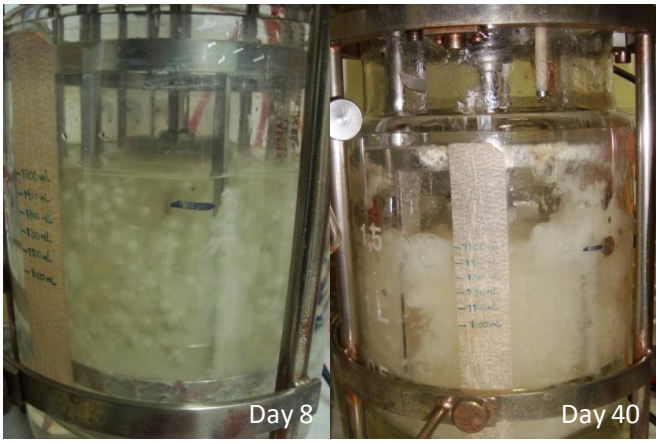


Figure 4-21 Appearance of the pellets of *P. chrysosporium* during the fed-batch and continuous experiment in the bioreactor.

The morphology of the pellets and their size were determined throughout the experiment; however to discuss this aspect only a few examples are shown in Figure 4-22 (days 1, 20, 40 and 70). A constant size throughout the experiment, in the range of 3.2-4.0 mm, was maintained. The release of aggregates of pellets was observed at day 40. New pellets with a compact aspect were formed from the fragments of fungal mycelia (Figure 4-22, day 70). Despite the excessive fungal growth, no clogging problems were observed throughout the 70 days of operation in the STR.

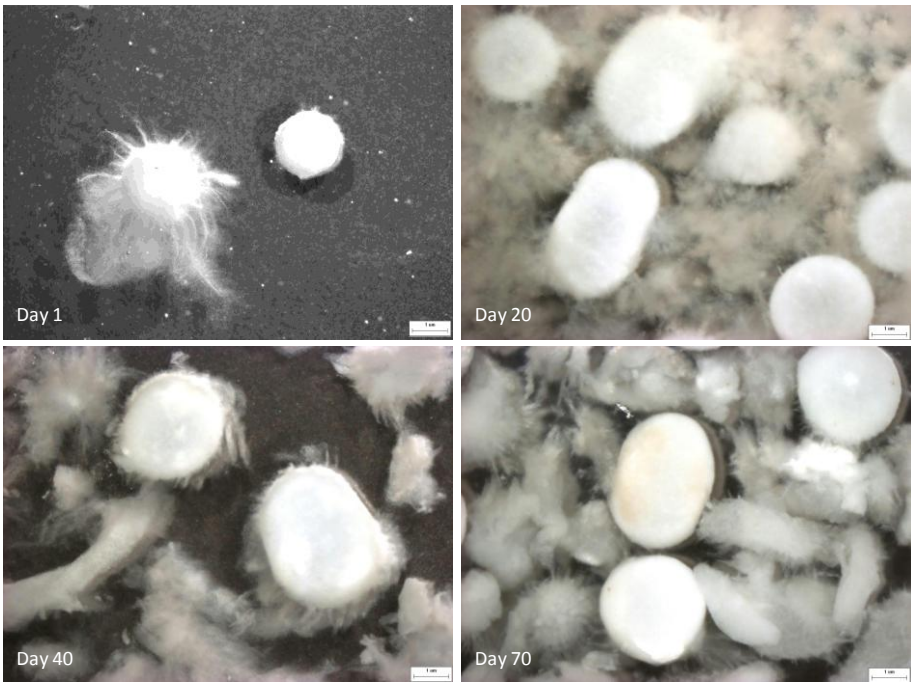


Figure 4-22. Pellet morphology by stereomicroscope during the aerated experiment

4.3.3. Operation of a continuous stirred tank reactor for the degradation of the DCF, IBP, NPX, CBZ and DZP

A continuous operation (nutrients and pharmaceutical compounds) was performed in this experiment for the removal of DCF, IBP, NPX, CBZ and DZP by pellets of *P. chrysosporium*. The configuration of the STR operated with a continuous feeding was the same as in the stage II of the previous experiment (Figure 4-23). A continuous air flow was also used in this experiment and the reactor was operated during 50 days.

The feed medium was added with a glucose concentration in a range between 4 g/L and 6 g/L during the experiment. Glucose was detected below 2 g/L in the effluent until day 30; then an increase until 3 g/L was observed (Figure 4-23a). This increase is related to the necessary changes in the addition rates. Since the HRT was of 24 hours, the feeding rate of glucose was maintained in a range between 150 mg/L·h and 250 mg/L·h (Figure 4-23b). Glucose consumption was negligible during the first days of experiment; it was until day 5 when a high consumption was observed. Despite this, from day 30 until the end of the experiment the fungus was able to consume about 60% of the glucose added into the reactor (Figure 4-23b).

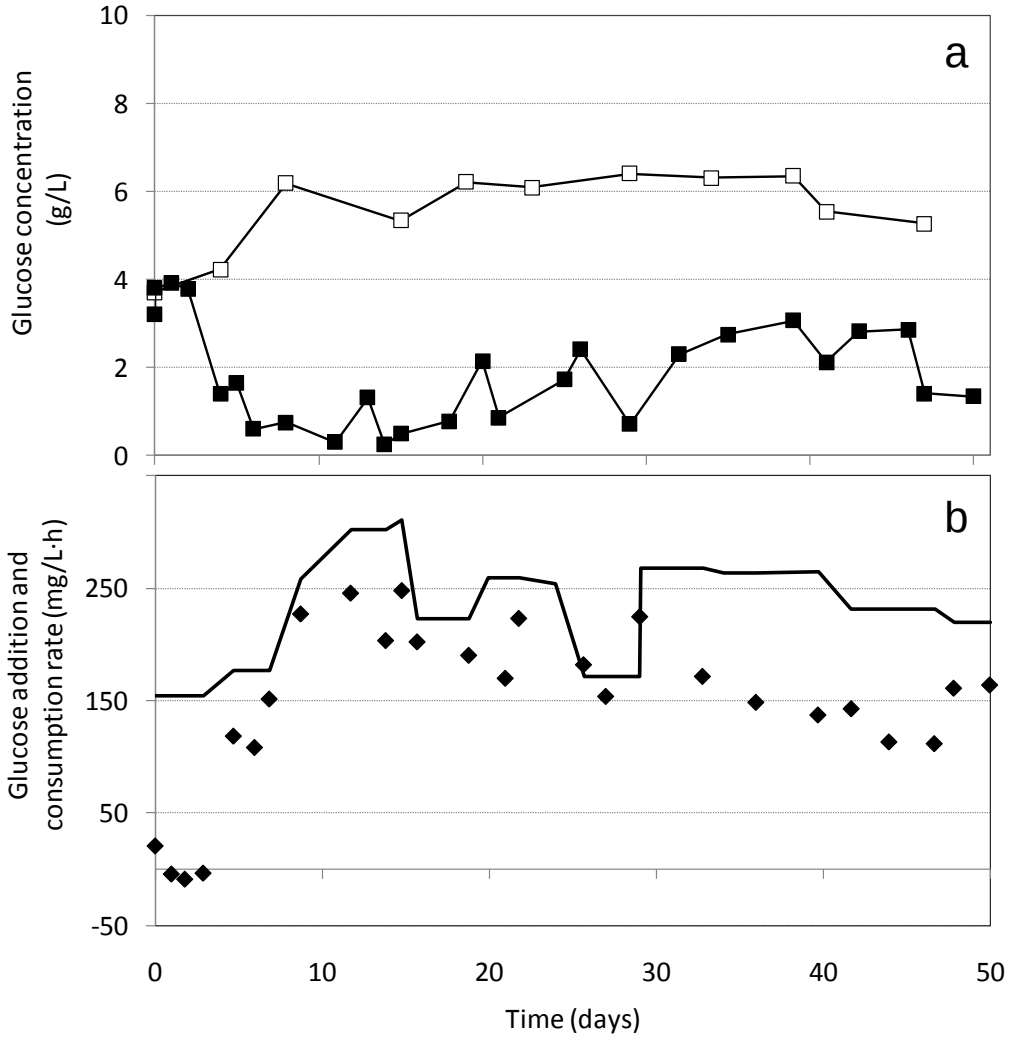


Figure 4-23. Glucose concentration (a) as its addition and consumption rates (b) during the continuous experiment in a STR. Symbols: feed medium (□), reactor effluent (■), glucose addition rate (—), glucose consumption rate (♦)

As shown in Figure 4-24a, the pH value in the reactor increased from 4.5 until 5.4 during the start of the operation; then, it was maintained below 5. The pH was not controlled through the 50 days of operation. Low enzymatic activities were detected throughout the experiment; however, a high peak of activity of MnP (200 U/L) was observed during the last days of the experiment (Figure 4-24b).

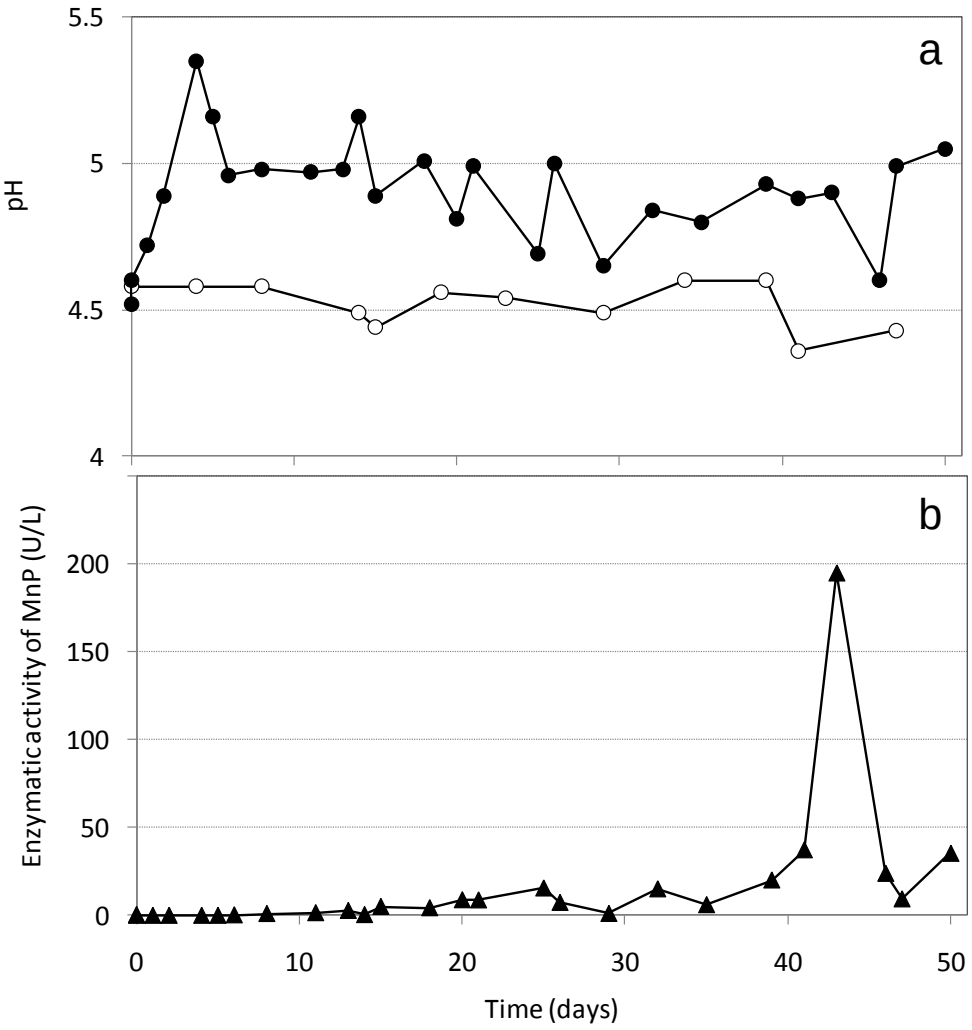


Figure 4-24. pH value (a) and enzymatic activity (b) during the continuous experiment in a STR. Symbols: feed medium (○), reactor effluent (●), enzymatic activity (▲).

In this experiment, a continuous air flow was maintained to aerate the reactor, for this reason the maximum concentration of dissolved oxygen detected was 7.9 mg/L (Figure 4-25a). At the beginning of the experiment, a high level of oxygen was measured; then, a decrease until concentrations below 2 g/L during the period between day 7 and 38 was detected. Finally, an increase of this parameter was observed during the last days of the experiment. The content of TOC is also shown in Figure 4-24b. When the operation started, concentrations of organic carbon were similar in the feed medium and the effluent. In the former this content was maintained between 2 g/L and 3 g/L; while in the outlet stream of the reactor the TOC was detected below 2 g/L until the end of the experiment (Figure 4-25b). The concentration of total nitrogen was detected below 0.05 g/L in the feed medium and the reactor effluent; also the peroxide content was measured finding concentrations below 2 mg/L over the 50 days of experiment (data not shown).

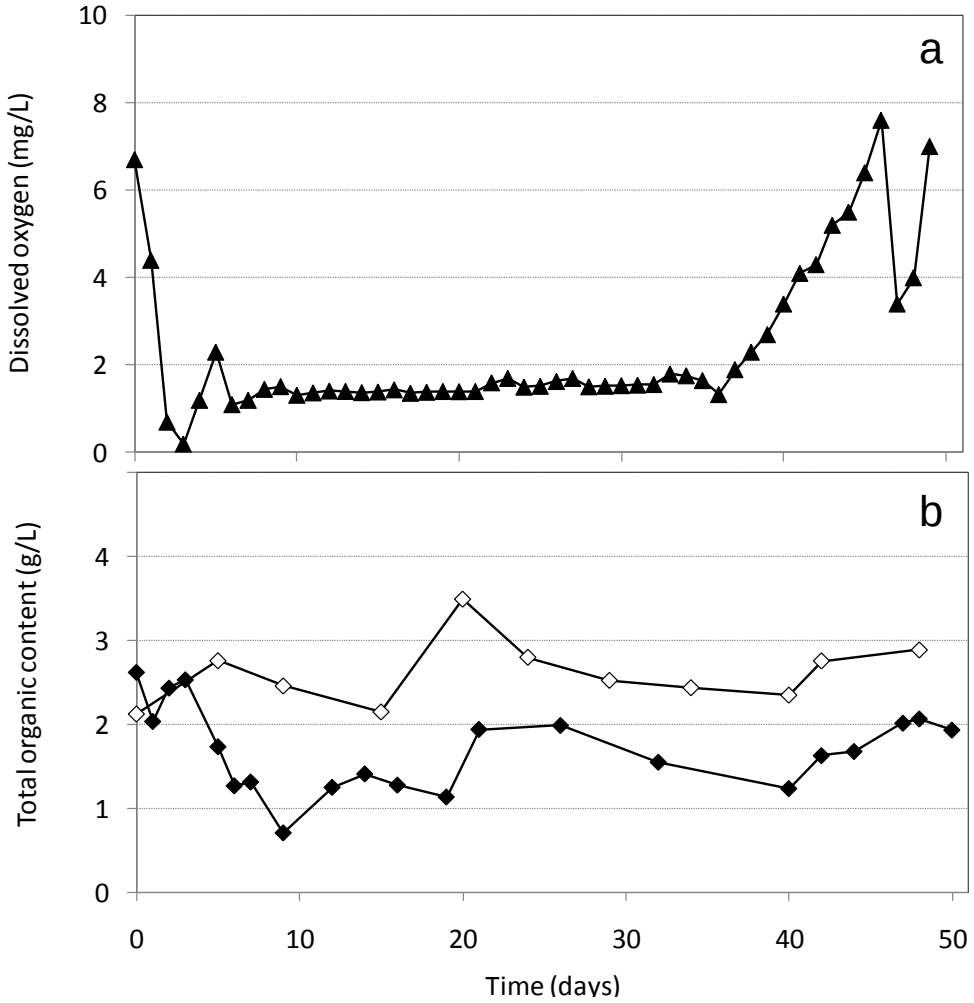


Figure 4-25. Dissolved oxygen (a) and total organic carbon (b) during the continuous experiment in a STR. Symbols: dissolved oxygen (▲), feed medium (◊), reactor effluent (♦)

The addition and consumption rates of the three anti-inflammatories drugs are shown in Figure 4-26. The drug DCF was not removed when the experiment started (day 1); however, high consumptions were observed during the first two weeks of experiment, except at day 12 (Figure 4-26a). From day 15 until the end of this experiment, this drug was easily degraded by the fungus *P. chrysosporium.* Ibuprofen degradation showed the same behavior than DCF drug, since low removals were detected at the start of the bioreactor, excepting at day 12. This decrease in the removal efficiency of the anti-inflammatory drugs might be caused for an adjustment of the reactor volume with fresh medium just before the sample was withdrawn. Finally, highly removals were achieved in the period between day 15 and day 50 (Figure 4-26b).

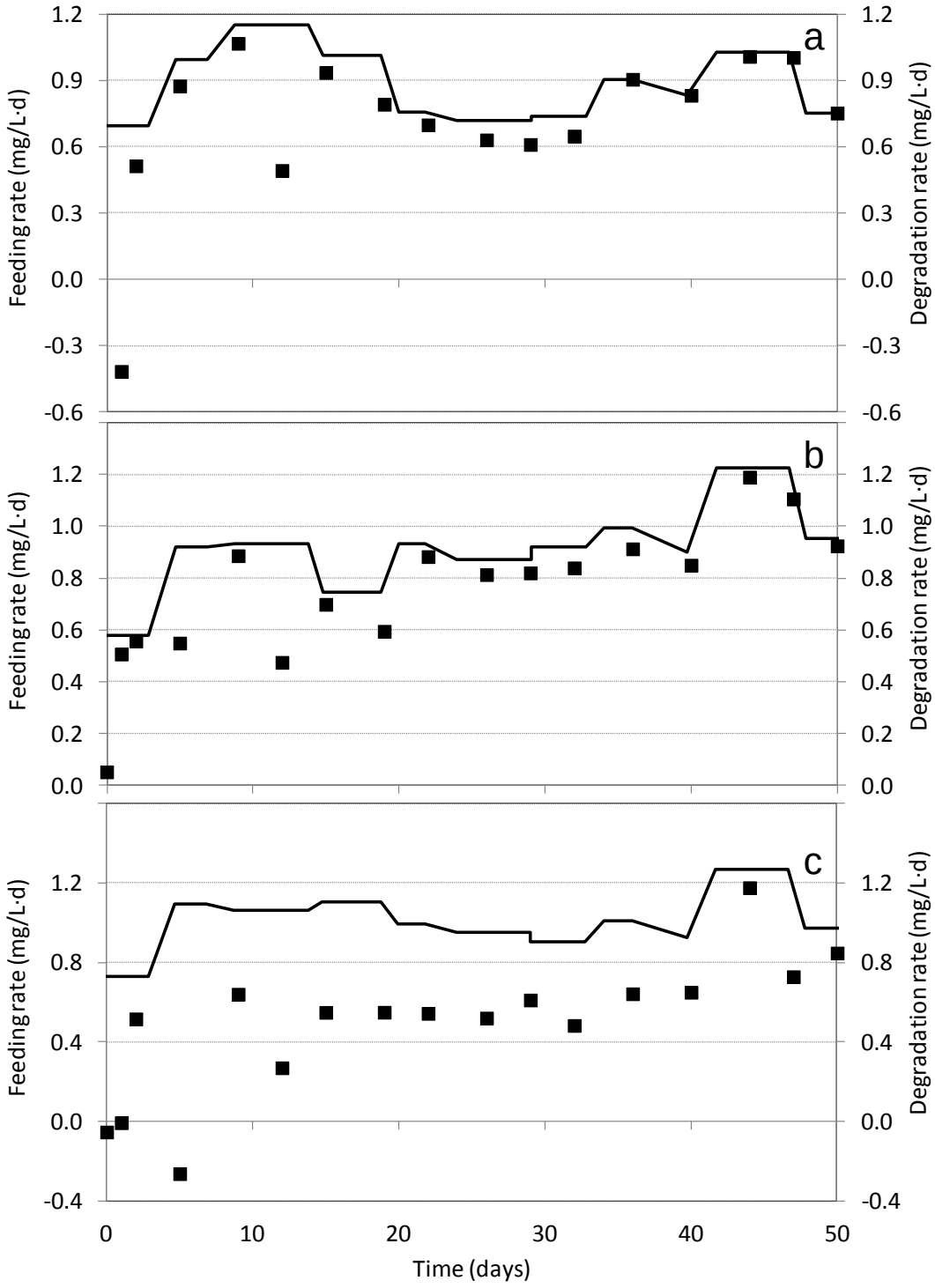


Figure 4-26. Feeding rates (—) and degradation rates (◊) of DCF (a), IBP (b) and NPX (c) during the continuous experiment in a STR.

Removal of NPX was negligible during the first days of experiment; however, partial degradations below 70% until day 15. In addition, at day 12 the decrease in the removal efficiency that was observed for DCF and IBP was also detected in this case. A removal percentage of 50% was maintained in the period between days 15 and 40; then a slight increase of the elimination percentages was detected up to 92% and 87% of degradation at days 44 and 50, respectively (Figure 4-26b).

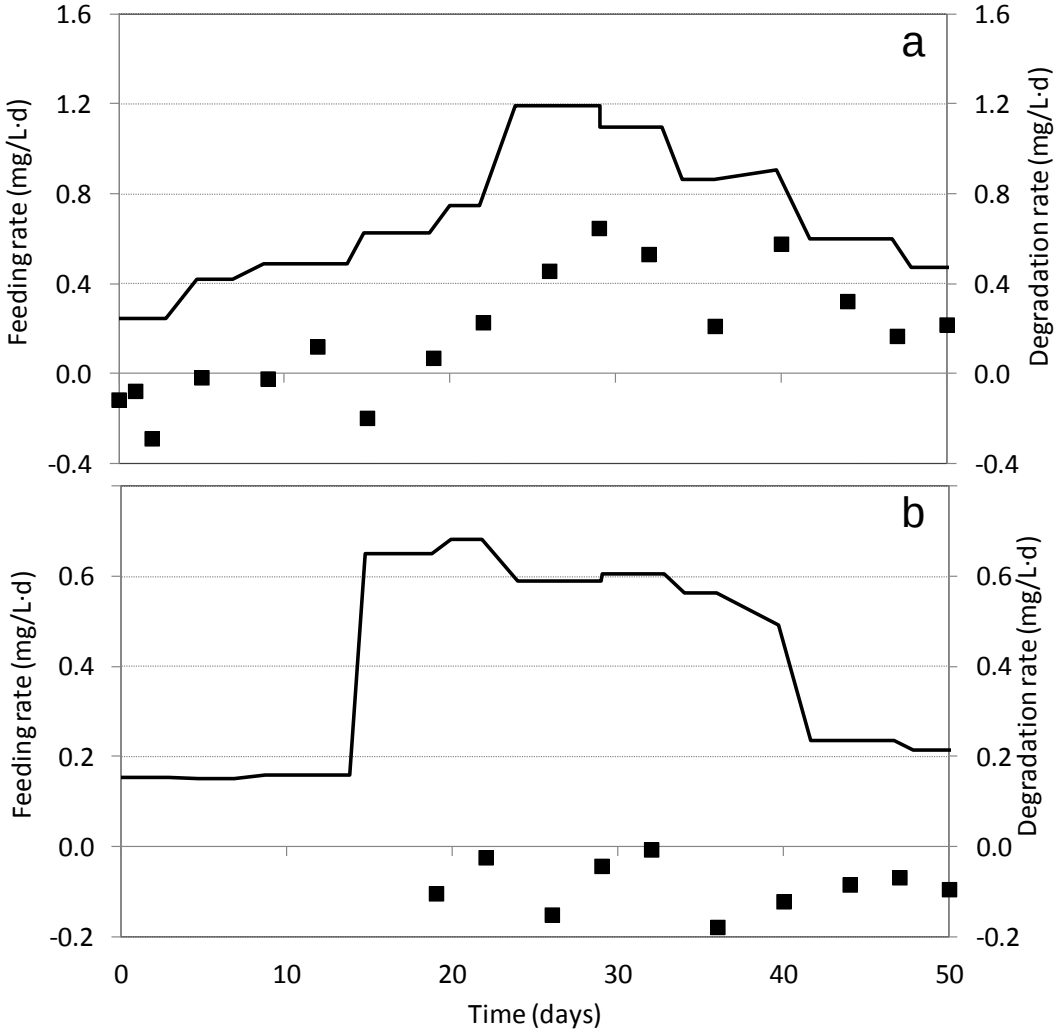


Figure 4-27. Feeding rates (—) and degradation rates (◊) of CBZ (a) and DZP (b) during the continuous experiment in a STR

Contrarily to the anti-inflammatory drugs, the elimination of CBZ was maintained almost negligible during the first two weeks of assay (Figure 4-27a). From day 20 until day 50, the removal of CBZ was maintained between 24% up to 63% of degradation. On the other hand, it was impossible to detect the drug DZP during the first days of assays; this could be due to an analytical problem. Therefore, the feeding rate of this compound was

increased until 0.6 mg/L·d until day 40; then the feeding rate was the same as the original addition (around 0.2 mg/L·d). This strategy does not improve the degradation of DZP, but at least the compound was detected by GC-MS (Figure 4-27b).

The degradation percentages achieved for each of the pharmaceutical compounds used in this experiment are shown in Figure 4-28. Anti-inflammatory drugs were easily degraded by fungal pellets in the STR during a continuous operation. On the other hand, the degradation of compounds such as CBZ was improving as the assay progressed whereas DZP was not degraded in this experiment.

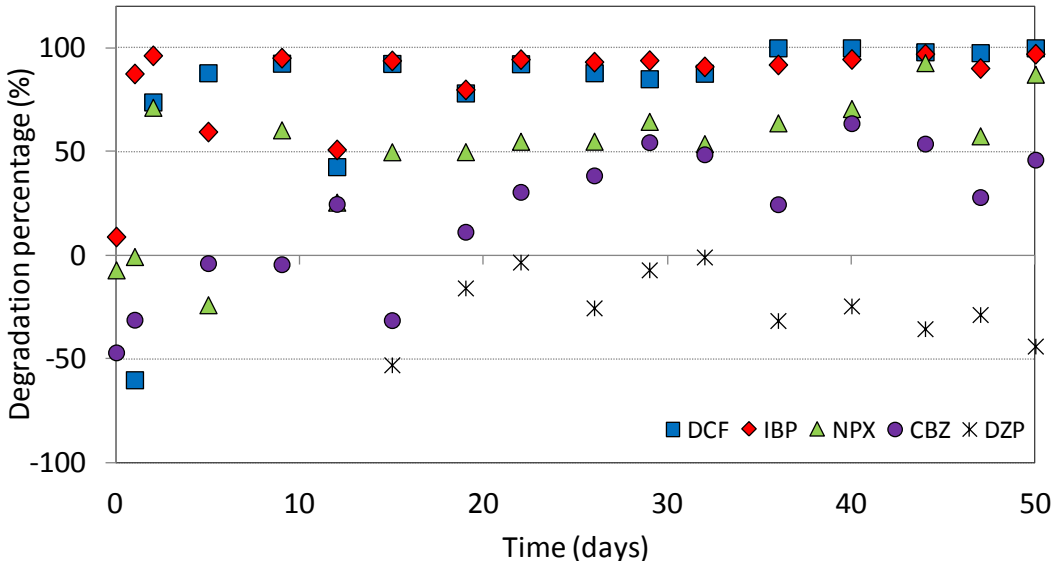


Figure 4-28. Degradation tendency of DCF, IBP, NPX and CBZ during the continuous experiment in a STR. Symbols: DCF (■), IBP (◆), NPX (▲), CBZ (●), DZP (*).

The adsorption of the pharmaceutical compounds onto the fungal biomass was analyzed (Table 4-7). Low quantities of the three anti-inflammatory were detected in the pellets of *P. chrysosporium* meanwhile the compound DZP showed the highest concentration per gram of biomass. All the compounds were detected in concentrations almost negligible in the vessel of the bioreactor.

Table 4-7 Residual concentration of pharmaceuticals onto biomass and reactor vessel

	Pharmaceutical concentration	
	Biomass (mg drug/g biomass)	Vessel (mg drug/cm^2 vessel)
DCF	0.000	$2.7\ E^{-7}$
IBP	0.003	$1.1\ E^{-6}$
NPX	0.012	$3.0\ E^{-6}$
CBZ	0.014	$4.7\ E^{-6}$
DZP	0.028	$1.1\ E^{-5}$

BDL (below detection limit)

The STR was operated during 50 days with pellets of the fungus *P. chrysosporium* using a continuous air flow. The appearance of the vessel reactor is shown in Figure 4-29. At the beginning of the experiment the reactor has an optimal mixture; however, an excessive mycelium growth was observed after 28 days of operation (Figure 4-29, day 28). For this reason, approximately 500 mL of medium (including pellets and free mycelium) were removed from the reactor and replaced with fresh medium. The content of the biomass recovered was of 7.3 g/L. At day 29, the reactor showed an appearance similar to the start of the reactor. Finally, after 50 days of operation the reactor showed again an excessive growth with a final concentration of biomass of 8 g/L.

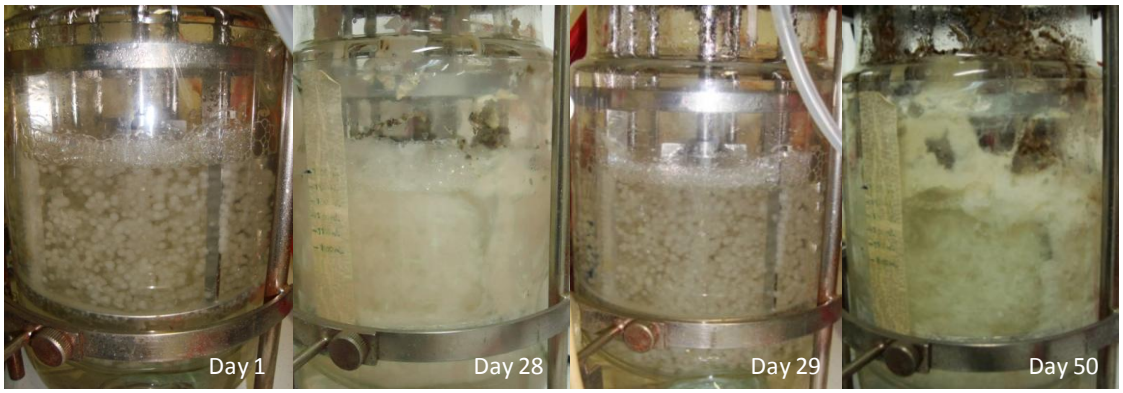


Figure 4-29. Appearance of the STR during the continuous experiment

Also, the morphology and appearance of the pellets were determined during the experiment (Figure 4-30). The continuous air flow caused an excessive fungal growth. Pellets from the inoculum showed a slight formation of hyphal branches with a size of 9.3 mm (Figure 4-30, day 1) and this appearance was also observed at day 10. In the following days, fragments of fungal mycelia formed pellets of smaller size: 7 mm (days 30 and 50).

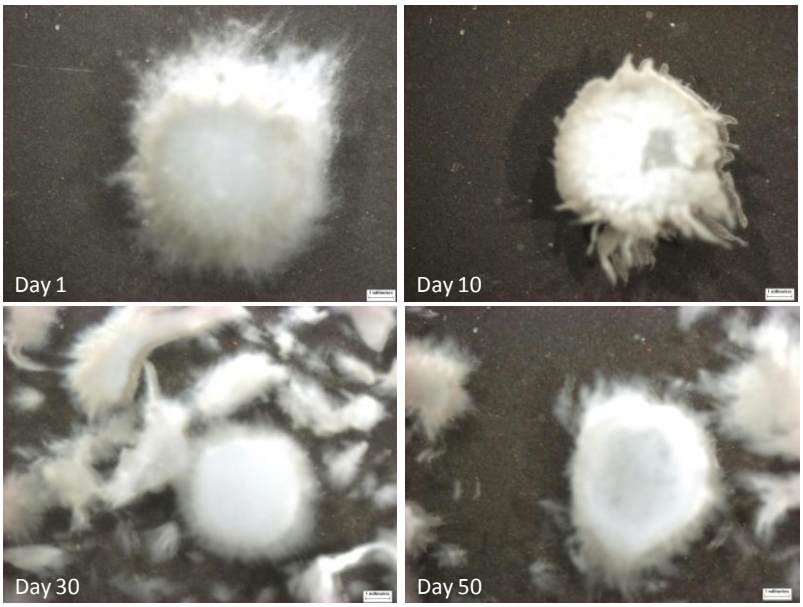


Figure 4-30. Pellet morphology by stereomicroscope during the aerated experiment

4.4. Discussion

In this chapter the degradation of five pharmaceutical compounds (DCF, IBP, NPX, CZB and DZP) was carried out by action of pellets of *P. chrysosporium* in a scale-lab reactor. To achieve a better understanding of the viability of this process, it was conducted a strategy based in two operational strategies: fed-batch mode and continuous operation. The former was conducted to evaluate the feasibility of the process and to establish the optimal conditions which enable and promote the oxidative action of this microorganism. On the other hand, the continuous configuration of the bioreactor was used establishing a continuous input and output flows; the main drawback of this process is the stability of the operation of a continuous bioreactor with filamentous fungi, whose degradative ability will depend on the maintenance of the conditions similar to those that regulate the secondary metabolism.

Once considered the two operational strategies (fed-batch and continuous), the selection of the bioreactor type to perform the degradation assays was required. Thereby, a stirred tank reactor (STR) was selected for the assays conducted in this chapter; this type of bioreactor works predominantly in a steady-state since volume and temperature are not time-depending. Moreover, the fungus *P. chrysosporium* was used in this chapter since it is a well-known WRF which has demonstrated its degradative potential over a variety of environmentally persistent compounds via its ligninolytic system (Mester and Tien, 2000; Miura et al., 2004). This potential was also showed in this doctoral thesis, since this fungal strain showed a high removal of pharmaceuticals by free pellets (results from

chapter 3). Although the anamorph of *Bjerkandera* sp. R1 showed similar or even higher removal percentages than *P. chrysosporium*, this strain was discarded due to its excessive growth which could cause clogging problems affecting the viability of the process during a continuous operation in a STR.

Three degradation experiments were conducted in this chapter: in a first experiment, a fed-batch operation in a STR was conducted for 30 days to carry out the degradation of the three anti-inflammatory drugs. Additionally, the influence of gas supply, either continuous aeration or periodic pulsation of oxygen, on the process efficiency was evaluated. In a later experiment, the bioreactor was operated in a fed-batch mode (stage I) for 26 days and thereafter changed to continuous mode until day 70 (stage II) and finally, a continuous experiment was carried out for more than 50 days to confirm the degradation of the five pharmaceuticals considered.

The operation of the STR remained stable during the three experiments for periods of time between 30 and 70 days, despite the fact that they are operated with filamentous fungi which often have an excessive mycelium growth that might cause clogging problems into the reactor. Several variables were analyzed during the operation of the STR in order to monitor the evolution of the microbial metabolism. The fungus *P. chrysosporium* was able to achieve a great consumption rate of nutrients under the three different feeding strategies used. The ligninolytic system of this microorganism is activated during the secondary metabolism; when the nutrients are in low concentration, the primary metabolism and proteases production can be avoided. The pH was maintained in values near to the optimal (4.5) during the first days of each experiment; however, this parameter can be influenced by the presence of organic acids endogenously produced by the fungus or free ammonia, obtained from the decomposition of proteins by extracellular proteases. It is important to ensure a high oxygen level throughout the degradation process, since it can improve the enzymatic production of MnP and also the removal of the pollutants; however, low enzymatic activities were detected during the first two experiments. Despite this, it was possible to remove the pharmaceutical compounds both in the reactors operated with air or oxygen supply as well as with fed-batch or continuous feeding. When the bioreactor started the operation with a continuous feeding a higher activity of MnP was reached. Other authors have removed this type of compounds with low levels of MnP enzyme suggesting that the degradation by fungal cultures could take place intracelularlly by the action of cytochrome P450 system (Hata et al., 2010; Marco-Urrea et al., 2009; Marco Urrea et al., 2010a).

According to the degradation of the five pharmaceutical compounds (DCF, IBP, NPX, CBZ and DZP), the addition rates of these drugs have been controlled due to the

conditions of operation, avoiding their accumulation into the bioreactor and improving their removal. Anti-inflammatory drugs are easily removed using several technologies, including in sewage treatment plants (STPs) (Huber et al., 2005; Zhang et al., 2008; Ziylan and Ince, 2011). Other pharmaceutical compounds, like CBZ and DZP, need oxidative treatments for its degradation since they are not removed in conventional STPs (Esplugas et al., 2007; Calisto and Esteves, 2009; Kim and Tanaka, 2009). The degradation of DCF, IBP, NPX and CBZ by fungal action has been studied achieving high removal percentages; in fact, the use of ligninolytic enzymes like Laccase enhances the removal of these compounds (Marco-Urrea et al., 2009; Hata et al., 2010; Lloret et al. 2010; Marco-Urrea et al., 2010b; Zhang and Geiβen, 2010; Eibes et al., 2011;).

In this chapter, the drugs DCF, IBP and NPX achieved from partial to total degradation depending on the feeding operation of the bioreactor. When the bioreactor started with a fed-batch operation, these compounds were totally removed regardless of the gas supply (air or oxygen); while when the reactor operation was changed to a continuous feeding, in all the studied cases, the elimination efficiency decreased over the progress of the experiment. Finally, when the bioreactor started with a continuous operation, the removal of the three anti-inflammatory took a longer period of time. Another important result of this chapter was the slight accumulation of NPX that takes place between days 12 and 22 in the aerated and oxygenated reactor. There is no clear evidence that explains this fact, but it may be due to a decline in the glucose consumption observed in this period resulting from a slowdown in the fungus metabolism. Moreover, it may also be attributed to an accumulation of either NPX or its degradation products, since Lindqvist et al. (2005) have reported higher concentration of this compound in effluents of STPs than in the influents, which is attributed to a possible hydrolysis of the metabolites, forming the parent compound. This behavior was also observed during the fed-batch stage, however when a continuous feeding was added to the reactor, a negligible accumulation of this compound was observed. The degradation of recalcitrant compounds such as CBZ and DZP was almost negligible; only the former achieved partial removal percentages during the fed-batch stage and total removal during the continuous stage of the second experiment performed in this chapter. As the anti-inflammatory drugs, it seems that a fed-batch operation enhances the removal of this compound while during a continuous operation the fungus needs more time to reach a complete depletion of this drug. The differences between the degradation percentages achieved for the five pharmaceutical compounds may be related to their physicochemical properties. Ibuprofen is a readily biodegradable drug perhaps due to its chemical structure with one aromatic ring; while DCF has two aromatic rings connected by an N-H functional group as well as the presence of two chlorine atoms (Ziylan and Ince, 2011). Moreover, recalcitrant

compounds such as CBZ and DZP have a very small K_{biol} (<0.01 L/g·SS·d and <0.1 L/g·SS·d, respectively) indicating that they are hardly biodegradable (Joss et al., 2006).

Regarding with the time-course degradation experiments, it is important to note that several degradation rates were achieved at different stages of the fungal fermentation. This indicates that the fungal age have an effect over the degradation of some compounds such as DCF and CBZ. With respect to the adsorption of the drugs in the biomass, this process depends on the physicochemical properties of each compound, as their octanol-water partition coefficient (K_{ow}) and their acid dissociation constant (pK_a) (Suárez et al., 2008). From the selected pharmaceutical compounds in this chapter, CBZ and DZP are more likely to be attached on the surface of solids. This is in accordance with the results of the adsorption experiments performed in this chapter since both compounds were detected in higher concentrations in the pellets than the anti-inflammatory drugs.

Concerning to the feeding conditions, the lowest concentration of pharmaceutical compounds were detected during the fed-batch experiment, even when in this experiment a large amount of pharmaceutical compounds was added. Typically during a fed-batch operation the fungus growth is activated after each nutrients pulse; also there is an improvement of the degradation rates of the target compounds after each pulsation. In contrast, during a continuous operation, the removal efficiency of the pharmaceutical compounds decreases throughout the assay. Moreover, the size of the pellets may also influence the adsorption of these compounds, since the surface to adhere will depend on the size of the fungal mycelium. On the other hand, the adsorption may also be related to the efficiencies of degradation and the accumulation of the drugs, since the higher concentration of these compounds per gram of biomass was detected in those experiments where the compounds were not completely degraded. Feeding strategy could also have a positive effect over the pellets morphology, since in this study it was found that when the bioreactor started the operation with a continuous addition of nutrients, the pellets showed a higher size than those obtained when the experiment started with a fed-batch operation; also, a great amount of this pellets maintained the spherical and compact shape until the last day of experiment and only a few percentage of these pellets have fragmented and formed aggregates.

With respect to the effect of the gas supply, fungal growth under high oxygen level conditions controls pellet morphology and enzyme production and was found to be optimal for a delignification process (Moreira et al., 1996; Rothschild et al., 1999; Miura et al., 2004). For instance, Moreira et al. (1996) obtained spherical shape and hyphae-free pellets when applying oxygen pulsations, favouring in this way fungal morphology.

Otherwise, when a continuous air flow was used an excessive fungus growth was observed. Disrupted pellets release mycelium fragments on the medium which in turn forms aggregates in the reactor. This is in accordance with the results of this chapter, where significant differences of pellets morphology where observed, depending on the gas supply. The application of pulses of oxygen favoured the pellets morphology which in turn stabilized the operation of the STR; however, in the fed-batch reactor with a continuous air flow, there were no clogging problems even though the pellets showed a filamentous growth. This could impede a continuous operation where it is necessary to maintain a constant output flow; despite this, it is important to note that during the continuous operation of the STR with air an overgrowth of the mycelium was observed but no clogging were detected into the reactor. Although this, it was necessary to remove biomass at day 28 from the reactor which started its operation in continuous. Concerning to the effect of the oxygen level associated with the degradation of the pharmaceutical compounds, there were no significant differences between the aerated and the oxygenated; therefore a configuration using air can be an optimal alternative for removal of these compounds since it reduces operating costs.

4.5. Conclusions

In this chapter the degradation of several pharmaceutical compounds by pellets of *P. chrysosporium* in a STR was analyzed under a fed-batch and a continuous operation. Also the influence of a continuous air flow and oxygen pulses over the morphology of the pellets as well as the degradation efficiencies were analyzed. According to the obtained results during the fed-batch experiment, the addition of oxygen pulses may control the growth of fungal biomass, thus facilitating the operation of the reactor with high removal percentages for DCF, IBP and NPX (80% - 99%). Meanwhile, the aerated reactor presented excessive growth, with removal rates of the three anti-inflammatories in the range of 65%-99%. These findings prove that the oxidative capability of this microorganism for the anti-inflammatory drugs is not restricted to an oxygen environment, as generally accepted. In the case of the second experiment, the three anti-inflammatory drugs mentioned above were used, but also an antiepileptic was analyzed (CBZ). The results showed that these compounds can be partially or totally removed during the fed-batch stage after a short period of time; this degradation efficiency is related to the culture age. When a continuous feeding was added, the elimination of these drugs was maintained stable until day 55, then a decrease of the removal was observed for all the cases. Finally, during the third experiment, a continuous feeding was used for the removal of DCF, IBP, NPX, CBZ and also DZP. When this type of operation was used since the beginning of the experiment, the elimination of the anti-inflammatory drugs took more time than during a

fed-batch experiment. The drug CBZ was partially removed at the end of the operation; while DZP was not removed throughout the experiment. It seems that a higher elimination of these pharmaceutical compounds can be achieved during a fed-batch experiment. Despite this, from the results of this chapter it can be concluded that the optimal configuration could be a fed-batch bioreactor operating for a few weeks; then a change to a continuous feeding might enhance and maintain the removal of these compounds for longer periods of time.

Chapter 5

Operation of fixed-bed and stirred tank reactors with immobilized *Phanerochaete chrysosporium* for the removal of five pharmaceutical compounds: DCF, IBP, NPX, CBZ and DZP

Summary

Fixed-bed and stirred tank reactors with immobilized *Phanerochaete chrysosporium* were applied for the removal of three anti-inflammatory drugs (DCF, IBP, NPX), an antiepileptic drug (CBZ) and a tranquilizer (DZP). The influence of gas supply either as a continuous flow of air or as pulses of oxygen on the efficiency of the process was evaluated in the fixed-bed configuration. This type of bioreactor was proven to maintain a stable and feasible operation for 100 days with total removal of the three anti-inflammatory drugs regardless of the aeration system. In the case of the antiepileptic compound, high removal percentages (from 60% up to 89%) were obtained until day 40 under both aeration conditions. Thereafter, the efficiency of degradation decreased to 50% in the aerated reactor and to a larger extent, between 20% and 50% in the reactor with oxygen pulsation. Conversely, removal of diazepam was favored in the oxygen reactor with remarkable degradation percentages higher than 60% after 100 days while values below 40% in the air reactor. Under both aeration conditions, low levels of MnP activity were detected, slightly higher (up to 30 U/L) in the oxygen reactor. The other assessed configuration, stirred tank reactor with immobilized fungus, attained stable operation for 50 days. A complete removal of DCF and IBP was achieved even when negligible activities of MnP were detected; the anti-inflammatory NPX was almost totally degraded during the first days of assay (until day 3) but then a decrease of the removal efficiency was

observed. Carbamazepine showed a similar degradation tendency than NPX, but with low removal percentages. Finally, the removal of DZP was lower than in the fixed bed reactor (up to 40%) and it was negligible from day 30 until the end of the experiment. Also, in this chapter adsorption experiments were carried out and the results showed that low residual concentrations of all the selected compounds were detected in the fixed-bed reactors.

* Part of this chapter has been submitted as a research paper as:

Rodarte-Morales AI, Feijoo G, Moreira MT, Lema JM. (2011). **Operation of a stirred tank reactor (STR) and fixed-bed reactor (FBR) with *Phanerochaete chrysosporium* for the continuous degradation of pharmaceutical compounds.** Biochemical Engineering Journal (Submitted).

Outline

5.1. Introduction

In recent years the configuration of a fixed bed reactor (FBR) with immobilized ligninolytic fungi has been applied for the production of ligninolytic enzymes or for the removal of various pollutants, especially dyes and phenolic compounds (Linko et al., 1996; Moreira et al. 1997a; Moreira et al., 1998; Mielgo et al. 2002; Ehlers and Rose, 2005; Moldes et al., 2005; Rogalski et al., 2006; Sedighi et al., 2009). The operation of this type of bioreactors is affected by diverse environmental factors such as medium composition, culture age, feeding strategy (fed-batch or continuous), hydraulic residence time (HRT), aeration supply, among others (Moreira et al. 1996; Jiménez-Tobon et al., 1997, Rothschild et al., 1999; Kaushik and Malik, 2009).

Nowadays there are many types of supports that have been used to immobilize white rot fungi (WRF) such as agarose gel, nylon-web, silicon tubing, porous plastic material, sintered glass, kissiris (mineral glass foam material) and polyurethane foam (Linko et al., 1996; Moldes et al. 2005; Sedighi et al., 2009). The immobilization support typically has a large surface area that facilitates the adhesion of the microorganism. Concerning the operation of the bioreactor, immobilization of the fungus avoids the washout of the biomass and it also provides mechanical strength to the bioparticle, superior to that of free mycelia or pellets. This culture technique restricts the growth of the fungus to the external and inner surface of the support and thus, excessive extension of the mycelia is controlled avoiding clogging problems and maintaining the reactor operation stable for longer periods of time (Brauer, 1988; Elhers and Rose, 2005). Moreover, the use of a support favours mass transfer for oxygen and nutrients in contact with the bioparticle (Moreira et al., 1998). However, there are several important drawbacks of FBRs that have to be highlighted: the lack of homogeneity and mechanical stirring that may encounter mass transfer limitations, the compaction of the support, the formation of preferential pathways that imply that a fraction of the reactor is not operative and it is considered as a dead volume (Ehlers and Rose, 2005). A strategy to overcome these drawbacks may rely on the way how the gas is supplied into the reactor, either continuously or in pulses. A continuous air flow ensures a constant flow at low operation costs whereas the use of a pulsing oxygen flow may maintain high levels of dissolved oxygen at moderate costs and even control the extension of mycelia by means of the mechanical stress on the bioparticle (Dosoretz et al., 1990; Kenealy and Dietrich, 2004; Xiong et al., 2008).

In previous chapters of this thesis, the fungus *P. chrysosporium* demonstrated the ability to remove a wide range of pharmaceutical compounds, regardless of the culture technique considered (static, free pellets and immobilized cultures). In particular in the

case of immobilized culture, this strain was able to remove three anti-inflammatory compounds (DCF, IBP and NPX) and an antiepileptic (CBZ), whereas partial degradation of DZP was attained (chapter 3). In the case of pellet culture, this fungus was able to remove these compounds in a stirred tank reactor (STR); however, operation of free pellets reactor presented clogging problems due to excessive fungal growth (Moreira et al., 1996). An alternative configuration based on the use of a conventional STR may be that one operating with immobilized fungus. In principle, this option may be valuable to combine the advantages of the two bioreactor configurations: homogeneity of the culture medium, high surface area for fungal colonization with controlled growth, improved mass transfer of nutrients and oxygen, prevention of preferential pathways and clogging.

In this chapter three degradation experiments were conducted for the removal of five pharmaceutical compounds: DCF, IBP, NPX, CBZ and DZP by immobilized *P. chrysosporium*. This strain was selected for a number of reasons: it is a well known fungus able to degrade a wide variety of pollutants via its ligninolytic system, it is an accessible microorganism which simplifies the process, it is characterized by a low biomass growth compared with the other strains used in chapter 3 (the anamorph of *Bjerkandera* sp. R1 and *B. adusta*) and finally, this fungus has shown a great ability to remove several pharmaceutical compounds even at low enzymatic activities (chapters 3 and 4). The support chosen was polyurethane foam because it is cheap, readily available and has an adequate mechanical strength. The influence of gas supply either as a continuous flow of air or as pulses of oxygen on the efficiency of the process was also evaluated in a FBR. In parallel, a STR with immobilized fungus was operated to assess the efficiency of degradation in this configuration as well as the process stability.

5.2 Materials and methods

5.2.1. Microorganisms

The white-rot fungus used in this chapter was *Phanerochaete chrysosporium*. In chapter 2 a description of the preparation of the pre-inoculum for fixed-bed reactor (FBR) and stirred tank reactor (STR) is included.

5.2.2. Pharmaceutical compounds and chemicals

Pharmaceutical compounds considered in this chapter were DCF, IBP, NPX, CBZ and DZP from a stock solution prepared as indicated in chapter 2, at three different concentrations: 1 mg/L (DCF, IBP and NPX), 0.5 mg/L (CBZ) and 0.25 mg/L (DZP).

5.2.3. Degradation of DCF, IBP, NPX, CBZ and DZP in a continuous fixed-bed reactor using *P. chrysosporium* immobilized in polyurethane foam.

The description of the main characteristics of the FBR used was already detailed in chapter 2. In figure 5-1 the configuration of the continuous operation of the FBR is shown.

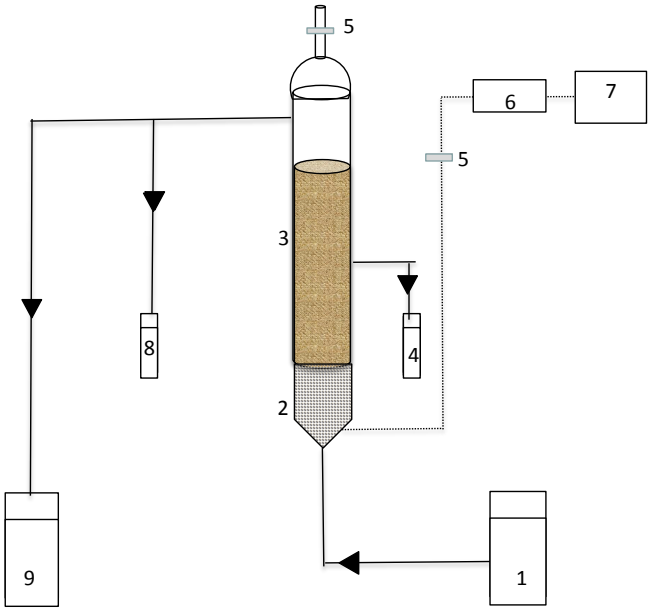


Figure 5-1. Configuration of a continuous operation of a fixed-bed reactor. Symbols: (1) Feed bottle, (2) glass spheres, (3) *P. chrysosporium* immobilized in polyurethane foam, (4) middle port, (5) filters, (6) pulsing device, (7) air or oxygen supply, (8) effluent from the upper port, (9) effluent reservoir.

Polyurethane foam with 3 or 4 day immobilized fungus corresponding to three flasks was transferred to the reactor. The feed medium was formulated on the basis of the Kirk culture medium, adapted to a continuous operation (Tien and Kirk, 1988) with the feeding rates specified in Table 5-1. Two ways of gas supply were considered: one reactor was supplied with a continuous air flow (1 L/min), whereas a second reactor was aerated with pulses of oxygen. The oxygen pulsing frequency was maintained at 0.0625 s^{-1}. Temperature was maintained at 30°C and pH was not controlled by acid or alkali addition and the level of dissolved oxygen was measured off-line by means of an external electrode (chapter 2). Degradation experiments under both aeration conditions were carried out for 100 days and the hydraulic retention time (HRT) selected for the operation was 24 h as calculated from the results presented in chapters 3 and 4. Samples were withdrawn twice per week from the upper port of the reactor and every 10 days from the middle port of the reactor.

Table 5-1. Feeding rates in the operation of the fixed-bed (FBR) and stirred tank reactors (STR)

Compounds	Oxygen FBR Feeding rate (mg/L·h)	Air FBR Feeding rate (mg/L·h)	Air STR Feeding rate (mg/L·h)
Glucose	125-250	125-250	125-250
Ammonium tartrate	0.6-2.08	0.6-1.04	0.6
KH_2PO_4	83.3	83.3	83.3
$MgSO_4$	20.8	20.8	20.8
$CaCl_2$	4.2	4.2	4.2
Sodium acetate	113.3	113.3	113.3
$MnSO_4$	3.5	3.5	3.5
Trace elements*	0.4	0.4	0.4
Tiamine*	0.04	0.04	0.04
DCF, IBP, NPX	0.042	0.042	0.042
CBZ	0.021	0.021	0.021
DZP	0.010	0.010	0.010

* (mL/L·h)

5.2.4. Degradation of DCF, IBP, NPX, CBZ and DZP in a continuous stirred tank reactor (STR) using *P. chrysosporium* immobilized in polyurethane foam

The description of the main characteristics of the STR used in this experiment was included in chapter 2 (Figure 2-3 and 2-4). The configuration of the continuous operation of the STR using *P. chrysosporium* immobilized in polyurethane foam is shown in Figure 5-2. The reactor vessel (2 L) was filled with active fungus previously immobilized in polyurethane foam (3 - 4 days) corresponding to four Erlenmeyer flasks. From the beginning of the experiment until day 9 the reactor was filled with 1.5 L of modified Kirk medium (Tien and Kirk, 1988) with the feeding rates described in Table 5-1; then, from day 10 until the end of the experiment the volume of the reactor was increased until 1.75 L in order to maintain a homogeneous agitation. The feed medium was added in the upper port of the reactor vessel while the effluent was withdrawn from the bottom of the reactor. A continuous air flow (0.5 – 3 L/min) was used during this experiment and the temperature was maintained at 30°C by circulation of thermostatized water through the reactor jacket. The experiment was carried out for 50 days with an HRT of 24 h and samples were withdrawn twice per week.

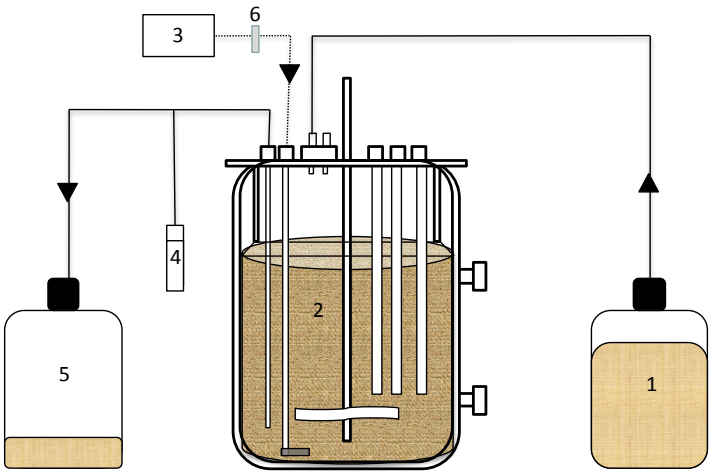


Figure 5-2. Configuration of a continuous operation in a STR using immobilized fungus. Symbols: (1) Feed bottle, (2) *P. chrysosporium* immobilized in polyurethane foam, (3) air supply, (4) sampling point, (5) effluent reservoir, (6) filter.

5.2.5. Determination of the residence time distribution (RTD) in a fixed-bed reactor (FBR)

The protocol to determine the residence time distribution (RTD) in both FBRs was described in chapter 2.

5.2.6. Adsorption of pharmaceutical compounds on the fungal biomass

The potential adsorption of pharmaceuticals on the immobilized fungus was carried out to discard any possible adsorption or volatilization process of the pharmaceutical compounds. The methodology to carry out these experiments was described in chapter 2.

5.2.7. Analytical techniques

A description of the analytical techniques used in this study was indicated in the Materials and methods chapter.

5.3. Results

5.3.1. *P. chrysosporium* immobilized in a fixed-bed reactor with a continuous air flow for the degradation of DCF, IBP, NPX, CBZ and DZP

Degradation of five pharmaceutical compounds was carried out using a FBR with immobilized *P. chrysosporium*. A continuous air flow was used as the aeration system and the configuration of the reactor is shown in Figure 5-3. As in the experiments conducted in chapter 4 of this doctoral thesis, several parameters were measured throughout the

experiments. The FBR and the STR with immobilized fungus were operated under a continuous regime in order to study the ability of *P. chrysosporium* to degrade the pharmaceutical compounds considered in this chapter. The use of immobilized fungus in a FBR provides an easy operation for longer periods of time with a high mass transfer for nutrients and oxygen; also, the fungal growth can be controlled. To achieve a stable operation it is necessary to monitor the nutrients concentration inside the column in order to avoid the formation of undesirable metabolites such as proteases which promotes cell lysis. Also, the pH value may be maintained in values near to 4.5, which is the optimal pH for the activation of the ligninolytic system, which is also related with high oxygen levels. To analyze the efficiency of the bioreactor, it is necessary to determine the hydrodynamics of the FBR.

Figure 5-3. Fixed-bed reactor configuration with *P. chrysosporium* immobilized in polyurethane foam and a continuous air flow. Symbols: (1) feed medium reservoir, (2) feed addition port, (3) middle port, (4) upper port, (5) air supply, (6) air supply port, (7) effluent reservoir, (8) thermostatized water.

The glucose concentrations of the feed medium and of the output streams (middle and upper ports of the reactor) are presented in Figure 5-4 as well as the calculated feeding and consumption rates of glucose. The glucose concentration detected in the middle port of the reactor was similar to that of the upper port (Figure 5-4a), indicating a high degree of homogeneity in the bioreactor.

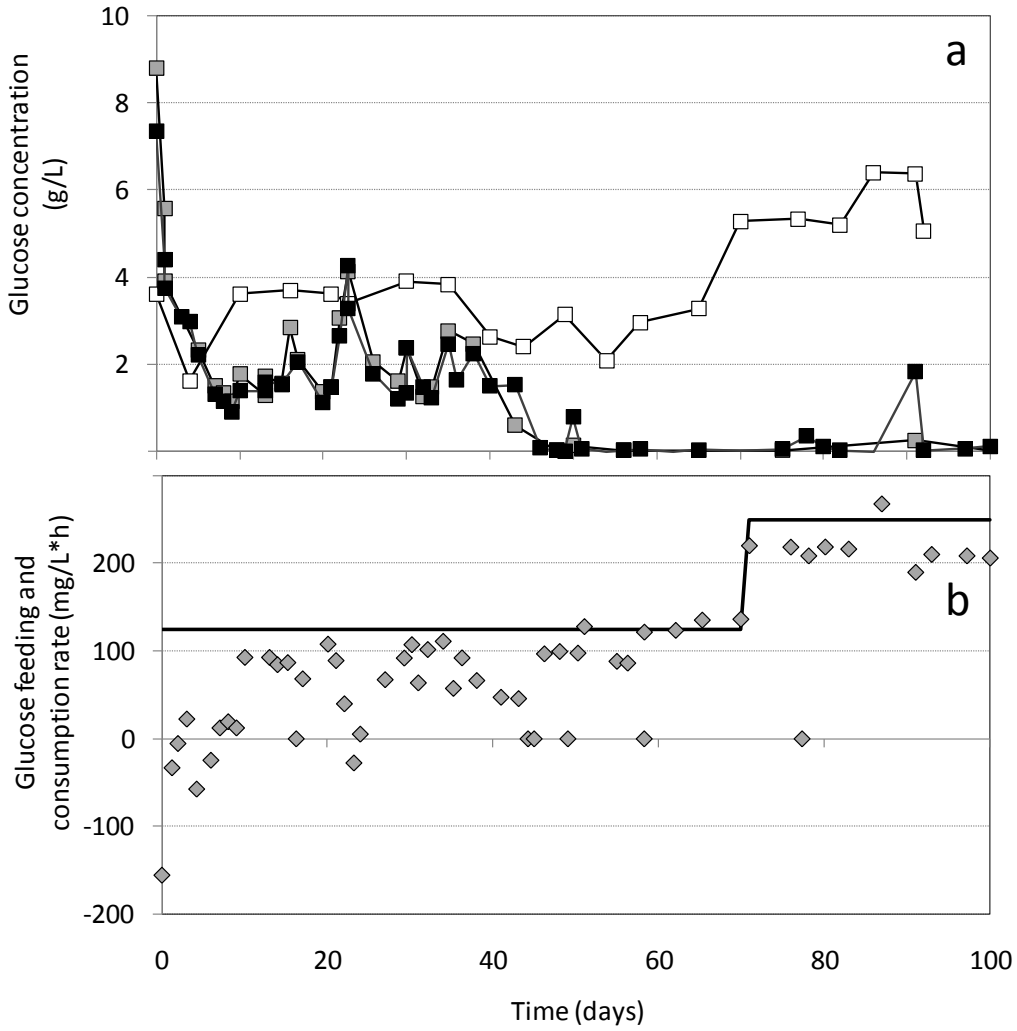


Figure 5-4. Glucose concentration (a) and glucose feeding and consumption rate (b) in the fixed-bed reactor Symbols: glucose – feed medium (□), glucose – reactor middle port (▣), glucose – reactor upper port (■), glucose feeding rate (—), glucose consumption rate (◇).

Two feeding addition rates were considered during the operation: 125 mg/L·h until day 70 and 250 mg/L·h from day 71 until the end of the experiment (Figure 5-4b) in order to maintain low levels of glucose in the effluent to avoid the secretion of proteases. As can be observed, the consumption of glucose was maintained low until day 15; however, from day 20 (except for very few days) the feeding and consumption rates achieved similar values, indicating that the fungus *P. chrysosporium* was able to consume most of the

glucose added in the feed stream. In Figure 5-5a values of pH in the reactor and the feed medium are shown. Although the feed medium was prepared with a pH of 4.5, the value of pH of the effluent stream was maintained between 4.5 and 5.5 throughout the experiment. Regarding the enzymatic activity, this parameter was detected below 10 U/L throughout the experiment (Figure 5-5b). Concentration of hydrogen peroxide was detected in a range between 0.5 and 2 mg/L throughout the experiment (data not shown). Both variables indicate that production of oxidative enzymes, specifically oxidases and MnP as peroxidase takes place during the operation although at low levels. Dissolved oxygen concentration was maintained in a range between 6–8 mg O_2/L (Figure 5-6a).

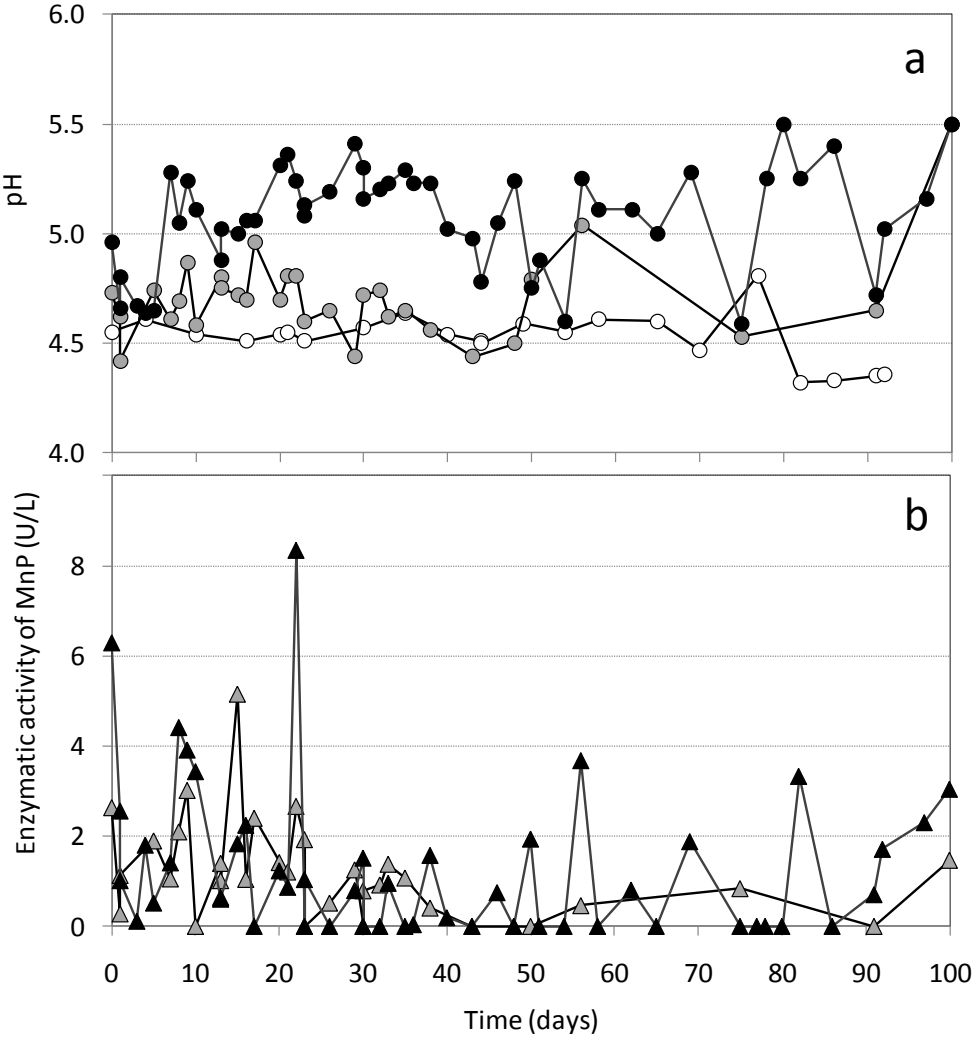


Figure 5-5.pH detected (a) and enzymatic activity of MnP (b) in the fixed-bed reactor Symbols: pH – feed medium (○), pH – reactor middle port (◎), pH – reactor upper port (●), MnP – reactor middle port (△), MnP – reactor upper port (▲).

The content of total organic carbon (TOC) was measured in the feed medium with values below 2 g/L until day 65 and then an increase up to 2.5 g/L coincident with the change in the feeding rate at day 75. Moreover, the TOC was determined in the middle port and the effluent of the reactor with concentrations in a range between 0.66–1.77 g/L (Figure 5-6b). The total nitrogen content was maintained below 0.1 g/L in the feed medium as in the effluent of the reactor.

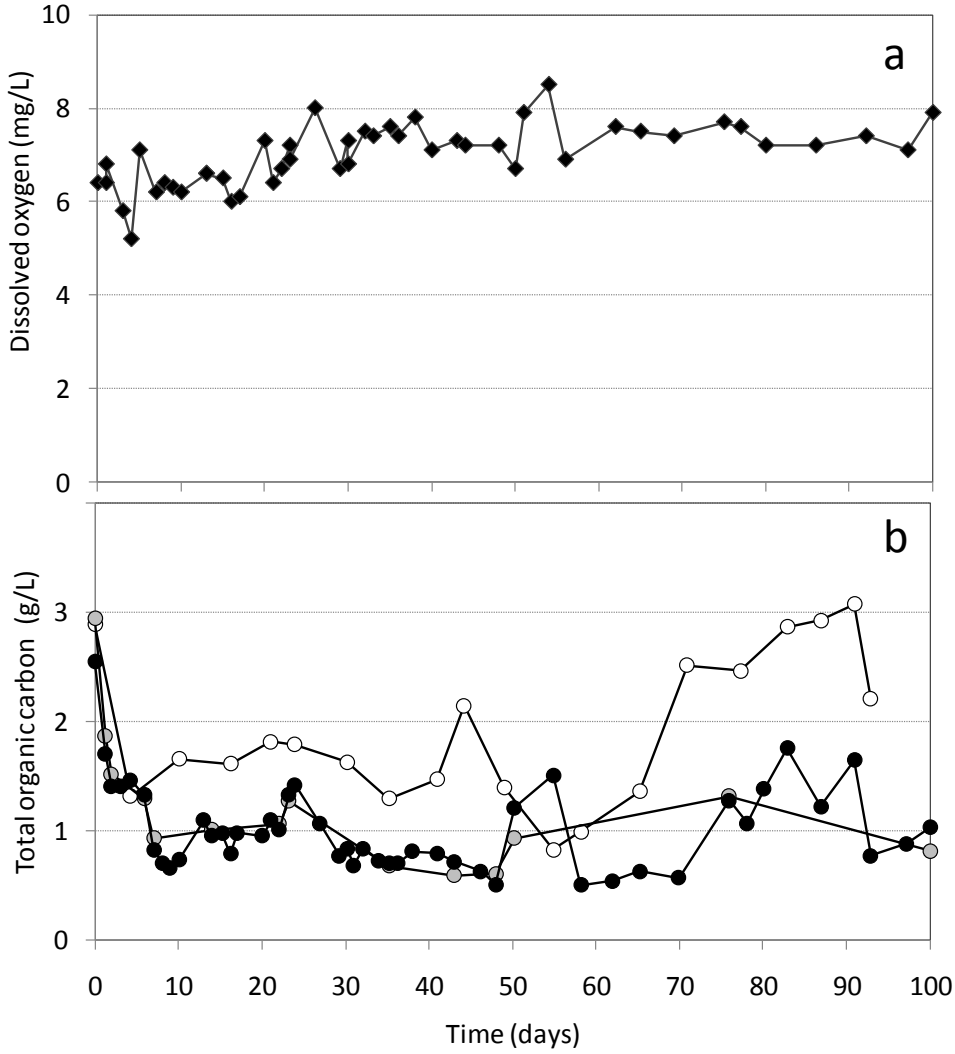


Figure 5-6.Dissolved oxygen (a) and total organic carbon (b) detected in the fixed-bed reactor Symbols: dissolved oxygen (◆), TOC – feed medium (○) TOC – reactor middle port (◍), TOC – reactor effluent (●).

Regarding the visual appearance of the bioreactor, only the support placed at the bottom of the reactor was submerged at the beginning of the experiment and was maintained semi-submerged for two weeks (Figure 5-7). The exterior of the polyurethane

foam presented a slight sporulation in the hollow spaces of the reactor during the first two weeks of experiment.

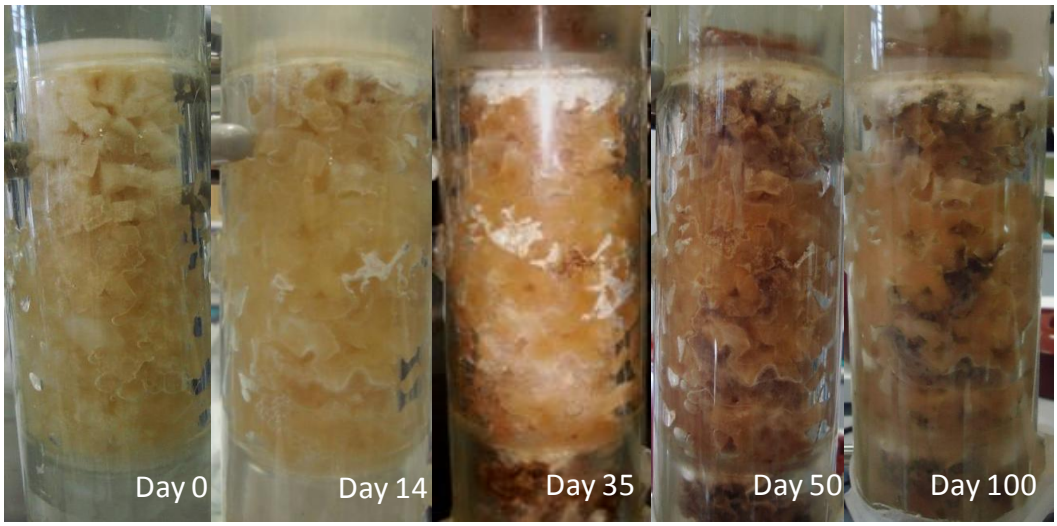


Figure 5-7. Appearance of the fixed-bed reactor with a continuous air flow

A slight darkening of the support through the column was observed at day 35, being more intense in the bottom. In parallel, spores were observed in the upper zone of the reactor and the fungus attained to grow on the surface of the porcelain plate. Dark spots in several regions of the support were observed after 50 days of experiment, maintaining this appearance until the end of the experiment. Biomass concentration at the end of the experiment was of 1.89 g in the reactor, which means a concentration of 11.3 g/L.

The study of the hydrodynamic behavior of the aerated FBR was carried out by the determination of the residence time distribution (RTD). A stimulus-response technique was carried out using lithium chloride (LiCl) as a tracer. The results were adjusted to the tank-in-series model (Levenspiel, 1999) and the obtained curve is shown in Figure 5-8. The hydraulic behavior of this reactor was similar to 6 complete stirred tanks, intermediate behavior between completely mixed and plug flow.

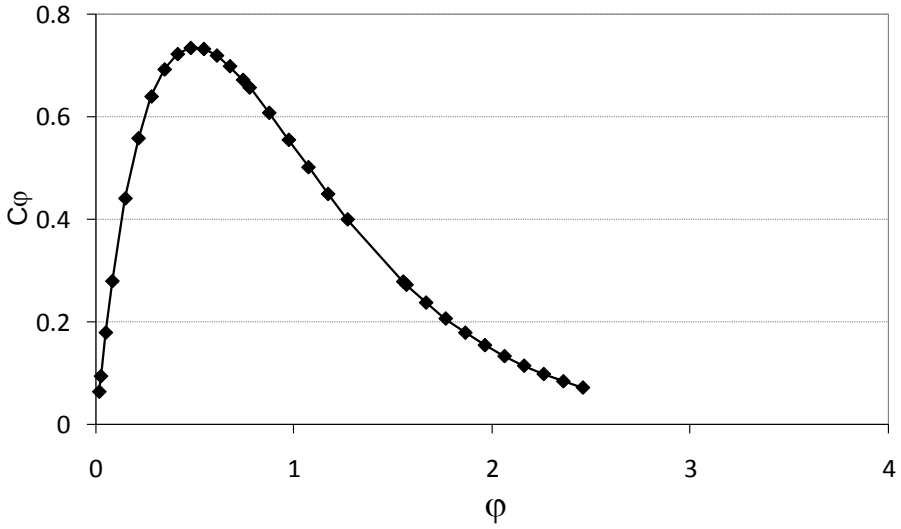


Figure 5-8. Residence time distribution (RTD) of the aerated fixed-bed reactor

The comparison of the feeding and consumption rates of the anti-inflammatory drugs is presented in Figure 5-9. DCF, IBP and NPX were largely removed throughout the experiment. In fact, the removal of DCF and IBP was maintained higher than 90% in both effluent ports of the reactor (Figure 5-9a and 5-9b) while NPX showed a removal percentage in a range between 78%-92% in the middle port and higher than 84% in the upper port (Figure 5-9c). The efficiency of degradation was maintained stable during 100 days (Figure 5-11).

The other compounds considered (CBZ and DZP) presented higher recalcitrance with partial degradation (Figure 5-10). The highest removal of the antiepileptic in the medium port was observed from the beginning of the experiment until day 41 (from 60% up to 85%); whereas it decreased below 30% during the rest of the experiment, except for the period between days 50 and 90, where no degradation was detected in the medium port (Figure 5-10a). Higher degradation percentages were obtained in the upper port, ranging from 50% up to 90% during the first half of the experiment; while during the rest of the assay, partial removals were detected (from 23% up to 50%). The measurement of the concentration of CBZ in the middle and upper ports evidenced gradients of concentration through the reactor column (Figure 5-11a).

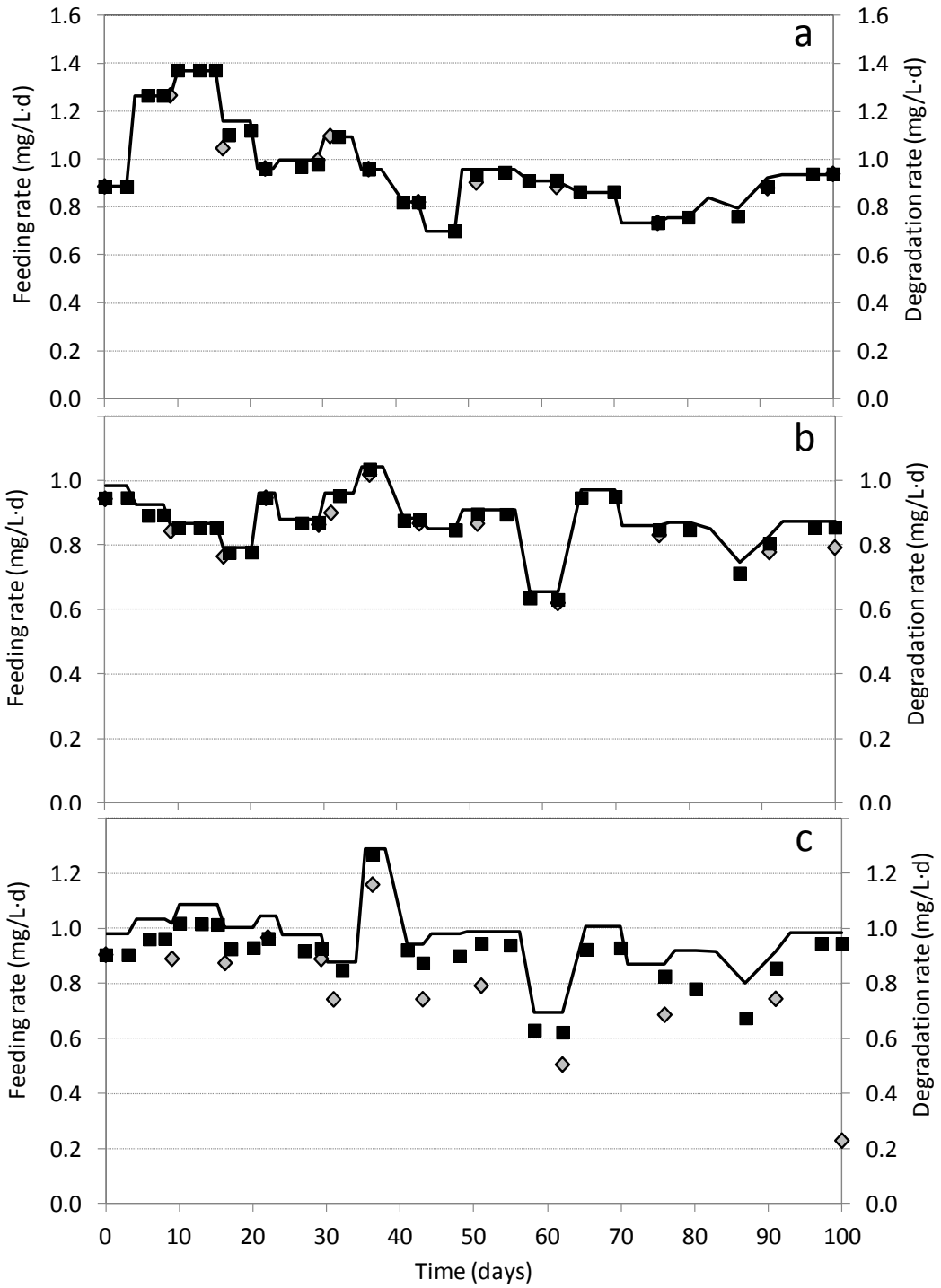


Figure 5-9. Feeding and degradation rate of DCF (a), IBP (b) and NPX (c) in a fixed bed reactor with *P. chrysosporium* immobilized in polyurethane foam. Symbols: feed medium (—), reactor middle port (◇), reactor effluent port (■).

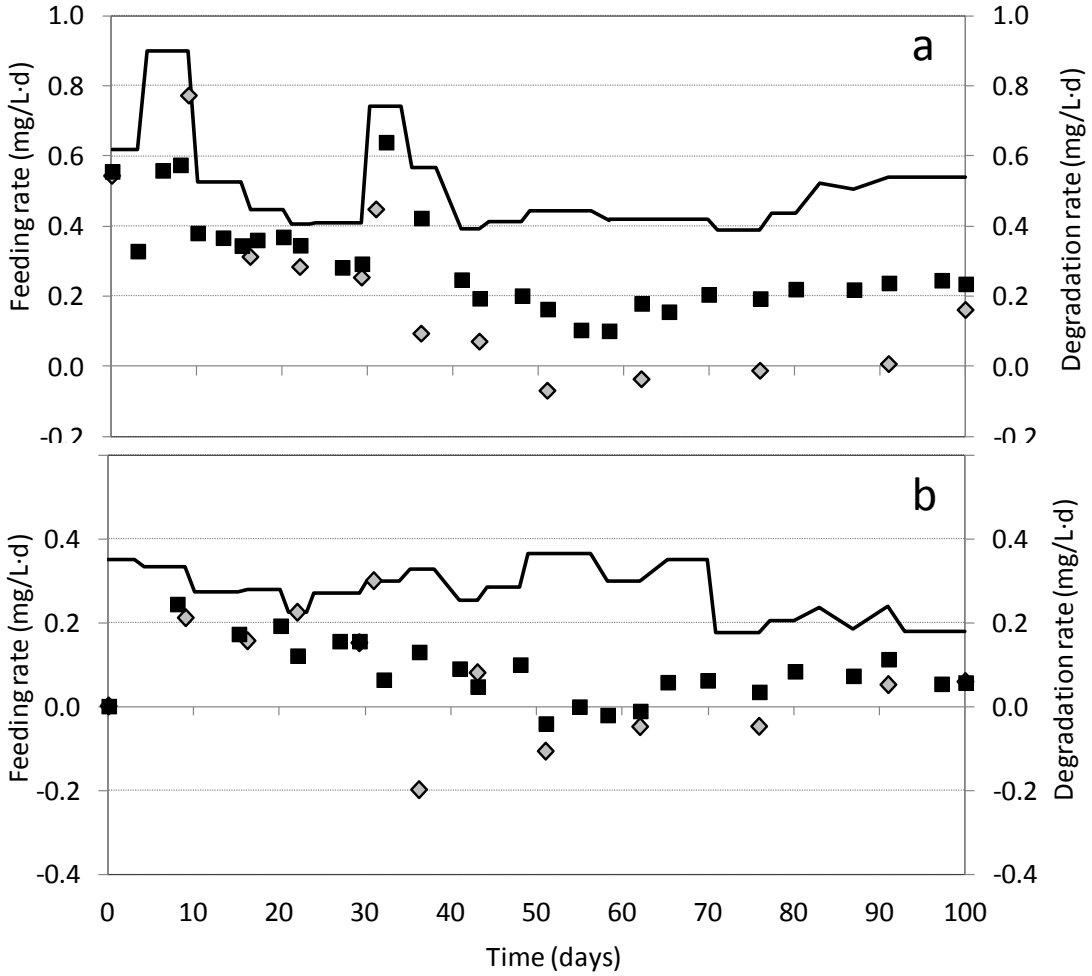


Figure 5-10. Feeding and degradation rate of CBZ (a) and DZP (b) in a fixed bed reactor with *P. chrysosporium* immobilized in polyurethane foam. Symbols: feed medium (—), reactor middle port (◇), reactor effluent port (■).

Diazepam was not detected by GC-MS during the first days of experiment, which may be attributed to an adsorption process onto the polyurethane foam. No significant differences in the degradation of DZP were observed between both effluent ports of the reactor, with removal percentages in a range between 45% and 72% until day 30. Then, during the rest of the experiment the removal was maintained between 14% and 40% until day 50. The period between days 50 and 60 the degradation was negligible in both ports, extending this behavior in the medium port until day 90. The upper port achieved a partial degradation below 46% from day 60 until the end of the assay; meanwhile in the middle port the degradation was detected in percentages between 14% and 32% during the last 10 days of experiment (Figure 5-10b).

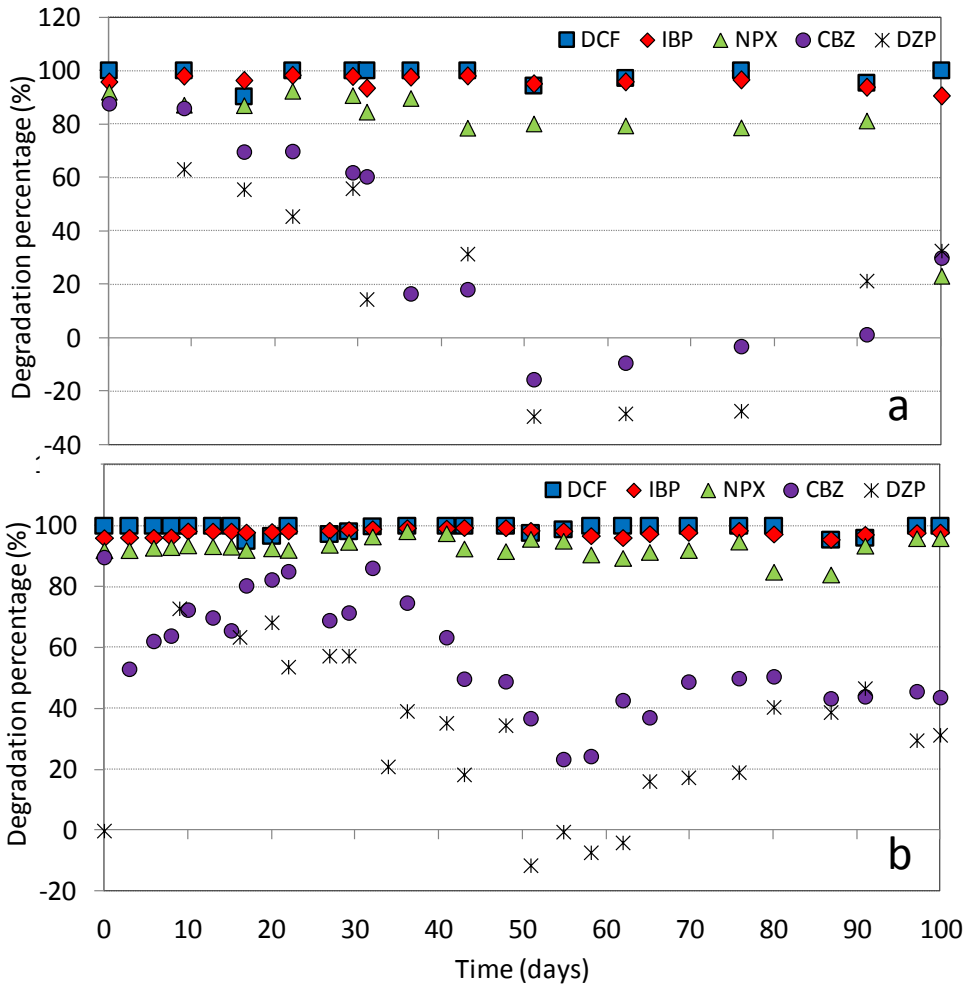


Figure 5-11. Degradation tendency of DCF, IBP, NPX, CBZ and DZP in the middle port (a) and the upper port (b) of the fixed-bed reactor with *P. chrysosporium* immobilized in polyurethane foam.

The degradation tendency of each of the selected pharmaceutical compounds is shown in Figure 5-11. It is noteworthy that the anti-inflammatory compounds are removed in both ports of the FBR (medium and upper port); however, compounds such as CBZ and DZP showed a different behaviour, depending on the port where the sample was withdrawn.

At the end of the assay in the FBR, the support was taken from the column and divided in three parts: the upper, the medium and the bottom of the column. With the upper and bottom fractions, an extraction with acetonitrile was carried out in order to determine the residual concentration. In addition, using the three fractions, the determination of the biomass content was conducted resulting in 1.89 g of total biomass in the column with 4.05 g of polyurethane foam (equivalent to 11.3 g/L). The results of the

adsorption experiment are shown in Table 5-2. Low concentrations (below 0.066 mg of drug per g of biomass) of the considered pharmaceutical compounds were detected in the polyurethane foam.

Table 5-2. Residual concentration of pharmaceuticals onto biomass and polyurethane foam

	Pharmaceutical concentration	
	Upper zone (mg drug/g biomass)	Bottom zone (mg drug/ g biomass)
DCF	0.000	0.031
IBP	0.012	0.033
NPX	0.049	0.066
CBZ	0.017	0.013
DZP	0.034	0.029

5.3.2. *P. chrysosporium* immobilized in a fixed-bed reactor with oxygen pulses for the degradation of DCF, IBP, NPX, CBZ and DZP

The effect of the gas supply on the degradation of the pharmaceutical compounds: DCF, IBP, NPX, CBZ and DZP, was assessed in the FBR operated with oxygen pulses. The configuration of the reactor is shown in Figure 5-12.

Glucose concentrations as well as the addition and consumption rates of glucose are shown in Figure 5-13. The concentrations of glucose in both outlet streams were similar until day 36; however, since almost negligible values were detected, the feed was added also through the medium port. This strategy was conducted in order to maintain the glucose in a low concentration but not limited into the reactor; this promotes the viability of the process and avoids cell lysis. The concentration of glucose in the upper port increases until 3.6 g/L after the new feeding strategy and it was maintained below 3 g/L until the end of the experiment (Figure 5-13a).

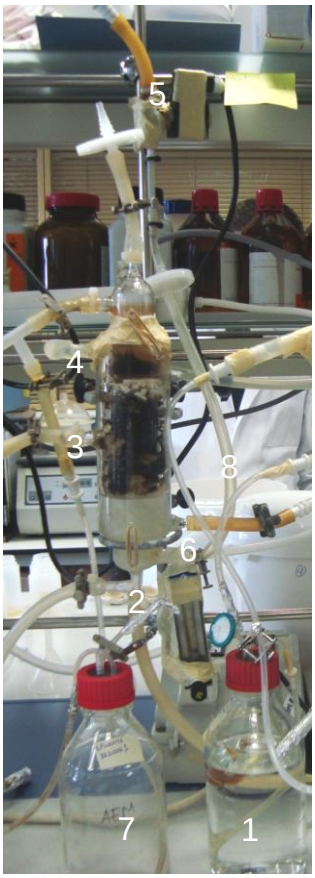


Figure 5-12. Fixed-bed reactor configuration with *P. chrysosporium* immobilized in polyurethane foam with oxygen pulses. Symbols: (1) feed medium bottle, (2) feed addition port, (3) reactor medium port, (4) reactor effluent port, (5) pulsing device, (6) oxygen supply port, (7) effluent bottle, (8) thermostatized water.

The feed medium was supplemented into the reactor by means of two addition rates: 250 mg/L·h throughout the experiment except for the period between days 9 and 19, where the addition rate was of 125 mg/L·h (Figure 5-13b). The decrease of the feeding rate was conducted since the fungus was unable to deplete all the glucose present in the feed flow and consequently secondary metabolism was avoided. Low consumption rates were detected until day 10 in the effluent of the reactor. After the implementation of two feeding ports (day 36) a slight decrease of the glucose consumption was observed; despite this, nearly total consumption of glucose was achieved during the second half of the experiment (Figure 5-13b).

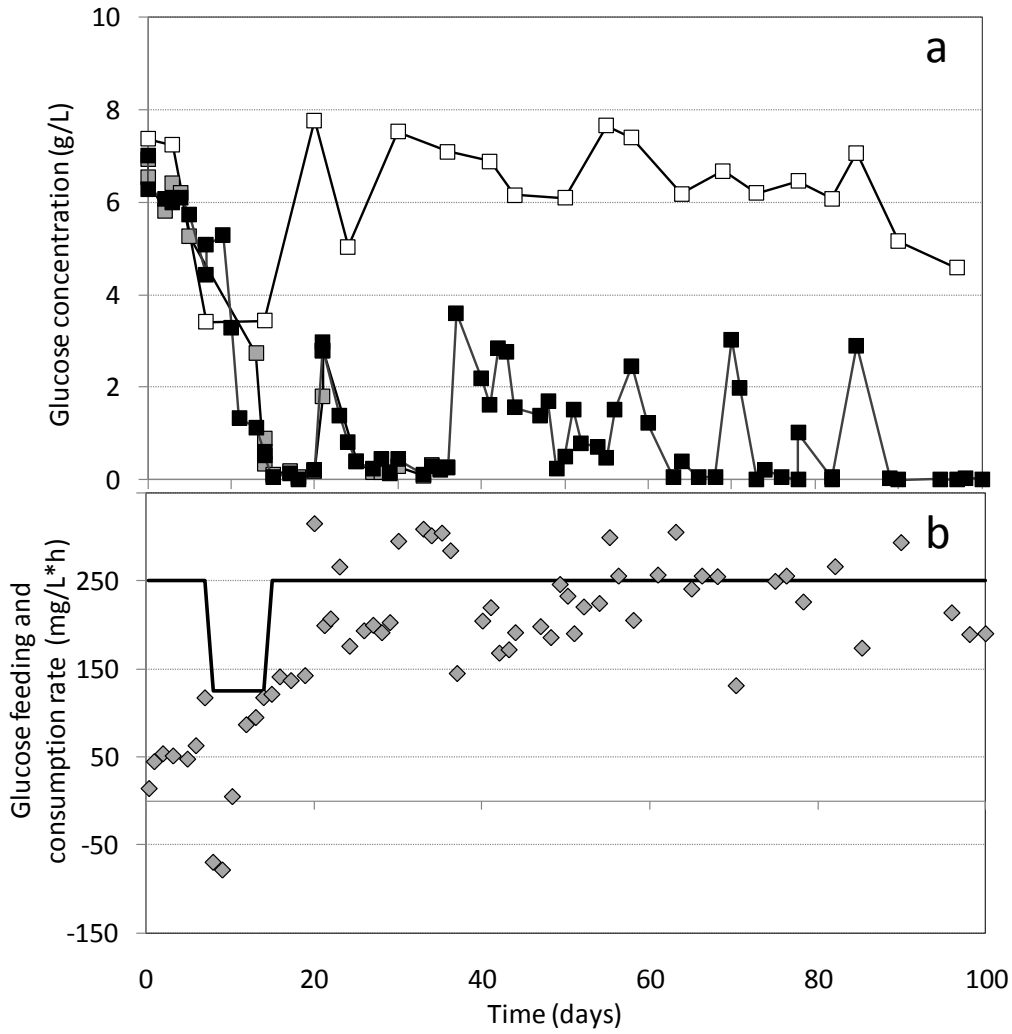


Figure 5-13. Glucose concentration (a) and glucose feeding and consumption rate (b) in the fixed-bed reactor Symbols: glucose – feed medium (□), glucose – reactor medium port (▣), glucose – reactor effluent (■), glucose feeding rate (—), glucose consumption rate (◇).

The pH of the feed medium was maintained approximately in 4.5 throughout the experiment. Moreover, the pH was detected between 4.7 and 5.7 in both effluent ports until day 36 (Figure 5-14a). Due to these high values of pH, the implementation of a second feeding addition port was also necessary, since high values of pH are related to protease secretion. Low enzymatic activities were detected until day 69 (below 10 U/L); then two peaks were detected at days 70 and 90 achieving a maximum MnP activity of 30 U/L (Figure 5-14b) while the concentration of hydrogen peroxide was detected between 0.5 and 2 mg/L throughout the experiment (data not shown).

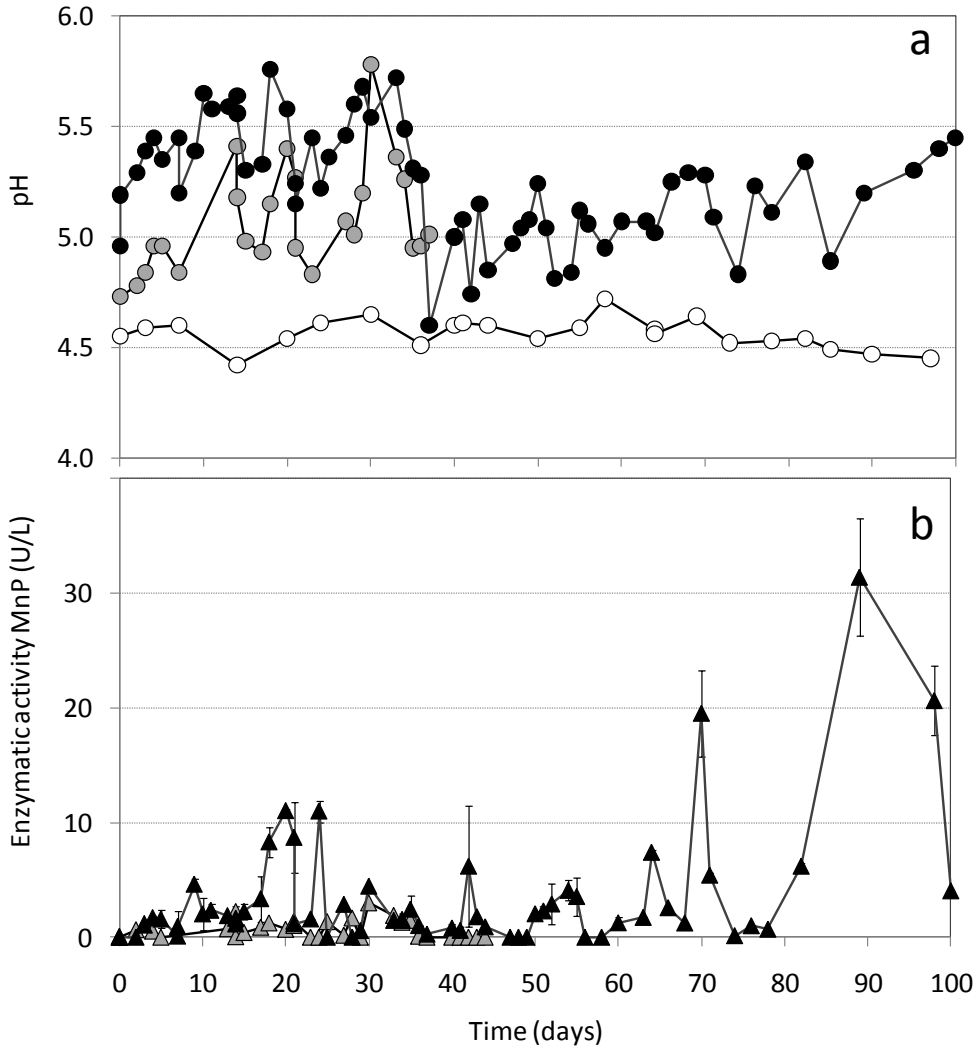


Figure 5-14.pH detected (a) and enzymatic activity of MnP (b) in the fixed-bed reactor Symbols: pH – feed medium (○), pH – reactor medium port (◎), pH – reactor effluent (●), MnP – reactor medium port (△), MnP – reactor effluent (▲).

A pulsing device was used to supply oxygen in a pulsed flow with a pulsation frequency of 0.0625 s^{-1}, which means that the electrovalve opens 1 and closes for 15 s. This frequency was used in order to maintain the maximum concentration of dissolved oxygen in the reactor, which was maintained in a range between 15 mg/L and 23 mg/L until day 20; then remained below 20 mg/L during the rest of the experiment (Figure 5-15a). The measurement of this parameter was conducted off-line with an external electrode; it was necessary to wait approximately 5 minutes in order to obtain enough sample volume to perform the measurement, for this reason the values are indicative.

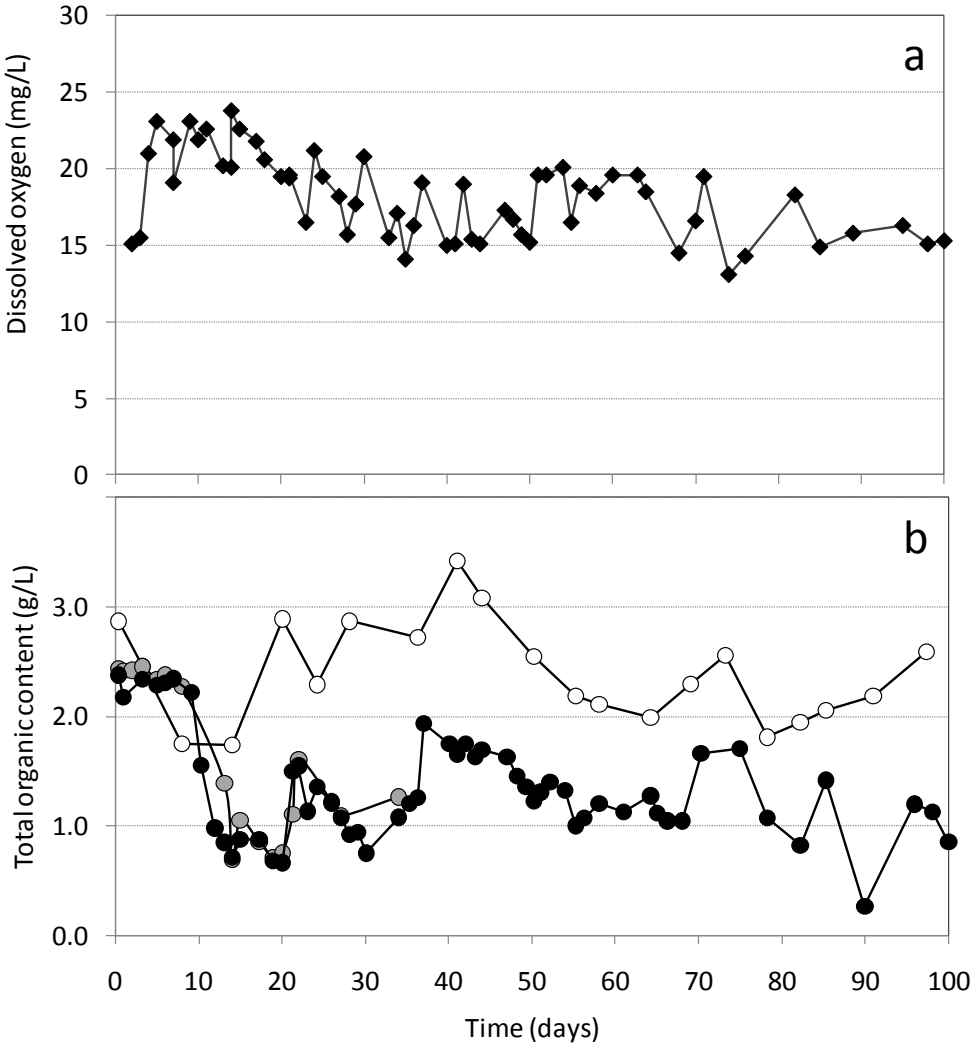


Figure 5-15.Dissolved oxygen (a) and total organic carbon (b) detected in the fixed-bed reactor Symbols: dissolved oxygen (◆), TOC – feed medium (○) TOC – reactor medium port (◍), TOC – reactor effluent (●).

There was no difference between the concentrations of total organic carbon (TOC) detected in the middle and upper ports (at least until day 36). A concentration of TOC in a range between 2-3 g/L was detected in the feed medium; while in the outlet streams less than 2 g/L were measured (Figure 5-15b). The concentration of total nitrogen was maintained below 0.15 g/L in all the withdrawn samples during 100 days.

Regarding the appearance of the support, its aspect was similar to that of the aerated FBR at the beginning of the experiment (Figure 5-16). Preferential pathways formed by the action of the feed and gas flows were observed after two weeks; also a slightly darkening of the upper zone and spores of *P. chrysosporium* were observed.

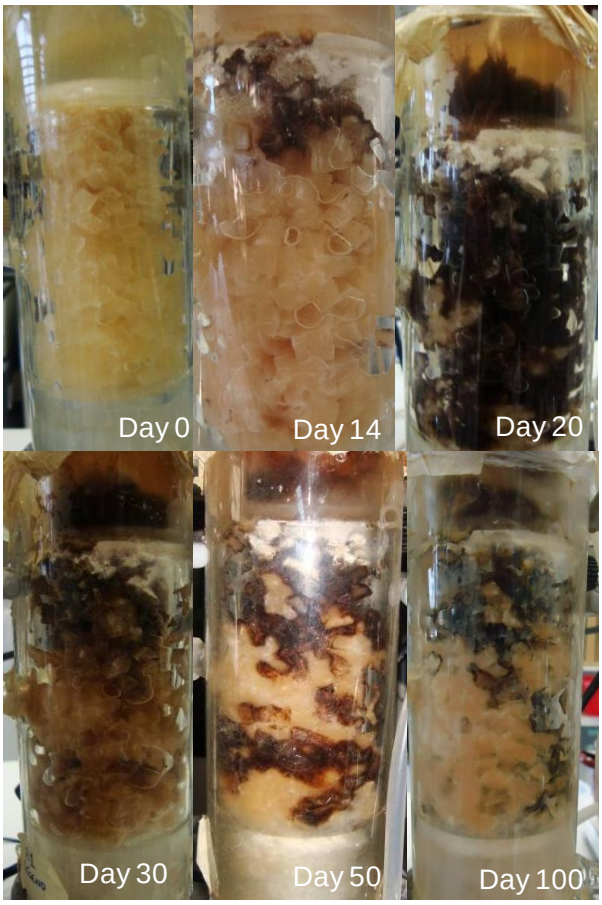


Figure 5-16. Appearance of the fixed-bed reactor with oxygen pulses

Polyurethane foam presented a complete darkening and also hyphal growth above the porcelain plate was observed after 20 days. It is noteworthy that after the addition of a second feeding port (at day 36), the darkening of the support at the bottom and the middle of the column disappeared; this was due to the fact that the feed medium was added into the reactor by this zone. The appearance of the reactor support from day 50 until the end of the experiment was similar, except for the fading of some darkening zones. The final concentration of the biomass was of 1.07 g in the column, which means a concentration of 6.41 g/L.

As in the previous experiment of this chapter, the study of the hydrodynamic behavior of the oxygenated FBR was performed by the determination of the residence time distribution (RTD) using lithium chloride as a tracer. The results concerning to the adjusted curve to the tanks-in-series model by Levenspiel (1999) are shown in Figure 5-17. The hydraulics of the reactor corresponds to 1.6 complete stirred tanks.

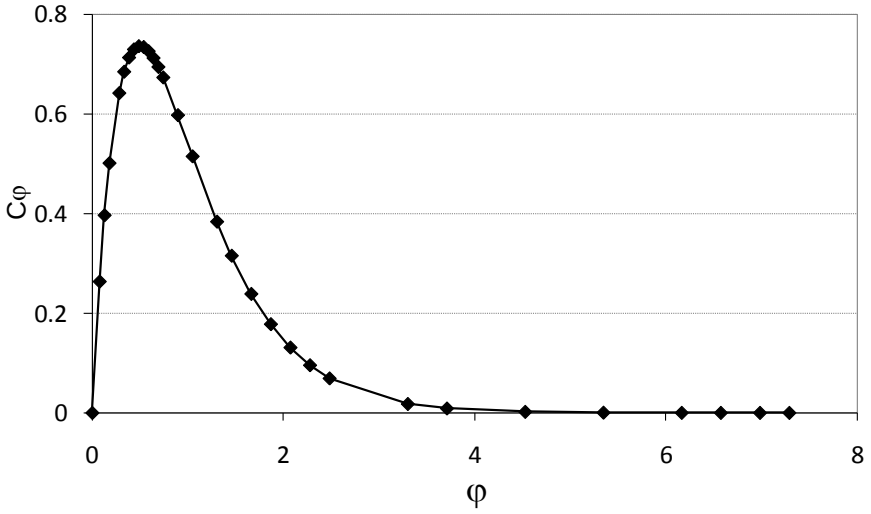


Figure 5-17. Residence time distribution (RTD) of the oxygenated fixed-bed reactor

The results of the degradation of DCF, IBP and NPX in the pulsed fungal bioreactor are shown in Figure 5-18. There was no significant difference between the feeding and degradation rates in the specific case of DCF (Figure 5-18a). Above 83% of removal in the upper port was detected for IBP and NPX; however in the medium port the highest removals were detected in the case of the former (> 92%) than the latter (> 69%).

Regarding the degradation of CBZ, only partial removal was achieved (Figure 5-19a). Until day 30 there was no differences between both outlet streams with degradations between 40% and 80%; however, at day 36 lower removals were detected in the upper port (< 10%). The degradation of CBZ was maintained with decreasing tendency from the middle of the experiment until the end achieving eliminations from 20% up to 60% (Figures 5-19a and 5-20).

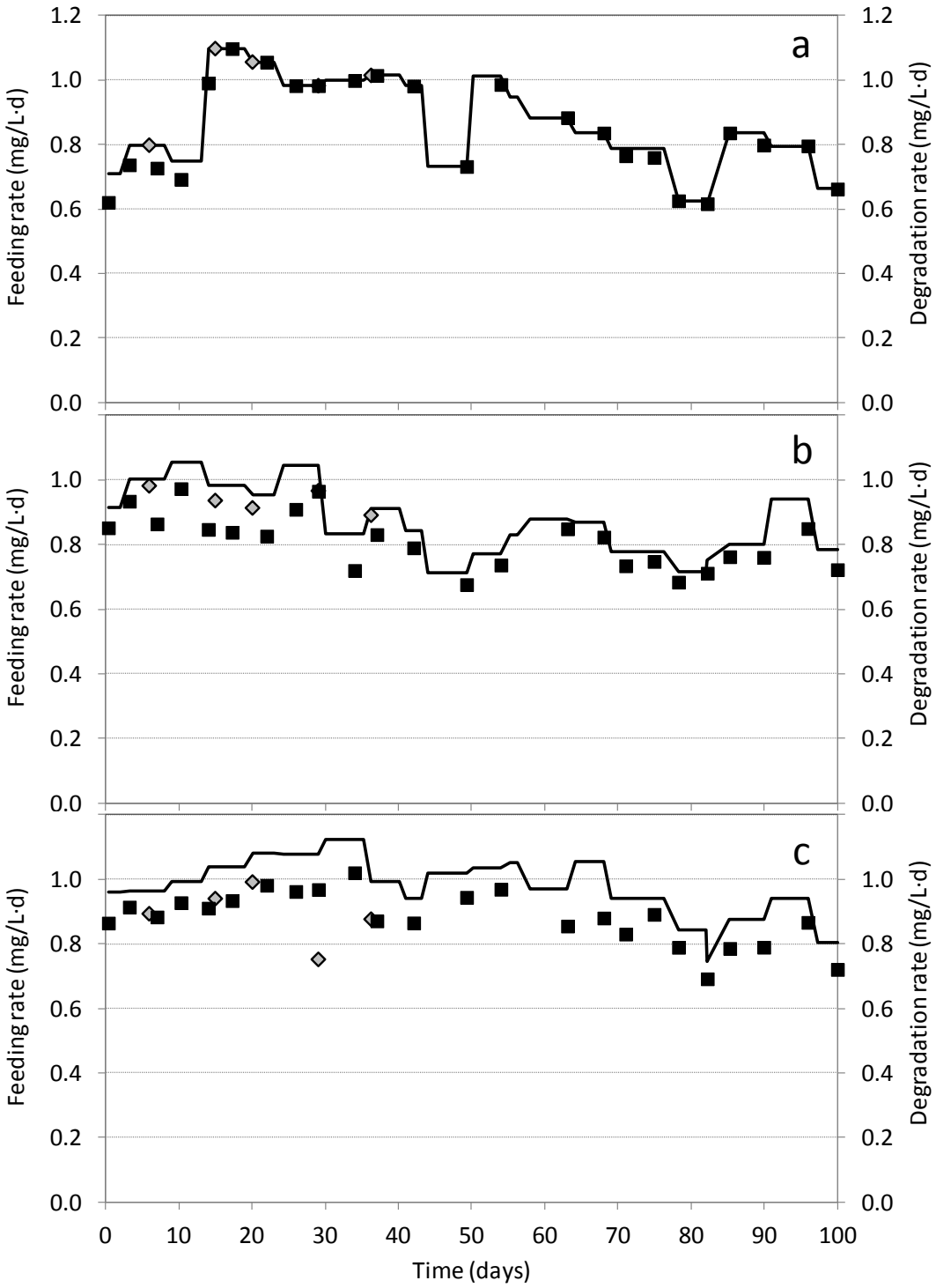


Figure 5-18. Feeding and degradation rate of DCF (a), IBP (b) and NPX (c) in a fixed bed reactor with *P. chrysosporium* immobilized in polyurethane foam. Symbols: feed medium (—), reactor medium port (◇), reactor effluent port (■).

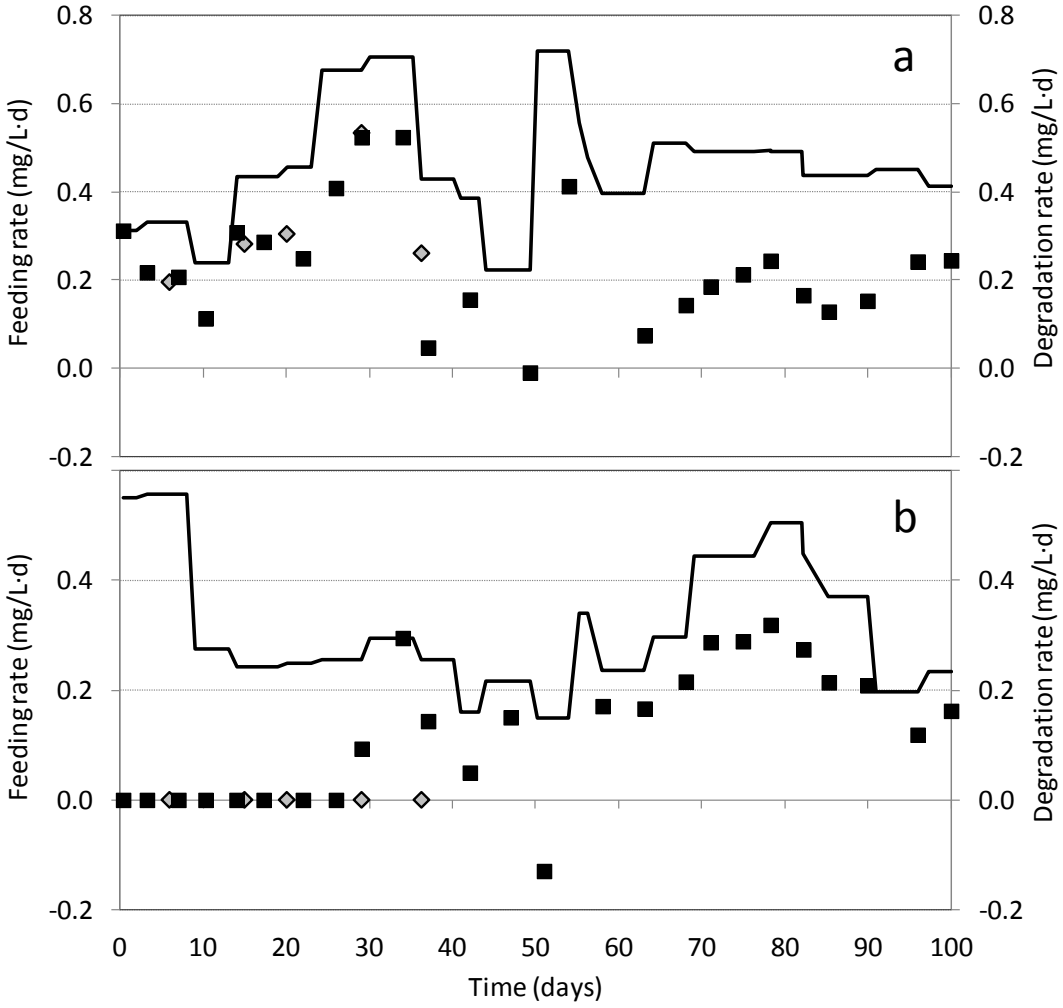


Figure 5-19. Feeding and degradation rate of CBZ (a) and DZP (b) in a fixed bed reactor with *P. chrysosporium* immobilized in polyurethane foam. Symbols: reed medium (—), reactor medium port (◇), reactor effluent port (■).

A different case was the degradation of DZP, since this compound was not detected until day 28 neither in the medium port nor the upper port of the reactor. This could mean its total removal; however, due to its low biodegradability and its physicochemical properties, this behavior might be due to an adsorption process. Diazepam started to appear in the upper port at day 28 with an increasing removal tendency over the experiment progressed. A maximum elimination of 72% was achieved and the degradation was maintained stable around 60% during the last days of experiment (Figure 5-19b).

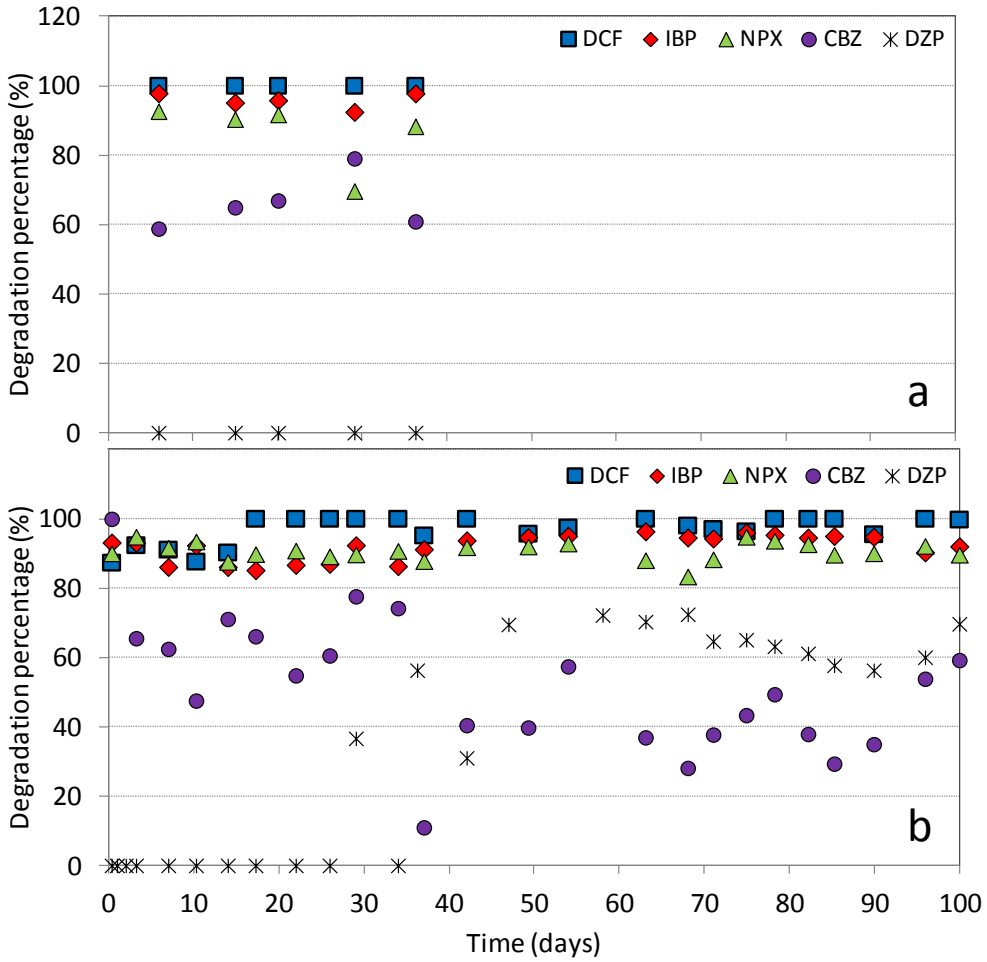


Figure 5-20. Degradation tendency of DCF, IBP, NPX, CBZ and DZP in the medium port (a) and the upper port (b) of the fixed-bed reactor with *P. chrysosporium* immobilized in polyurethane foam.

The degradation tendency of the five pharmaceutical compounds in the oxygen pulsed bioreactor is shown in Figure 5-20. The anti-inflammatory drugs and the antiepileptic drug maintained a high elimination (>60%) until day 36 in the medium port; after this day this port was used as feeding port. Moreover, the drug DZP was not detected in this port (Figure 5-20a). Contrary to these results, better degradations were observed in the upper port; furthermore, the three anti-inflammatory compounds maintained their removal tendency until the end of the experiment. The case of CBZ and DZP was different, since the degradation of the former tends to decrease while the removal of the latter increase over the experiment progressed (Figure 5-20b).

As in the FBR with air, the support was divided into three parts in order to carry out the determination of the residual concentration of the target compounds and to

measure the amount of biomass within the reactor. The final content of biomass was of 1.07 g which according to the bioreactor volume it was equivalent to 6.4 g/L. The results of the residual concentration of pharmaceutical compounds are shown in Table 5-3. All the compounds were detected in a concentration in a range between 0.005- 0.026 g per gram of biomass. Comparing these results with those obtained in the FBR with a continuous air flow, lower amounts of DCF, IBP and CBZ were detected in the oxygenated FBR; NPX was detected in higher amounts at least in the upper zone of the column while similar results were found for DZP.

Table 5-3. Residual concentration of pharmaceuticals compounds onto biomass and polyurethane foam

	Pharmaceutical concentration	
	Upper zone (mg drug/g biomass)	Bottom zone (mg drug/ g biomass)
DCF	0.002	0.001
IBP	0.001	0.001
NPX	0.026	0.009
CBZ	0.002	0.001
DZP	0.010	0.006

5.3.3. *P. chrysosporium* immobilized in a stirred tank reactor (STR) with a continuous air flow for the degradation of DCF, IBP, NPX, CBZ and DZP

The configuration of a STR with immobilized *P. chrysosporium* is shown in Figure 5-21. The reactor was operated for 50 days; however, an increase of the reactor volume (from 1.5 L to 1.75 L) and an adjustment of the gas flow (0.5 L/min) were necessary at day 9 in order to keep the maximum agitation of the support.

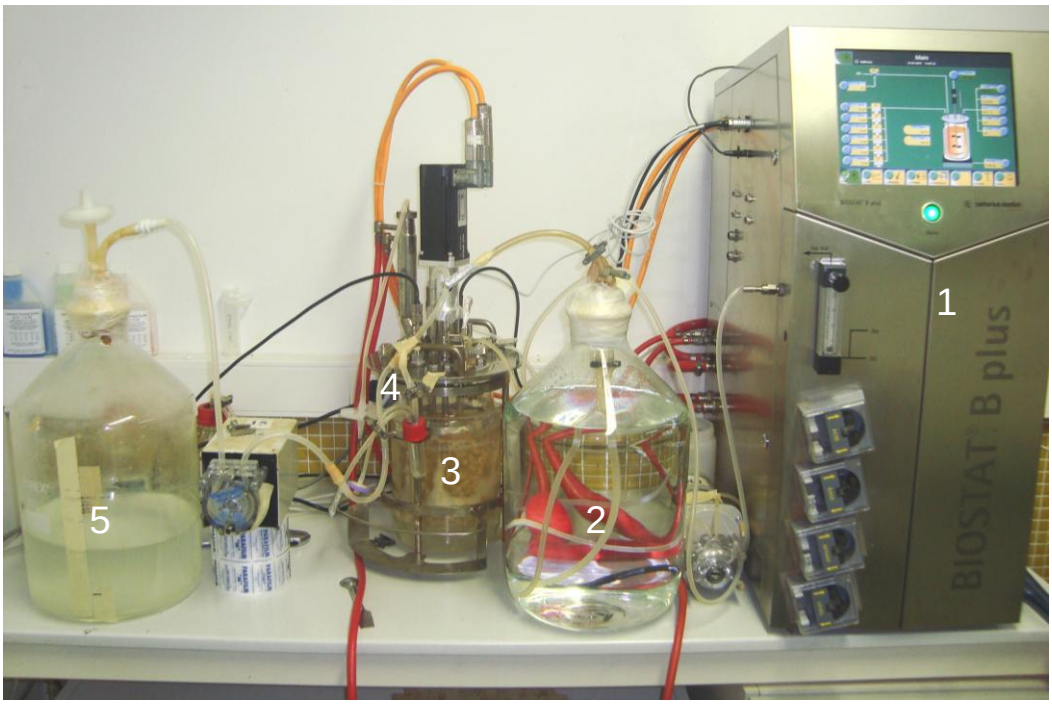


Figure 5-21. Stirred tank reactor (STR) configuration with *P. chrysosporium* immobilized in polyurethane foam and a continuous air flow. Symbols: (1) module operator service program, (2) feed medium port, (3) reactor vessel filled with immobilized fungus, (4) effluent sampling, (5) effluent reservoir.

Glucose concentration detected in the feed medium and the effluents are shown in Figure 5-21 as well as their feeding addition and consumption rates. The concentration of glucose in the effluent was higher or similar to that of the feed medium until day 10 (Figure 5-21a); for this reason a decrease in the feeding rate was decided: from 250 mg/L·h to 125 mg/L·h (Figure 5-22b). After this modification, glucose consumption was almost complete.

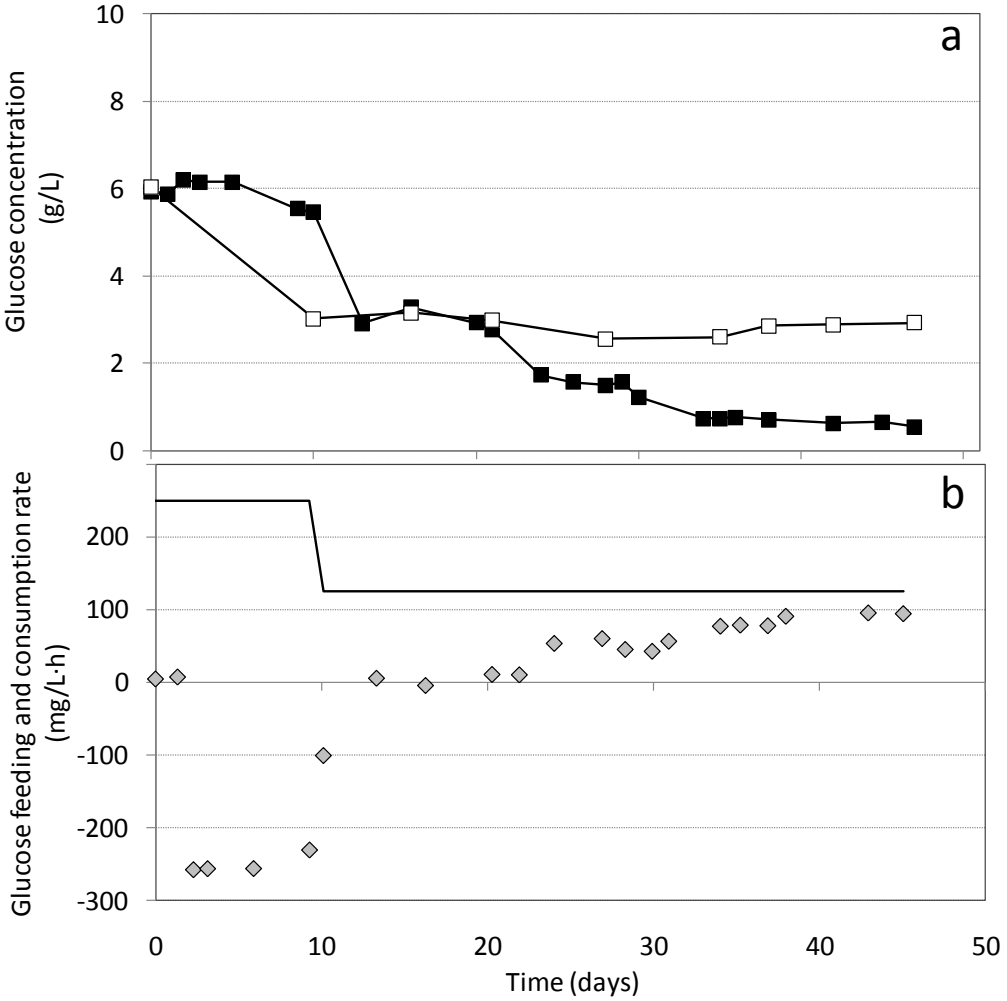


Figure 5-22. Glucose concentration (a) and glucose feeding and consumption rate (b) in a STR with *P. chrysosporium* immobilized in polyurethane foam. Symbols: glucose – feed medium (□), glucose – reactor effluent (■), glucose feeding rate (—), glucose consumption rate (◇).

The pH in the feed medium was maintained between 4.2 – 4.7 throughout the experiment. In the effluent, the pH increased until values around 5.0 after 30 days of experiment; then, a pH decay was observed (Figure 5-23a). No enzymatic activity was observed throughout the experiment (Figure 5-23b).

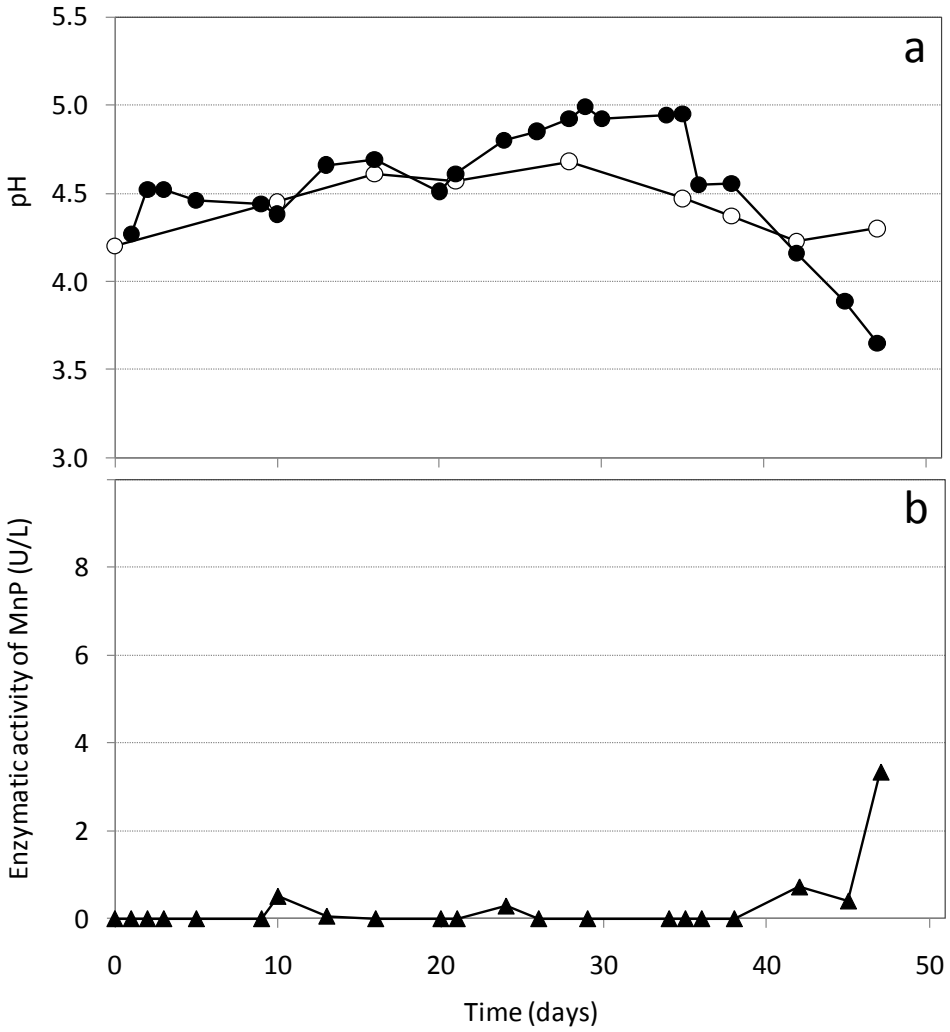


Figure 5-23. pH detected (a) and enzymatic activity of MnP (b)) in a STR with *P. chrysosporium* immobilized in polyurethane foam. Symbols: pH – feed medium (○), pH – reactor effluent (●), MnP - reactor effluent (▲).

The concentration of dissolved oxygen was maintained around 5 mg/L during the 50 days of experiment (Figure 5-24a). Also, total organic carbon (TOC) content was detected above 2 g/L when the feeding addition rate was 250 mg/L·h; while TOC was below 1.9 g/L with the lower feeding medium (Figure 5-24b). Total nitrogen content was measured below 0.05 g/L in all the samples and the content of hydrogen peroxide was maintained between 0.5 and 2 mg/L throughout the experiment (data not shown).

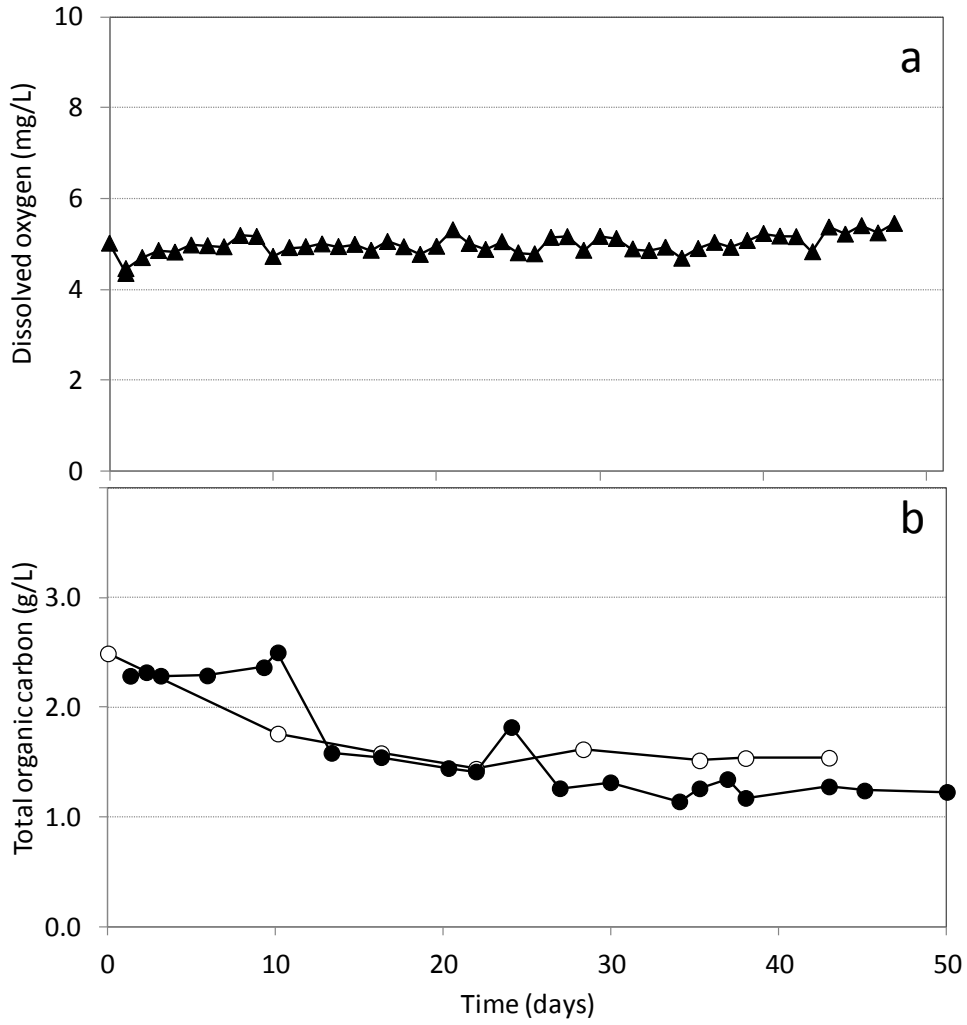


Figure 5-24. Dissolved oxygen (a) and total organic carbon (b) detected in a STR with *P. chrysosporium* immobilized in polyurethane foam. Symbols: dissolved oxygen (▲), TOC – feed medium (○), TOC – reactor effluent (●).

Concerning to the appearance of the immobilized fungus in the STR, until day 9 the volume was of 1.5 L with an agitation of 200 rpm and the gas flow was added in a range between 2-3 L/min; this caused that the polyurethane foam was kept retained in the surface of the medium avoiding a homogeneous mixture. For this reason, the volume of the reactor was increased until 1.75 L and the gas flow was decreased until 0.5 L/min; this allowed a homogeneous agitation of the support and high dissolved oxygen content.

Unlike the FBRs, there was no change in the aspect of the polyurethane foam in the STR, specifically the colour of the support. A slight mycelium growth was observed attached to the inner walls of the reactor vessel after 20 days of experiment. No loss of support cubes in the effluent was observed throughout the experiment, except at day 28,

where a small amount of polyurethane foam was recovered from the effluent reservoir. The extraction of the pharmaceutical compounds and the biomass determination were carried out with this support (Figure 5-25).

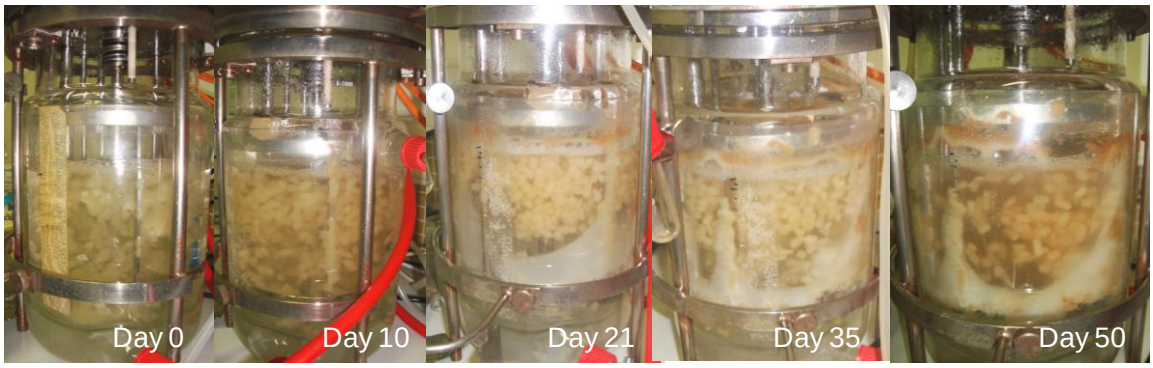


Figure 5-25. Appearance of *P. chrysosporium* immobilized in a stirred tank reactor with a continuous air flow.

At the end of the experiment, all the support and free mycelia was recovered from the reactor vessel; then an extraction with acetonitrile and the determination of the biomass was performed. Considering the amount of biomass measured at day 28 and the biomass content at the end of the experiment it was found 3.4 g of biomass in the STR, which means a concentration of 2 g/L.

The results regarding to the elimination of DCF, IBP and NPX are shown in Figure 5-26. The feeding and degradation rate of DCF and IBP were similar throughout the 50 days of assay, achieving removal percentages above 93% for both compounds (Figures 5-26a and b). A high removal of NPX was detected during the first 3 days of assay (above 90%); however, a decrease in the efficiency of elimination until percentages in a range between 65% and 77% were observed until the end of the assay (Figure 5-26c).

Regarding to the removal of the antiepileptic and the tranquilizer, these drugs are characterized by their recalcitrant behavior. Carbamazepine was partially removed at the beginning of the assay (until day 3) in a range between 50% and 61%; then, the degradation of this compound decreases until percentages below 30% maintaining this removal until the end of the assay (Figure 5-27a). The same behaviour was observed for the removal of DZP which was removed in a percentage below 40% until day 20; then the elimination of this compound was negligible. Despite this, during the period between days 35 and 50, the removal of DZP was maintained in a range between 18% and 47% (Figure 5-27b).

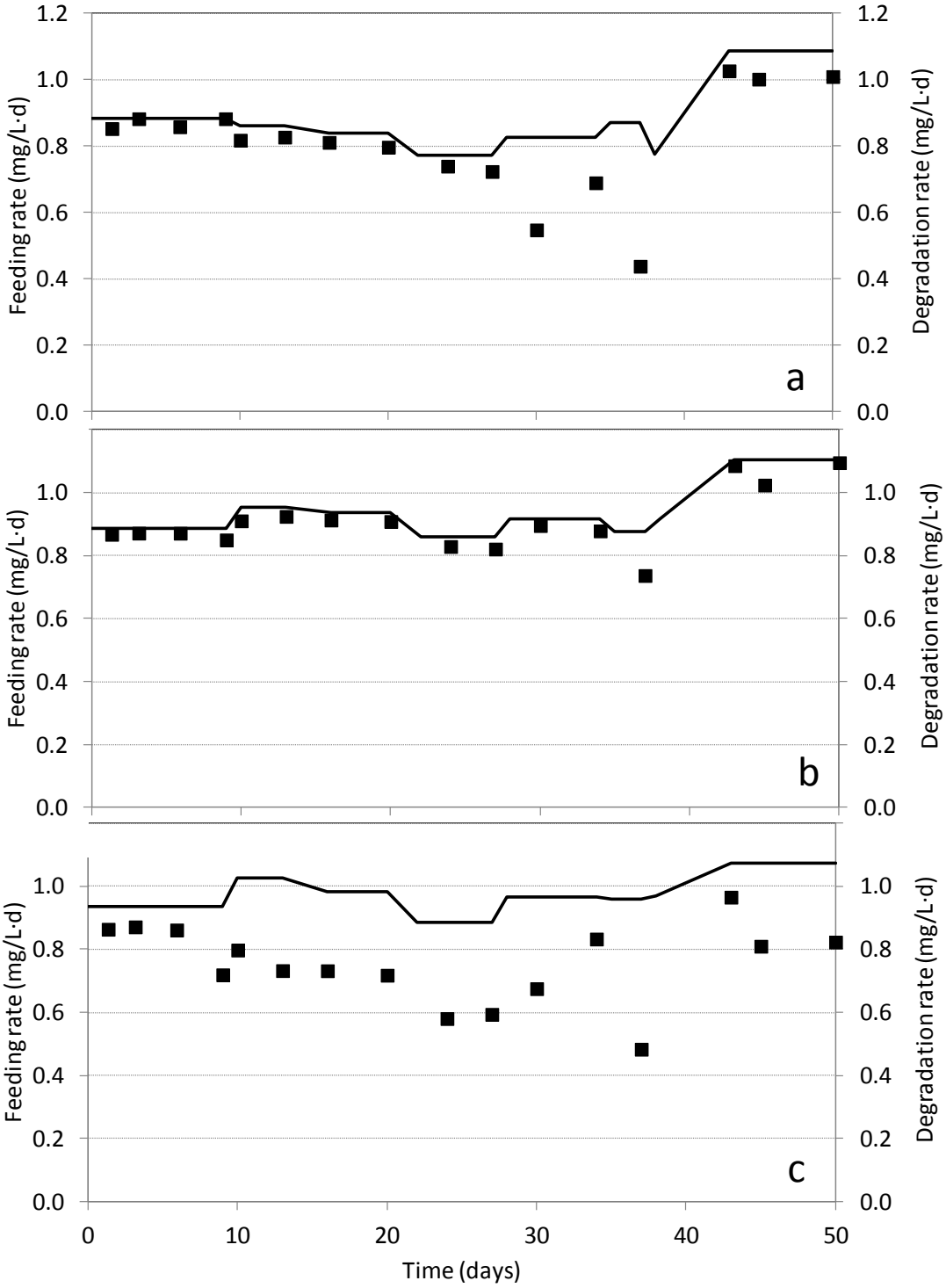


Figure 5-26. Feeding and degradation rate of DCF (a), IBP (b) and NPX (C) in a STR with *P. chrysosporium* immobilized in polyurethane foam. Symbols: feed medium (—), reactor effluent port (■).

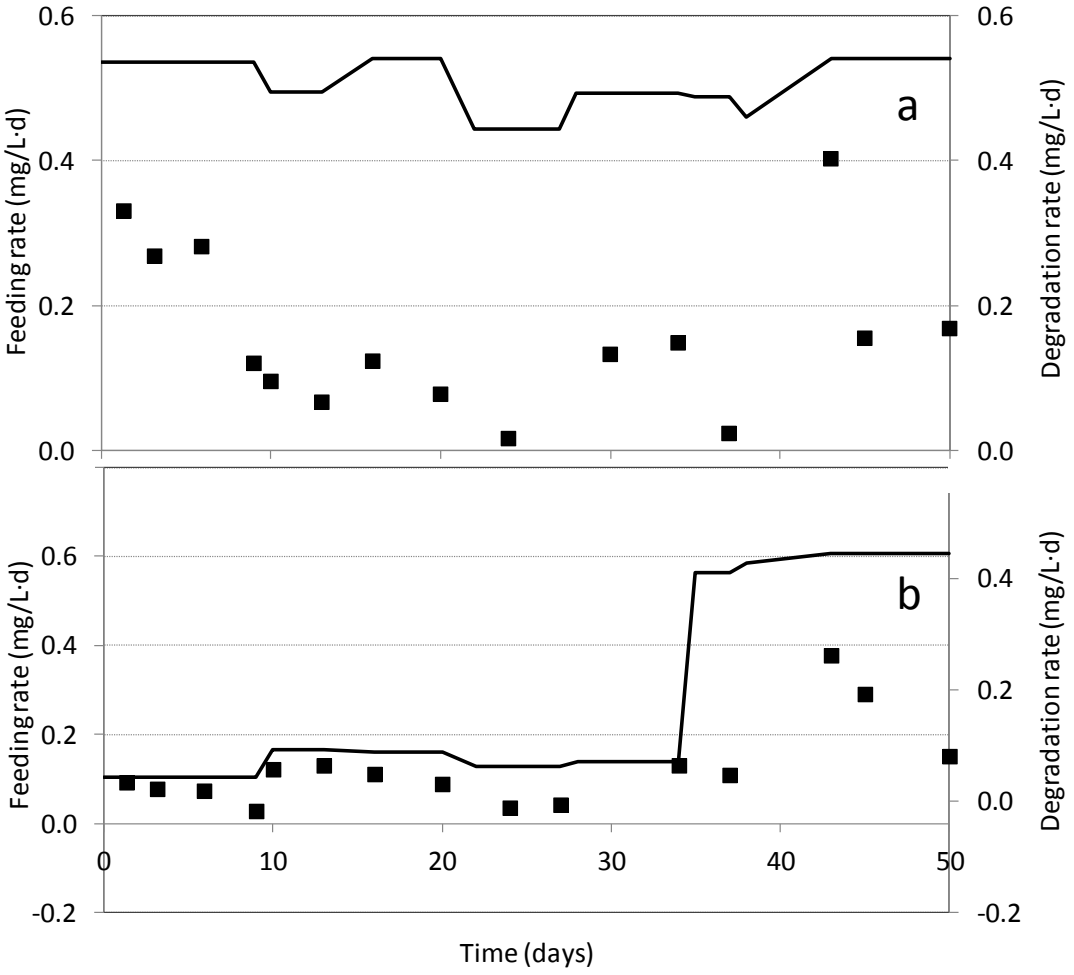


Figure 5-27. Feeding and degradation rate of CBZ (a) and DZP (b) in a STR with *P. chrysosporium* immobilized in polyurethane foam. Symbols: feed medium (—), reactor effluent port (■).

The degradation tendency of the considered compounds is shown in Figure 5-28. The elimination of DCF and IBP is remarkable since above 80% of this compounds were degraded by the fungus *P. chrysosporium*. Similar results were observed for the drug NPX which was highly removed at the beginning of the experiment; however, the degradation percentages were maintained between 60 and 80% during the rest of the assay. Concerning to CBZ, this compound showed a decrease tendency, from degradation around 60% until negligible percentages at day 30. From day 35 until the end of the experiment, the removal efficiency increases until 30%. Similar behavior was observed for DZP, since from day 35 until the end of the assay, the degradation of this compound increased.

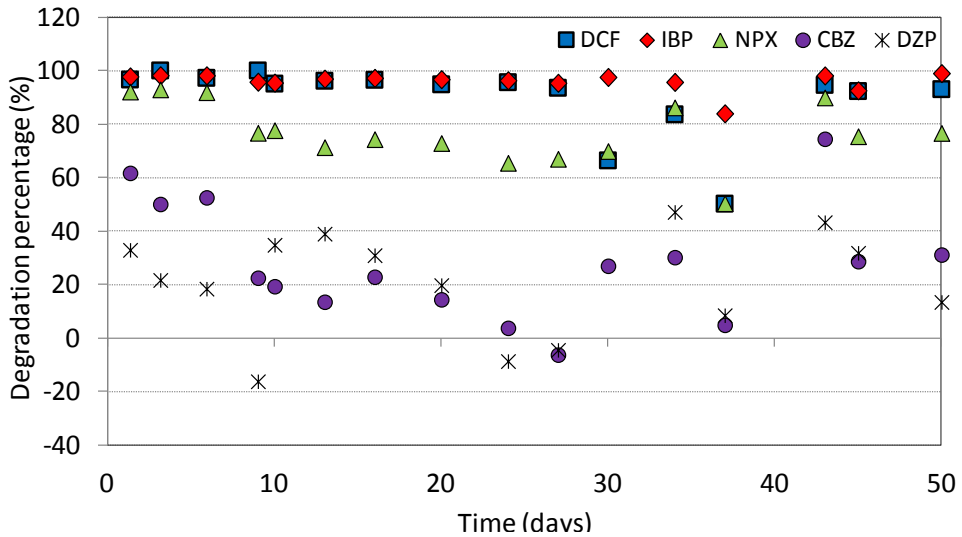


Figure 5-28. Degradation tendency of DCF, IBP, NPX, CBZ and DZP in the medium port (a) and the upper port (b) of the STR with *P. chrysosporium* immobilized in polyurethane foam.

At the end of the experiment, the determination of the residual concentration of the pharmaceutical compounds that could be adsorbed onto the polyurethane foam and the biomass was conducted. Therefore, all the support and free mycelium were taken out from the reactor and an extraction with acetonitrile was carried out. The results of this experiment are shown in Table 5-4. High amounts of all the considered compounds were found in the support. Diclofenac showed the highest amount (15.9 mg of drug per gram of polyurethane foam with biomass) followed by the drug NPX. From the rest of the compounds, IBP was the one with the lowest amount adsorbed. In the bioreactor vessel, almost negligible concentrations were detected for all the compounds.

Table 5-4. Residual concentration of pharmaceuticals onto biomass-polyurethane foam and the bioreactor vessel.

	Pharmaceutical concentration	
	Biomass and polyurethane foam (mg drug/g)	Vessel (mg drug/cm^2)
DCF	15.97	0.003
IBP	0.411	0.002
NPX	5.32	0.001
CBZ	1.43	0.0004
DZP	2.60	0.000

5.4. Discussion

Degradation of five pharmaceutical compounds (DCF, IBP, NPX, CBZ and DZP) was conducted in FBRs and STRs using *P. chrysosporium* immobilized in polyurethane foam. Also, two aeration strategies were studied in the FBR: a continuous air flow and oxygen pulses added by a pulsing device. Concerning to the continuous STR, the gas supply consisted in air. The operation of the bioreactors was maintained stable for 100 for the FBRs and 50 days for the STR.

Operational parameters of FBRs were monitored throughout the assays; glucose concentration was maintained in low values in order to promote the secondary metabolism of the fungus *P. chrysosporium* and consequently avoided a possible cell lysis by secretion of proteases. However, during the FBR with oxygen pulses, it was necessary to implement a second feeding addition port due to the increase of the pH value and the low consumption of glucose. This strategy was successful since the concentration of glucose was maintained below 3 g/L and the pH value was detected near to 5; however towards the end of this assay, an increase of the pH was observed. This increase was also detected in the FBR with continuous air flow, indicating the low viability of the bioreactor. Concerning to the assay conducted in a STR with immobilized fungus, the content of glucose inside the bioreactor was high during the first 10 days of assay, but a decrease of the feeding rate favour the consumption rate. The pH detected in this assay showed a decrease during the last days, this may be related with the secretion of organic acids. According to the effect of the gas supply over the enzymatic activity, high levels of oxygen favour the ligninolytic system of *P. chrysosporium*; this is in accordance with the results of this chapter since low values were detected in the FBR and the STR with continuous air flow while in the FBR with oxygen pulses higher activities were observed.

The support in FBRs showed large changes in its color during experiments. When oxygen pulses were added, the support presented a total darkening whereas only small black spots were observed in the aerated reactor. Moreover, this darkening was not observed during the STR experiment. The factors that might have caused this darkening or appearing of black spots have not been studied; however, Blanchette (1984) demonstrated that black spots within decayed wood are usually produced by several fungi and are always associated with delignification of wood. Also, in the study of Blanchette (1984) a total darkening was observed in those areas of wood where a delignification process occurred by several fungal strains; in addition, manganese oxides deposits were detected in those areas. A process of oxidation of manganese may have occurred during the FBRs experiments conducted in this chapter. It has been demonstrated that high levels

of oxygen promote lignin degradation and enzyme production (Dosoretz et al, 1990; Moreira et al. 1996; Jiménez-Tobon et al., 1997, Rothschild et al., 1999). Perhaps the level of oxygen in the bioreactor is related to the darkening of the support, since when a high concentration of dissolved oxygen was maintained a total support darkening was observed. Despite these conclusions, a further study would be necessary to clarify the causes of these black spots on the FBRs. Concerning to biomass content, high levels of oxygen tend to control fungal growth and this was demonstrated in the oxygenated FBR where it was detected almost the half of the biomass obtained in the aerated reactor. In contrast, the use of immobilized fungi instead of free mycelia in a STR controls the excessive fungal growth.

Anti-inflammatory drugs are characterized by their high biodegradability due to their pseudo first-order degradation constant (K_{biol}), while CBZ and DZP are classified as hardly biodegradable compounds (Joss et al., 2006). The removal of the three anti-inflammatory drugs have been studied in sewage treatment plants (STPs) achieving removals above 60% for all the compounds, although the removal of DCF is usually around 20-40% (Lindqvist et al., 2005; Zhang et al., 2008). Higher degradations have been reported using several technologies such as advanced oxidation processes (AOPs), membrane bioreactor (MBR), conventional activated sludge (CAS), among others (Huber et al., 2005; Hofmann et al., 2007; Sipma et al., 2010; Ziylan and Ince, 2011). Otherwise, low removals of recalcitrant compounds such as CBZ and DZP in STPs have been reported (Redshaw et al., 2008; Calisto and Esteves, 2009); however, the use of AOPs may improve the degradation of these compounds (Vogna et al., 2004; Esplugas et al., 2007; Kim and Tanaka 2009). Currently there is no research using white-rot fungi immobilized in FBRs and STRs for the removal of pharmaceutical compounds. Although in recent studies the use of ligninolytic fungi and their enzymes to remove pharmaceutical compounds during batch and fed-batch experiments have been performed achieving high removals, even when high concentrations were used (up to 10 mg/L) (Marco-Urrea et al . 2009; Marco-Urrea et al., 2010b; Rodríguez-Rodríguez et al., 2010). Furthermore, ligninolytic enzymes are able to degrade DCF, IBP and NPX in shorter periods of time (only a few hours) than during *in vivo* fungal cultures (Lloret et al. 2010; Marco-Urrea et al., 2010a; Zhang and Geiβen, 2010; Eibes et al., 2011); although, it was necessary 2 days for the removal of 66% of CBZ with the enzyme Laccase in a fed-batch experiment (Hata et al., 2010). Satisfactory results were found during the continuous removal of these compounds in both types of reactors, even high removals were found for recalcitrant compounds such as CBZ and DZP.

Regarding the effect of gas supply used (continuous air flow and oxygen pulses) on the degradation of pharmaceutical compounds in FBRs, the three anti-inflammatories were highly degraded regardless of the aeration supply. Moreover, the removal of CBZ

was enhanced when continuous air supply was used, maintaining a partial removal until the end of the experiment; while a degradation with a decreasing tendency was observed during the oxygenated FBR. Also, the use of oxygen pulses significantly improves the removal of DZP. On the other hand, a total removal of DCF and IBP in the STR with immobilized fungus was maintained throughout the 50 days of assay; while NPX was highly degraded at the beginning of the assay but a decrease of the removal efficiency was observed as the assay progressed. Carbamazepine was partially removed (up to 60%) in the STR at the beginning of the experiment, maintaining degradation percentages below 30% throught the 50 days of operation. Up to 40% of DZP was removed until day 20 in the STR; then, the removal was negligible until day 35, where an increase of the removal efficency was observed (up to 47%). Despite the low activities of MnP it was possible to achieve a total or partial removal of all the selected pharmaceutical compounds; this low concentration might be enough to degrade these compounds.

Another important aspect to take into account is the possible adsorption of these compounds in polyurethane foam. One strategy to counter this problem is to perform an extraction with acetonitrile. Anti-inflammatory compounds tend to be adsorbed on biomass or support due to their liphophilic character (K_{ow}) and their acid dissociation constant (pK_a) which are associated with the processes of sorption into the solids (Suarez et al., 2008). Low concentrations of all the compounds were found onto the support and the fungal biomass; however, significant differences were detected between the medium and the upper port as well as between the aerated and oxygenated FBRs. It seems that when a continuous air flow was added into the reactor, compounds such as DCF, IBP and NPX tend to be adsorbed in the bottom of the column; and contrary results were observed in the oxygenated FBR since higher residual concentrations of these compounds were detected in the upper zone of the column. Higher concentrations of CBZ were detected in the aerated FBR and regardless of the gas supply, similar concentrations were observed for DZP in both bioreactors. Contrary to the FBRs, when a STR was used with immobilized fungus, higher concentrations of all the considered compounds were found adsorbed in the support and the fungal biomass.

5.5. Conclusions

In this chapter the removal of five pharmaceutical compounds (DCF, IBP, NPX, CBZ and DZP) was conducted by the fungus *P. chrysosporium* immobilized in polyurethane foam. Using fixed-bed reactors (FBRs), the effect of a continuous air flow or oxygen pulses addition over the enzyme production and the degradation of pollutants were analyzed. Moreover, the removal of these compounds was also studied using the fungus immobilized in a stirred tank reactor (STR). Compounds such as DCF, IBP and NPX were

easily removed under both aeration conditions in the FBRs. Contrary to these results, recalcitrant compounds such as CBZ and DZP were partially degraded throughout the experiment. In the specific case of CBZ, the degradation of this compound was favoured when a continuous air flow was used; while the removal of DZP was maintained stable during the last 50 days of the oxygenated FBR. Concerning to the degradation of these compound in the STR, the results showed that DCF and IBP were effectively removed, while NPX was partially removed. Lower degradation percentages were achieved for CBZ and DZP compared with those obtained in the FBR. Low concentrations of the five pharmaceutical compounds were detected in the polyurethane foam from the FBRs indicating that these compounds were barely adsorbed under this configuration; however significant differences in the residual concentration were observed through the column of the FBR. Higher values were detected in the upper zone when oxygen pulses were added and the contrary occurred when a continuous air flow was added. High concentrations of DCF, IBP, NPX, CBZ and DZP were found in the support and the fungal biomass within the STR. Low levels of enzymatic activity were detected in the FBR under both aeration conditions; however, it was demonstrated that high concentrations of oxygen improves the enzymatic production, since the high activities were detected in the oxygenated FBR. In the case of the STR, it was no possible to detect enzyme production. A darkening process that might be related with the oxidation of manganese and the oxygen level was observed in the polyurethane foam during the oxygenated FBR; meanwhile only small black spots were observed in the aerated reactor. This darkening process was not detected in the support from the STR.

Chapter 6

Assessment of the degradation products of anti-inflammatory drugs*

Summary

A tentative identification of the degradation products of DCF, IBP and NPX was performed in this chapter. Based on the chemical structure of the selected compounds as well as the available information from literature, three main degradation products were considered: 4-hydroxy-diclofenac (4-OH-DCF), 1-hydroxy-ibuprofen (1-OH-IBP) and 6-O-desmethyl-naproxen (6-O-DM-NPX). The chemical structure of these compounds suffered a derivatization process using N-methyl-N-(tert.-butyldimehtylsilyl)trifluoroacetamide (MTBSTFA) before the analysis by gas chromatography – mass spectrometry (GC-MS). Then, using the derivatized structure of each of the degradation products, a tentative fragmentation pattern related with the major quantification ions detected in the GC-MS chromatograms was suggested. In all the analyzed samples, the presence of 4-OH-DCF and 1-OH-IBP was detected; however, the peaks corresponding to the former showed a higher intensity than those corresponding to the latter; this means that IBP and its degradation product were more easily removed than DCF or 4-OH-DCF. Concerning 6-O-DM-NPX derived from the parent compound NPX, the degradation product was also detected in samples from the beginning of the experiment, indicating that this compound might be present in the stock solutions from a possible hydrolysis of NPX. A secondary peak with a quantification ion of m/z:285 was determined in analyzed samples, which could be related to the loss of the carboxylic group from the fragmented molecule of the derivatized 6-O-DM-NPX. It seems that all the chemical structures might suffer the loss of the tert-butyl-sylil derivatives, oxygen and hydrogen atoms; also, the loss of the carboxylic group may occur. To ensure the accurate identification of the degradation products of the three anti-

inflammatory drugs with the equipment used, standards are required in order to compare their mass spectrum (MS) and their retention time with those of samples.

*Part of this chapter has been published as:

Rodarte-Morales AI, Feijoo G, Moreira MT, Lema JM. (2011). **Operation of stirred tank reactors (STRs) and fixed-bed reactors (FBRs) with free and immobilized *Phanerochaete chrysosporium* for the continuous removal of pharmaceutical compounds.** Biochemical Engineering Journal (Submitted).

Outline

6.1 Introduction

In the year 1829, German scientists were able to isolate the first non-steroidal anti-inflammatory drug (NSAID): salicylate, which was recovered from willow bark. For 130 years, this anti-inflammatory was the only one in the market until the development of indomethacin in the 1960's decade. Over the last 50 years, several compounds with anti-inflammatory potential have been developed worldwide (Green, 2001). This type of drugs is a group of pharmaceuticals used for the treatment of inflammation and pain caused for several illnesses such as arthritis, bursitis, gout or swelling (Adler et al., 2006; Esplugas et al., 2007). This group of compounds belong to several chemical classes such as salicylates, propionic acid derivatives, acetic acid derivatives, fenamates, naphthylalkanone, indoles, oxicams, among others (Green, 2001; Ziylan and Ince, 2011). Some examples of these drugs are diclofenac (DCF), ibuprofen (IBP), indomethacin (INM), naproxen (NPX), mefenamic acid (MFA), piroxicam (PRX), ketoprofen (KPF), acetylsalicylic acid (ASA) and salicylic acid (SAL) (Ikehata et al., 2006; Ziylan and Ince, 2011).

Anti-inflammatory drugs are extensively consumed worldwide without prescription, achieving a global consumption of several hundreds of tons (Clara et al., 2005; Lindqvist et al., 2005; Fent et al., 2006; Zhang and Geiβen, 2010; Ziylan and Ince, 2011). Recent investigations have reported their presence in concentrations from ng/L up to µg/L in the aquatic environment, including sewage treatment plants (STPs) influents and effluents, as well as surface waters and groundwaters (Daughton and Ternes, 1999; Rodríguez et al., 2003; Vogna et al., 2004; Fent et al., 2006; Zuccato et al., 2006; Esplugas et al., 2007; Larsson et al., 2007; Zhang et al., 2007; Ziylan and Ince, 2011). The release of anti-inflammatory drugs requires special attention since it may be an environmental problem due to their toxicity effects and possible bioaccumulation in organisms in the aquatic environment (Cleuvers, 2004; Oaks et al., 2004; Brown et al., 2007).

After their administration, drugs can be metabolized in one or more metabolites through several chemical reactions (hydrolysis, oxidation, reduction, alkylation and dealkylation). Additionally, drugs are biotransformed into more water-soluble compounds by glucuronidation in the liver; this process is catalyzed by the enzymes UDP-glucuronosyltransferanses (UGTs), which covalently bind glucuronic acid from UDP-glucuronic acid (UDPGA) to molecules with hydroxyl, carboxyl, amine or thiol groups. Finally, these compounds are excreted in a mixture of the parent compound and/or their metabolites into the environment (via urine or faeces). It is noteworthy to mention that the original compound often is the minor component of the mixture (Weigel et al., 2004; Adler et al., 2006; Aresta et al., 2006; Mückter, 2006; Pérez and Barceló, 2007). This chapter will focus on the assessment of the degradation products of three anti-

inflammatory drugs: diclofenac (DCF), ibuprofen (IBP) and naproxen (NPX), after fungal transformation.

6.1.1. Degradation route of diclofenac and main degradation products

Diclofenac belongs to the anti-inflammatory class of the acetic acid derivatives and it has been reported to be metabolized in the human body to its hydroxylated (4'-hydroxydiclofenac (4'-OH-DCF), 5-hydroxydiclofenac (5-OH-DCF), 3'-hydroxydiclofenac (3'-OH-DCF) and 4',5-dihydroxydiclofenac (4',5-$(OH)_2$-DCF) or methoxylated forms (3'-hydroxy-4'-methoxydiclofenac (3'-OH-4-OMeDCF) and further conjugated to glucuronides. These metabolites have been found in urine and plasma samples in their free and glucuronide-conjugated forms (Scheurell et al., 2009). These compounds have been detected in STPs influents and effluents; being the concentration of the parent compound much higher than that of the metabolites (Stülten et al., 2008a and 2008b). The chemical structure of DCF and its metabolites are shown in Figure 6-1.

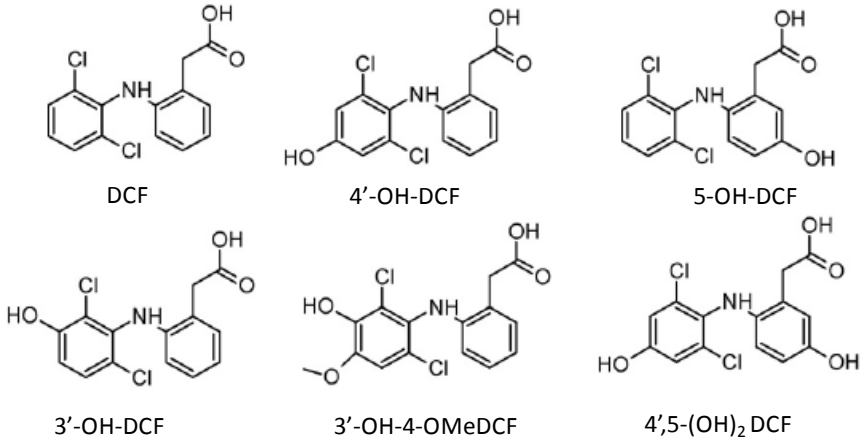


Figure 6-1. Chemical structure of DCF and its metabolites (Stülten et al., 2008a)

The natural degradation pathways of DCF and its metabolites may occur by a partial biodegradation and photodegradation in surface waters in extended periods of time in the environment (Ziylan and Ince, 2011). DCF and its degradation products are metabolized depending on the type of application: oral administration, intramuscular via or biliary excretion; also, during ointment application only 6% of DCF is resorbed through the skin, while the rest of the compound is washed away (Stülten et al., 2008a and 2008b). In Table 6-1 the excreted percentages of each compound for different types of administration are shown.

Table 6-1. Percentages of excretion of DCF and its main metabolites

Compound	Oral administration	Via intramuscular	Biliary excretion
DCF	-	6.5%	< 5%
4'-OH-DCF	16%	18.1%	10% – 20%
3'-OH-DCF	2%	1.4%	5% - 10%
5-OH-DCF	6.1%	8.2%	-
3'-OH-4-OMeDCF	<0.01%	-	-
4',5-$(OH)_2$-DCF	-	15.4%	-

(Stülten et al., 2008a and 2008b)

6.1.2. Degradation route of ibuprofen and main degradation products

Ibuprofen belongs to the class of the propionic acid derivatives (Green, 2001; Ziylan and Ince, 2011). These compounds have been detected in STPs effluents and the aquatic environment in concentrations between 0.7 μg/L and 3.5 μg/L (Weigel et al., 2004). After ingestion of IBP, only 15% of the parent compound is excreted as IBP while 26% and 43% are excreted as hydroxyl-ibuprofen (OH-IBP) and carboxy-ibuprofen (CX-IBP), the main degradation products of IBP. Concerning the formation of IBP degradation products in STPs, the formation of one or other of the OH-IBP and CX-IBP during the conventional activated sludge process (CAS) will depend on the aeration conditions: CX-IBP is mainly formed under anaerobic conditions, while the formation of OH-IBP is enhanced under high oxygen levels. Despite this, the total amount of both metabolites represented less than 10% of the parent compound, suggesting that the major source of these compounds is human excretion (Zwiener et al., 2002). After fungal treatment by *Trametes versicolor*, Marco-Urrea et al. (2009) identified three metabolites: 1- hydroxy-ibuprofen (1-OH-IBP), 2-hydroxy-ibuprofen (2-OH-IBP) and 1,2-dihydroxy-ibuprofen (1,2-$(OH)_2$-IBP). The structure of these compounds and the suggested pathway is shown in Figure 6-2.

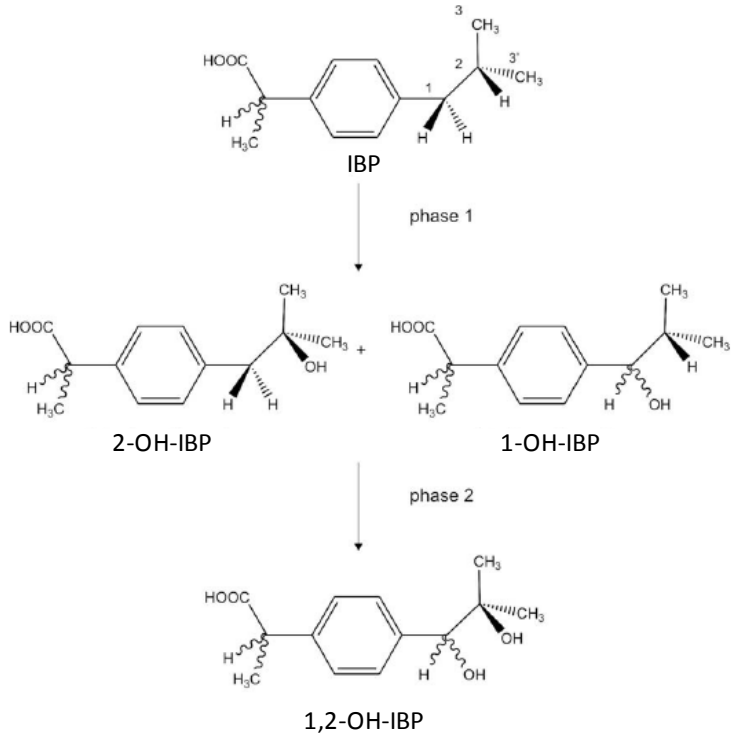


Figure 6-2. Suggested pathway of the degradation of IBP by the fungus *T. versicolor* (Marco-Urrea et al., 2009).

6.1.3. Degradation route of naproxen and main degradation products

The drug NPX belongs to the class of the propionic acid derivatives (Green, 2001; Ziylan and Ince, 2011). After oral administration, this compound can be partially metabolized to its 6-*O*-desmethylated metabolite and then both compounds are excreted via urine in unchanged or conjugated forms. The compound 6-O-desmethyl-naproxen (6-*O*-DM-NPX) is considered the major metabolite of the degradation of NPX (Aresta et al., 2006). The structure of NPX and its major metabolite is shown in Figure 6-3.

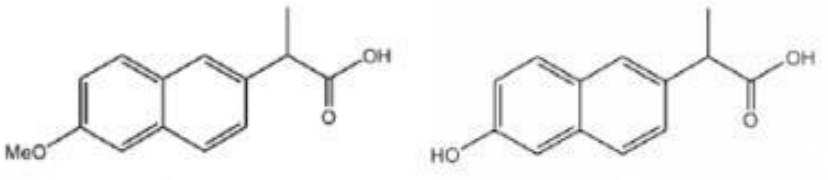


Figure 6-3. Chemical strucutre of NPX and its major metabolite (Adapted from Marco-Urrea et al., 2010b).

From 50% up to 80% of NPX is removed in CAS units; however, in some cases STPs effluents may contain higher concentrations of the parent compound than in the influent. This could be attributed to the fact that approximately 60% of NPX can be excreted as

hydrolysable conjugates which could be converted to the original compound increasing its concentration (Khan and Ongerth, 2004; Ziylan and Ince, 2011). Moreover, several photoproducts can be formed in the environment due to NPX and its metabolites are sensitive to sunlight. These photoproducts have been reported to present toxicity, genotoxicity and mutagenesis. In experiments with algae, rotifers and crustaceans, the parent compound was proved to be less harmful to the organisms than its metabolites (Isidori et al, 2005; Ziylan and Ince, 2011).

6.1.4. Derivatization of the anti-inflammatory drugs

DCF, IBP and NPX are nonvolatile and/or thermally unstable. For this reason, a derivatization process which converts the original compound into metabolites with much improved volatilies is necessary for GC-MS analysis. In this case, all the analyzed samples were derivatized with N-methyl-N-(tert.-butyldimehtylsilyl)trifluoroacetamide (MTBSTFA) which provides greater thermal and hydrolytic stability to the compounds by formation of methyl-terbutyl-silyl derivates (Rodríguez et al., 2003; Zhang et al., 2010). In Figure 6-4 the chemical structure of the derivatizant agent MTBSTFA is shown.

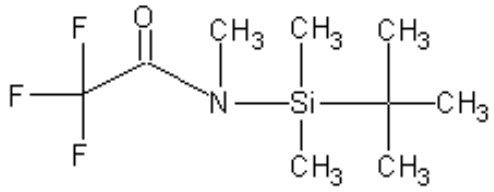


Figure 6-4. Chemical structure of MTBSTFA.

In this chapter, chromatograms obtained by gas chromatography–mass spectrometry (GC-MS) during the degradation experiments conducted in this doctoral thesis (chapters 3, 4 and 5) were analyzed in order to carry out a tentative identification of the main degradation metabolites of DCF, IBP and NPX: 4-OH-DCF, 1-OH-IBP and 6-O-DM-NPX. It is important to mention that a derivatization process was performed in all the samples. For this reason, the structure of the original compound and the derivatized compound were different. In section 6.3 a detailed description of this process is given.

6.2. Methodology

6.2.1. Samples analyzed

GC-MS chromatograms obtained in different degradation experiments were analyzed: fed-batch to continuous experiment with air flow and oxygen pulses in a STR (chapter 4, section 4.3.1), fed-batch to continuous experiment in a STR (chapter 4, section 4.3.2), continuous experiment in a STR (chapter 4, section 4.3.3) and continuous experiment with

air and oxygen in a fixed-bed reactor (chapter 5, sections 5.3.1 and 5.3.2). A detailed list of all the samples analyzed is included in Table 6-2. All the chromatograms were analyzed with the software Saturn GC-MS workstation version 5.52 (Varian, Inc., Walnut Creek, California, USA).

Table 6-2. GC-MS chromatograms analyzed

Experiment	Bioreactor	Type of culture	Samples (days)
Fed-batch (air and oxygen)	STR	Free pellets	Air (0, 10, 20 and 30) Oxygen (0, 10, 20 and 30; also three samples from the 24-h degradation time-course during the 3rd week of experiment)
Fed-batch to continuous (air)	STR	Free pellets	Fed-batch operation (0 and 20)[a] Continuous operation (29, 40, 52, 59 and 69)
Continuous experiment (air)	STR	Free pellets	0, 19, 33, 50 and the sample from the final extraction with ACN (day 50)
Continuous experiment (air and oxygen)	Fixed-bed reactor	Immobilized fungus	20, 40, 68, 100 and the sample from the final extraction with ACN (day 100)[b]

[a] Corresponding to 0, 8 and 24 hours of experiment
[b] Withdrawn samples from the top and the bottom of the column.

6.2.2. Procedure

To conduct the tentative identification of the major degradation products of the three anti-inflammatories considered, it was necessary to study the chemical structures of each compound. It is noteworthy that the mass spectrum (MS) of the original compound is different than the derivatized drug. The chemical structure will depend on the derivatizant agent used (MTBSTFA in this case). From the derivatized structures of each anti-inflammatory drug as well as their metabolites, it was assumed the way how these molecules might suffer a fragmentation process**.** Then, the GC-MS chromatograms were analyzed in order to find those peaks with similar MS to the expected after fragmentation of the parent compound to the metabolite.

6.2.3. Quantification of the metabolites

Since the standards of the identified metabolites were not commercially available, the formation of metabolites was estimated from the relative area (T_f/T_0) measured by GC-MS peaks of the corresponding metabolite at the analyzed day of experiment (T_f) and the relative area of the parent compound at the beginning of the experiment (T_0).

6.3. Results

Removal of three anti-inflammatory drugs by the fungus *P. chrysosporium* took place in all cases: static, free pellets and immobilized fungus, as well as two bioreactors configurations: stirred tank reactor (STRs) and fixed-bed reactors (FBRs) (chapters 3, 4 and 5). However, the identification of their main degradation products is not straightforward since in many cases the MS of the metabolites is very similar to its parent compound. Additionally, the standards of the suggested metabolites are not commercially available; therefore the detected peaks in the samples are supposed to correspond to those of the anti-inflammatory major metabolites reported in literature. Despite this, a tentative identification of the metabolites obtained was carried out by the analysis of GC-MS chromatograms. A better understanding about the way how the parent compounds could fragment to form its major metabolites may be achieved by using the data published about these compounds.

6.3.1. Derivatization of the samples

When anti-inflammatory drugs are derivatizated, the hydroxyl group from each molecule reacts with the MTBSTFA resulting in the loss of a hydrogen atom and the formation of methyl-terbutyl-sylil derivatives (Rodríguez et al., 2003). In Figure 6-6 the chemical structures of DCF, IBP and NPX, as well as their respective major metabolites after the derivatization process are shown.

Table 6-3. Derivatized structures of DCF, IBP and NPX and their major metabolites

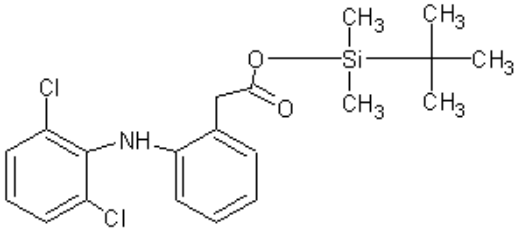
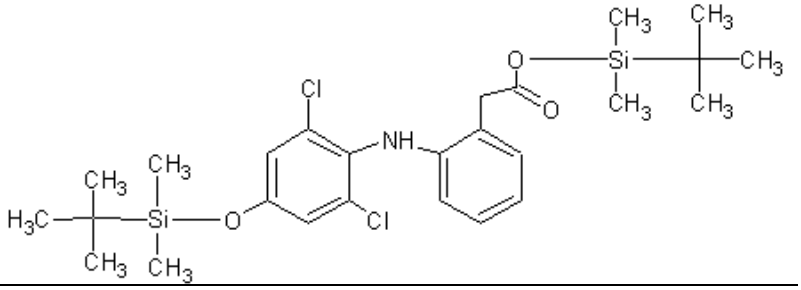
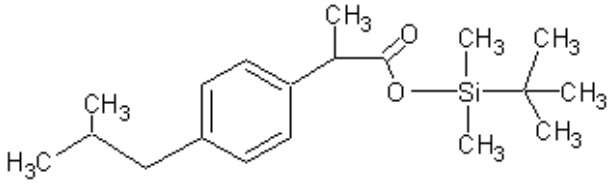
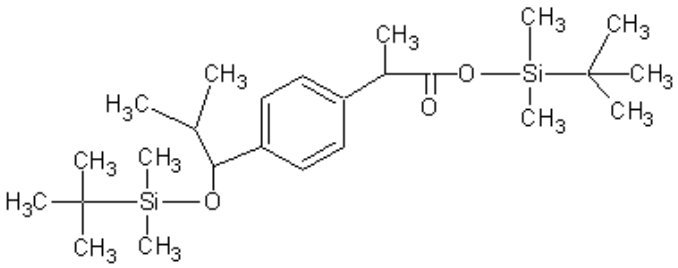
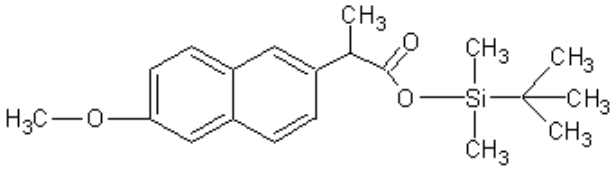
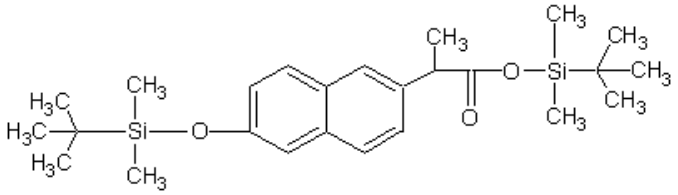
Compound (MS and quantification ion)	Chemical structure and their MS of fragmentation
DCF derivatized (m/z: 409) (m/z: 352+354+356)	
4-OH-DCF derivatized (m/z: 539) (m/z: 337+379)	

Table 6-5 (continued)

Compound (MS and quantification ion)	Chemical structure and their MS of fragmentation
IBP derivatized (m/z: 320) (m/z: 263)	
1-OH-IBP derivatized (m/z: 450) (m/z: 263+305+320)	
NPX derivatized (m/z: 344) (m/z: 287)	
6-O-DM-NPX derivatized (m/z: 444) (m/z: 313)	

6.3.2. Determination of the major degradation product of diclofenac: 4-hydroxy-diclofenac

The main degradation product of DCF is the hydroxylated metabolite (4-OH-DCF). This compound has a molecular ion of m/z: 539. After the loss of the tert-butyl-sylil derivative, as well as the carboxylated fragment, the main quantification ion is m/z: 380±1 (depending on the loss of a hydrogen atom). Moreover, a possible breakdown of the aromatic ring and the loss of a hydrogen atom is described in Figure 6-5, corresponding to a compound with a MS of m/z: 339±1. Please note that the loss of 1 or 2 additional hydrogen atoms gives a MS of m/z: 338±1. In Figure 6-6 the fragment pattern of 4-OH-DCF is shown. For this reason, the GC-MS chromatograms were studied in order to find those peaks with these quantification ions (m/z: 380+338).

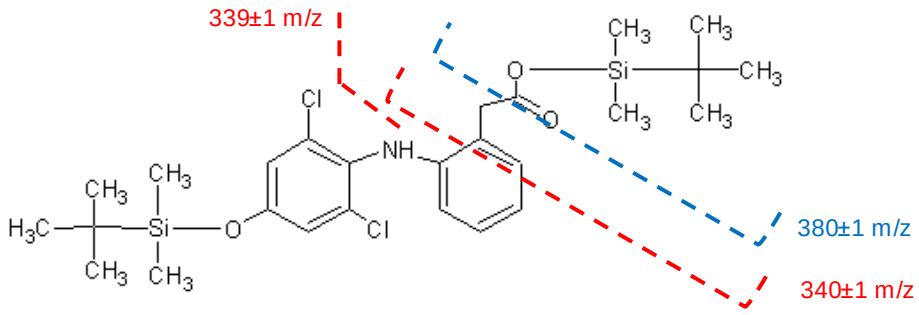


Figure 6-5. Metabolite 4-OH-DCF after the possible fragmentation.

Regarding the degradation of DCF, the selected ion monitoring (SIM) chromatogram for all the analyzed samples was of m/z: 352+254+256, which corresponds to the parent compound. Additionally, the ions m/z: 337+379 were selected since a peak with these major quantification ions was found in all the analyzed samples (Figures 6-6 to 6-10). It seems that degradation of DCF and the consequent formation of 4-OH-DCF by fungal transformation during a fed-batch experiment in a STR depends on the gas supply (chapter 4, section 4.3.1). When a continuous air flow was introduced into the reactor, the tentative metabolite was the only compound detected (Figure 6-6). On the other hand, under high levels of oxygen, both compounds were detected (Figure 6-7). Furthermore, during the third week of the experiment in the STR with oxygen, a degradation time-course of 24 hours was performed daily after the addition of a pulse of the pharmaceutical compounds. Figure 6-8 shows the GC-MS chromatogram corresponding to the 13th day of experiment. It shows an evident disappearance of the peak corresponding to DCF (m/z: 352+354+356), while the degradation product remains after 18 hours. Besides, the GC-MS chromatograms corresponding at day 0 and 20 of the fed-batch experiment in a STR (chapter 4, section 4.3.2), clearly show that the culture age have a great influence on the degradation of anti-inflammatory. At the beginning of the experiment, the fungus *P. chrysosporium* was not able to remove DCF but after 20 days this compound was completely degraded within 8 hours. Moreover, the appearance of 4-OH-DCF is remarkable on both days (data not shown). Furthermore, at day 26 this bioreactor changed its operation from fed-batch to continuous operation. The presence of 4-OH-DCF is noticeable from the beginning of this stage until day 52. However, a greater proportion of this compound was observed during the last days of operation as well as the presence of the parent compound (data not shown). Different results with respect to the presence of the DCF degradation product were observed when the STR started in continuous operation (chapter 4, section 4.3.3). The hydroxylated metabolite of DCF was detected in all the analyzed samples except at day 0; while DCF was only detected at the beginning of the experiment (Figure 6-9). Finally, the peak corresponding to the suspected metabolite

was not detected during the experiments in the fixed-bed reactor (chapter 5, section 5.3.1). However, at the end of the experiment an extraction with acetonitrile was carried out to the support and the results of the GC-MS showed a peak with a high signal with the same MS than those detected in the other experiments (m/z: 337) (Figure 6-10).

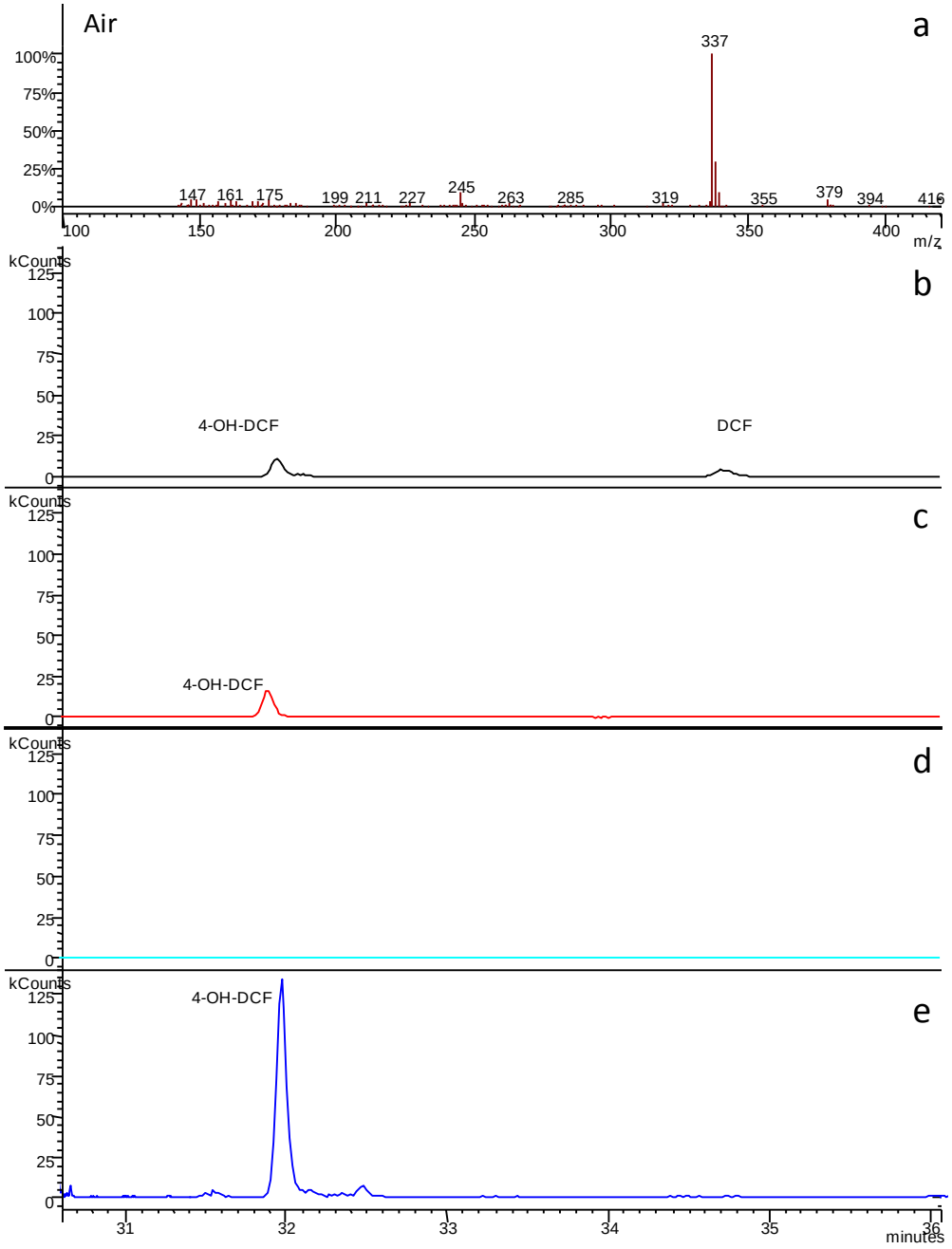


Figure 6-6. Degradation products of DCF during a fed-batch experiment in a STR with continuous air flow. Symbols: (a) tentative mass spectrum of 4-OH-DCF; SIM chromatogram m/z: 352+354+356+337+379 corresponding to: (b) day 0; (c) day 10; (d) day 20; (e) day 30 after an extraction with acetonitrile.

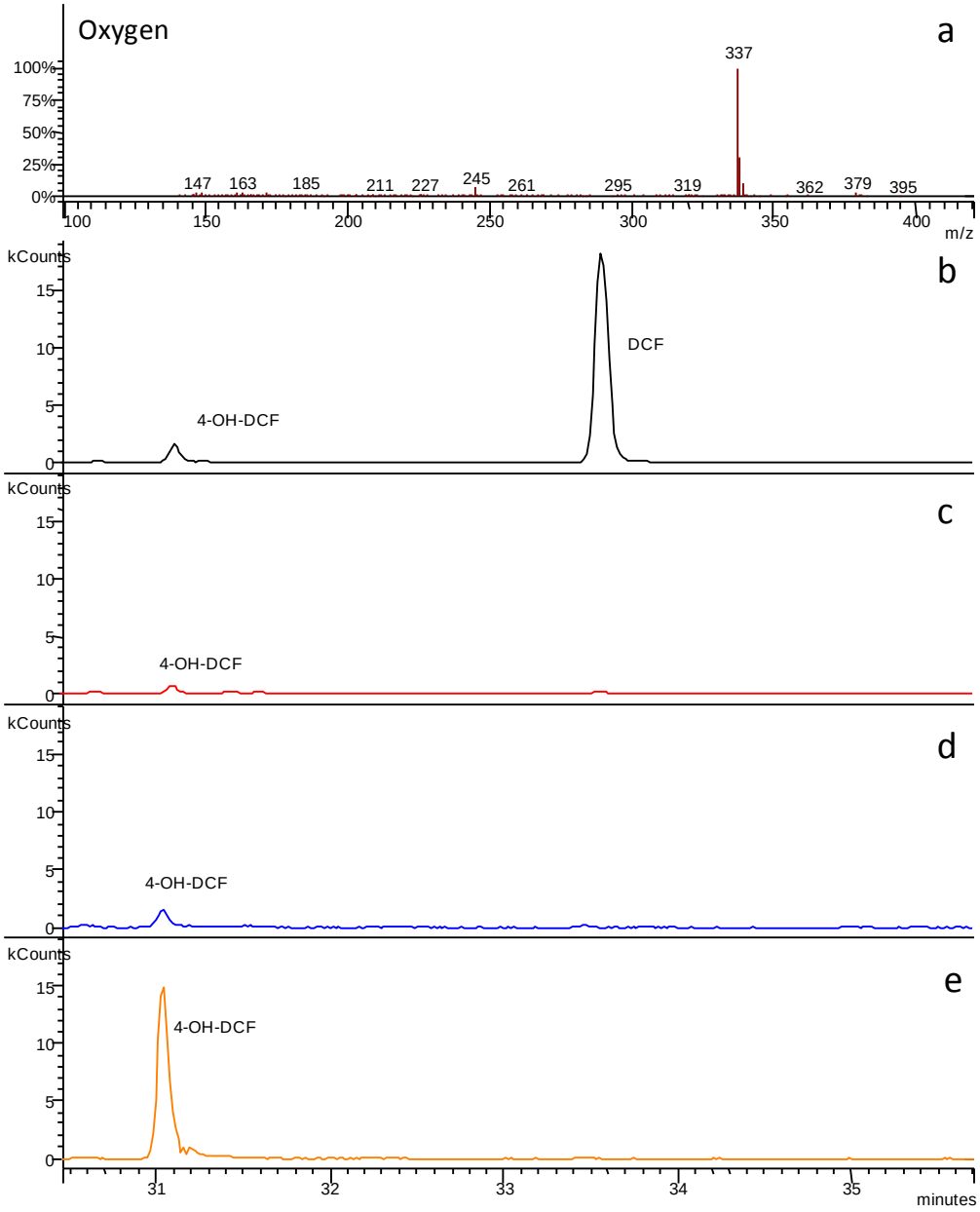


Figure 6-7. Degradation products of DCF during a fed-batch experiment in a STR with oxygen pulses. Symbols: (a) tentative mass spectrum of 4-OH-DCF; SIM chromatogram m/z: 352+354+356+337+379 corresponding to: (b) day 0; (c) day 10; (d) day 30; (e) day 30 after an extraction with acetonitrile.

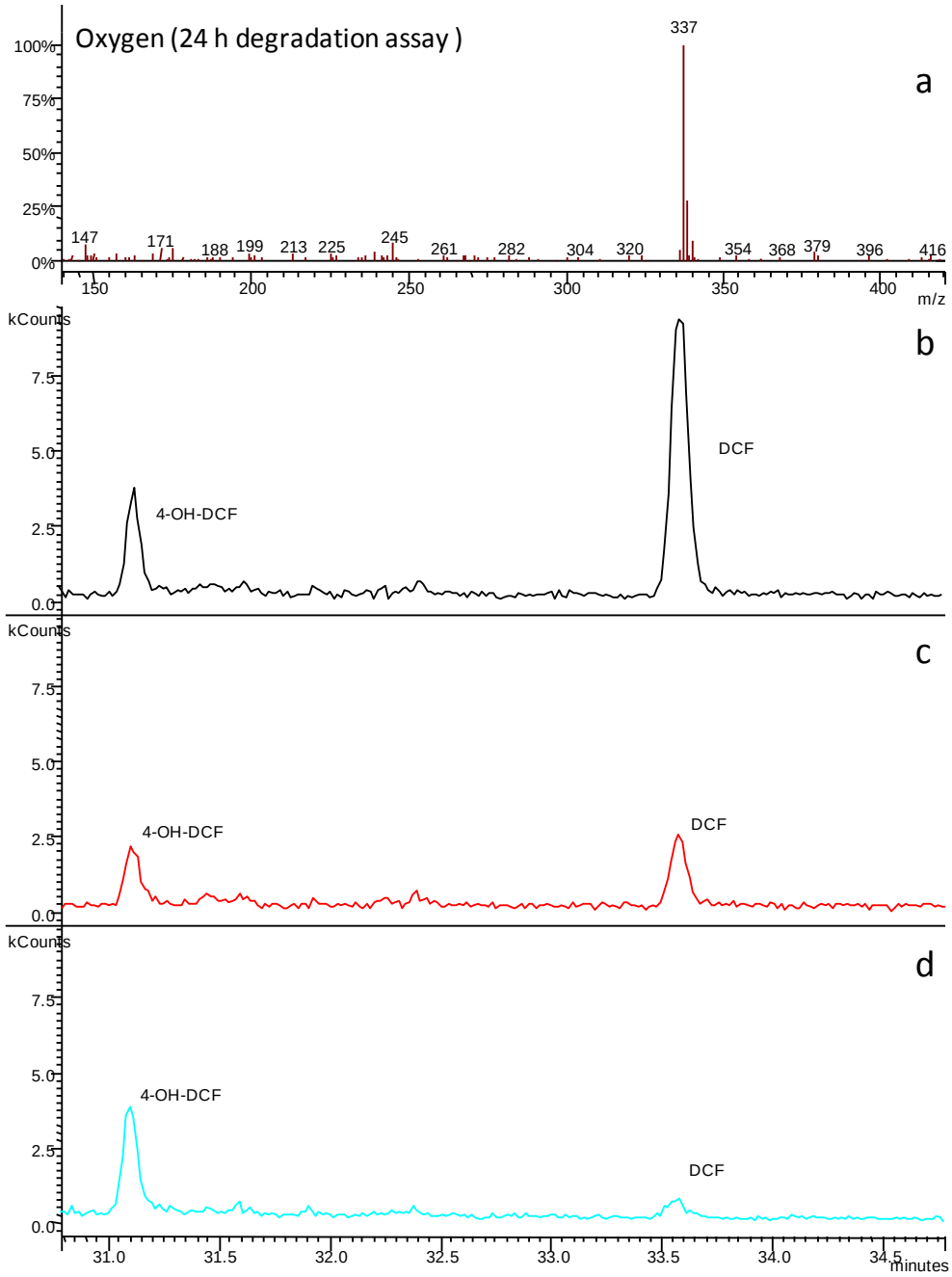


Figure 6-8. Degradation products of DCF during a fed-batch experiment in a STR with oxygen pulses (day 13). Symbols: (a) tentative mass spectrum of 4-OH-DCF; SIM chromatogram m/z: 352+354+356+337+379 corresponding to: (b) 0 h; (c) 4 h; (d) 18 h.

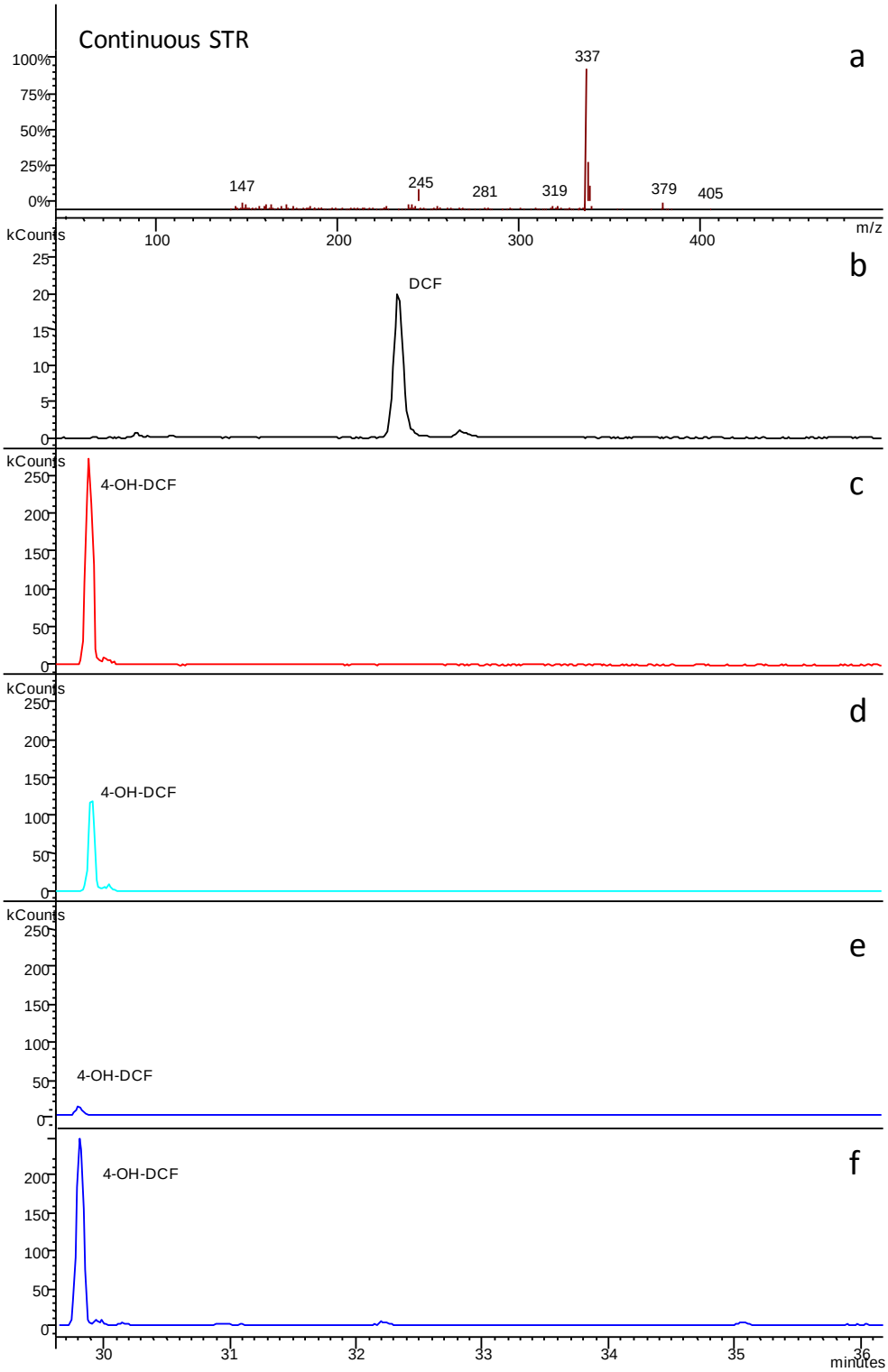


Figure 6-9. Degradation products of DCF during a continuous experiment in a STR. Symbols: (a) tentative mass spectrum of 4-OH-DCF; SIM chromatogram m/z: 352+354+356+337+379 corresponding to: (b) day 0; (c) day 19; (d) day 33; (e) day 50; (f) day 50 after a final extraction with acetonitrile.

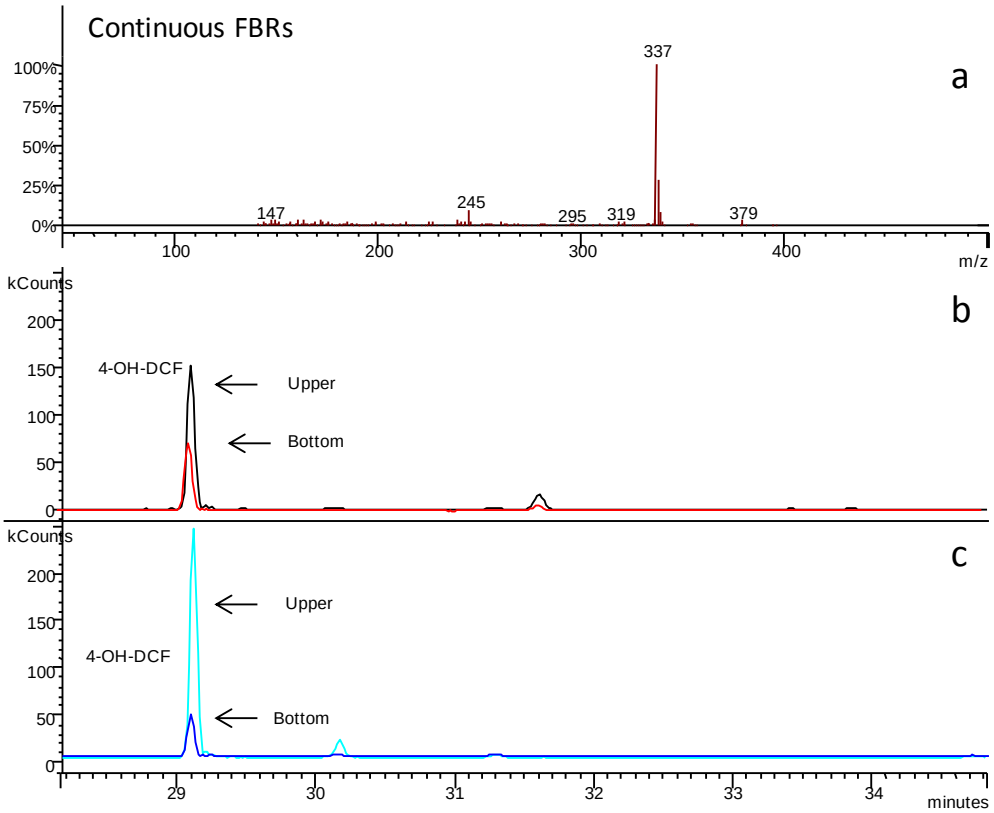


Figure 6-10. Degradation products of DCF during a continuous experiment in a fixed-bed reactor after a final extraction from the upper zone and bottom of the column (day 100). Symbols: (a) tentative mass spectrum of 4-OH-DCF; SIM chromatogram m/z: 352+354+356+337+379 corresponding to: (b) oxygen pulses; (c) continuous air flow.

6.3.3. Determination of the major degradation product of ibuprofen: 1-hydroxy-ibuprofen

According with several authors, the major IBP degradation product is 1-hydroxy-ibuprofen (1-OH-IBP) (Weigel et al., 2004; Ziylan and Ince, 2011). This compound is probably formed from the fragmentation of IBP as described in Figure 6-11. After derivatization of the parent compound, 1-OH-IBP has a molecular mass of m/z: 450 with a major quantification ion of m/z: 263 and also two identification ions such as m/z: 305 and m/z: 320. The loss of the tert-butyl-sylil groups and also both oxygen atoms might correspond to a quantification ion of m/z: 319±1. In addition, the breakdown of the carboxylated group provides a quantification ion of m/z: 305±1. Moreover, a compound with a major quantification ion of m/z: 263 implies the loss of the propane group (CH_3-CH_2-CH_3).

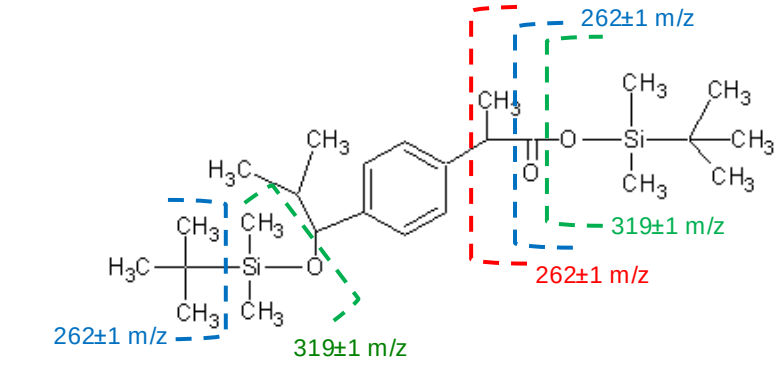


Figure 6-11. Degradation product 1-OH-IBP after the possible fragmentation.

The SIM chromatogram presented peaks at m/z: 263+305+320 (Figures 6-12 to 6-18). A peak with high intensity of ion of m/z: 263 was observed in all the analyzed cases; this peak could correspond with the IBP degradation product, 1-OH-IBP. As in the previous case, the influence of the gas supply on the formation of 1-OH-IBP was observed since peaks with higher intensity were found when the gas supply consisted of a continuous air flow (Figures 6-12 and 6-13). Additionally, during the monitorization of the operation of the bioreactor with oxygen pulsation, the degradation of IBP was remarkable. Despite this, a slight formation of 1-OH-IBP was observed (Figure 6-14).

This degradation product was also detected in a STR during the fed-batch operation at days 0 and 20. It is noteworthy to mention that the degradation of the parent compound is enhanced with the culture age and the peak corresponding to 1-OH-IBP has a greater signal at the beginning of the experiment than at day 20 (data not shown). During the continuous operation of this experiment, the presence of this degradation product was notable from day 20 until day 52. From that day until the end of the experiment, a decrease in the intensity of the peak was observed (data not shown). When the STR started the operation with a continuous feeding, the metabolite 1-OH-IBP was also detected (Figure 6-15). It is important to highlight the degradation of IBP throughout the experiment and the low signal of the peak detected after the extraction with acetonitrile.

In contrast with the degradation product of DCF, the presence of 1-OH-IBP was detected with similar signals in samples from the fixed-bed reactor under both aeration conditions (air and oxygen) (Figures 6-16 and 6-17). The peak corresponding to the parent compound was not detected during the aerated experiment and the degradation product peak was detected in higher intensity under air supply than oxygen pulses. This was also observed after the final extraction with acetonitrile where the peaks with higher areas were observed in the aerated fixed-bed reactor (Figure 6-18).

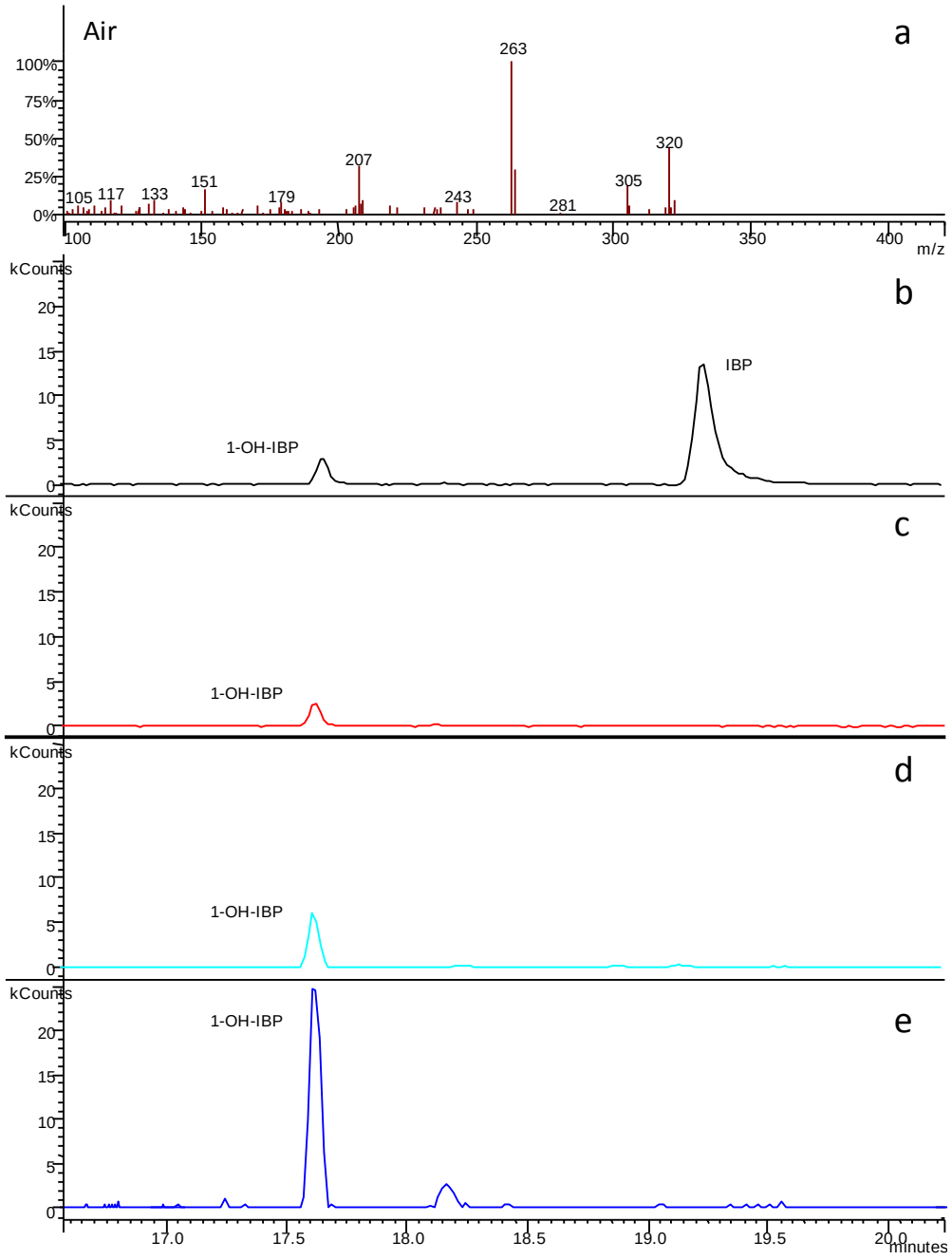


Figure 6-12. Degradation products of DCF during a fed-batch experiment in a STR with continuous air flow. Symbols: (a) tentative mass spectrum of 1-OH-IBP; SIM chromatogram m/z: 263+305+320 corresponding to: (b) day 0; (c) day 10; (d) day 20; (e) day 30 after an extraction with acetonitrile.

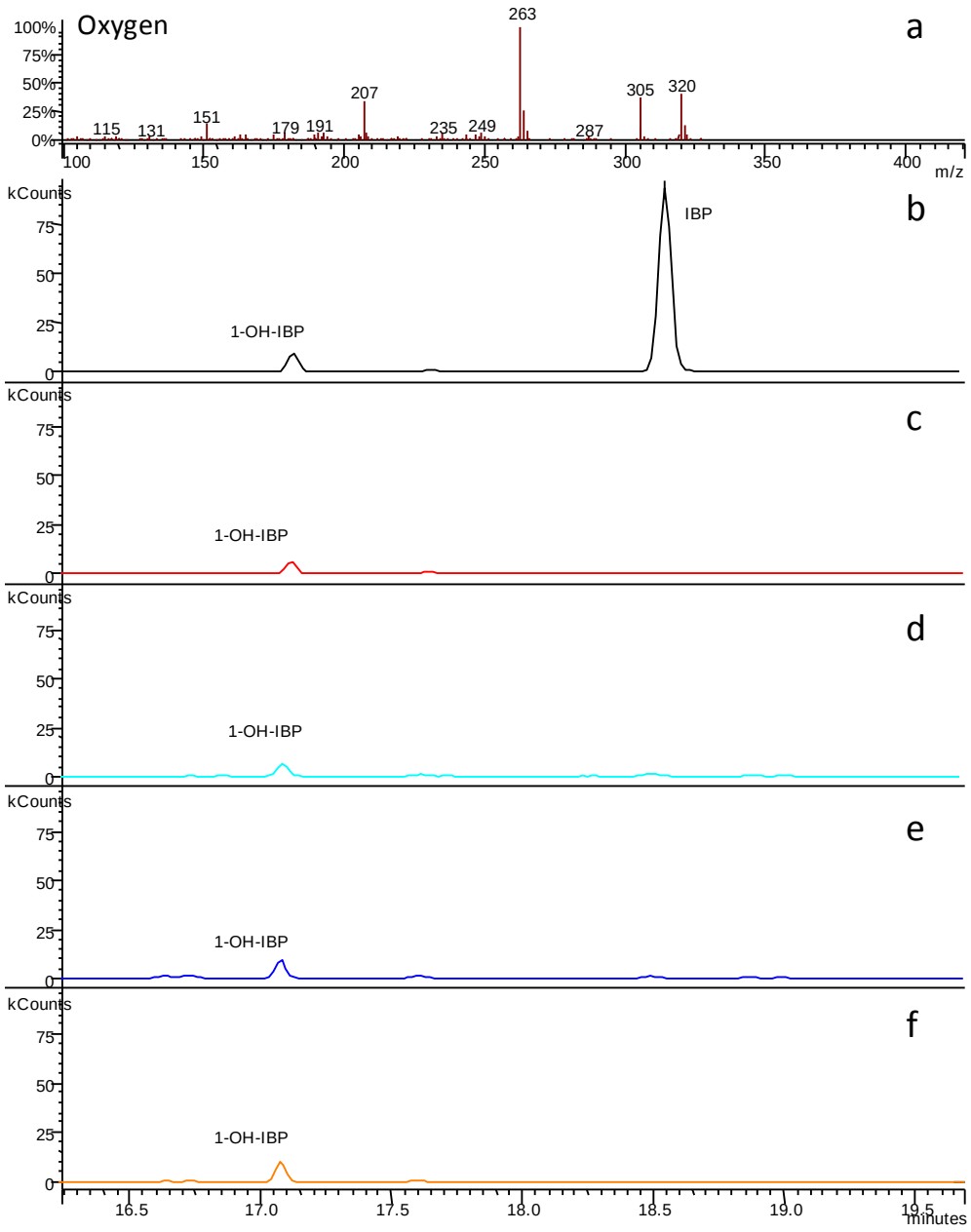


Figure 6-13. Degradation products of IBP during a fed-batch experiment in a STR with oxygen pulses. Symbols: (a) tentative mass spectrum of 1-OH-IBP; SIM chromatogram m/z: 263+305+320 corresponding to: (b) day 0; (c) day 10; (d) day 20; (e) day 30; (f) day 30 after an extraction with acetonitrile.

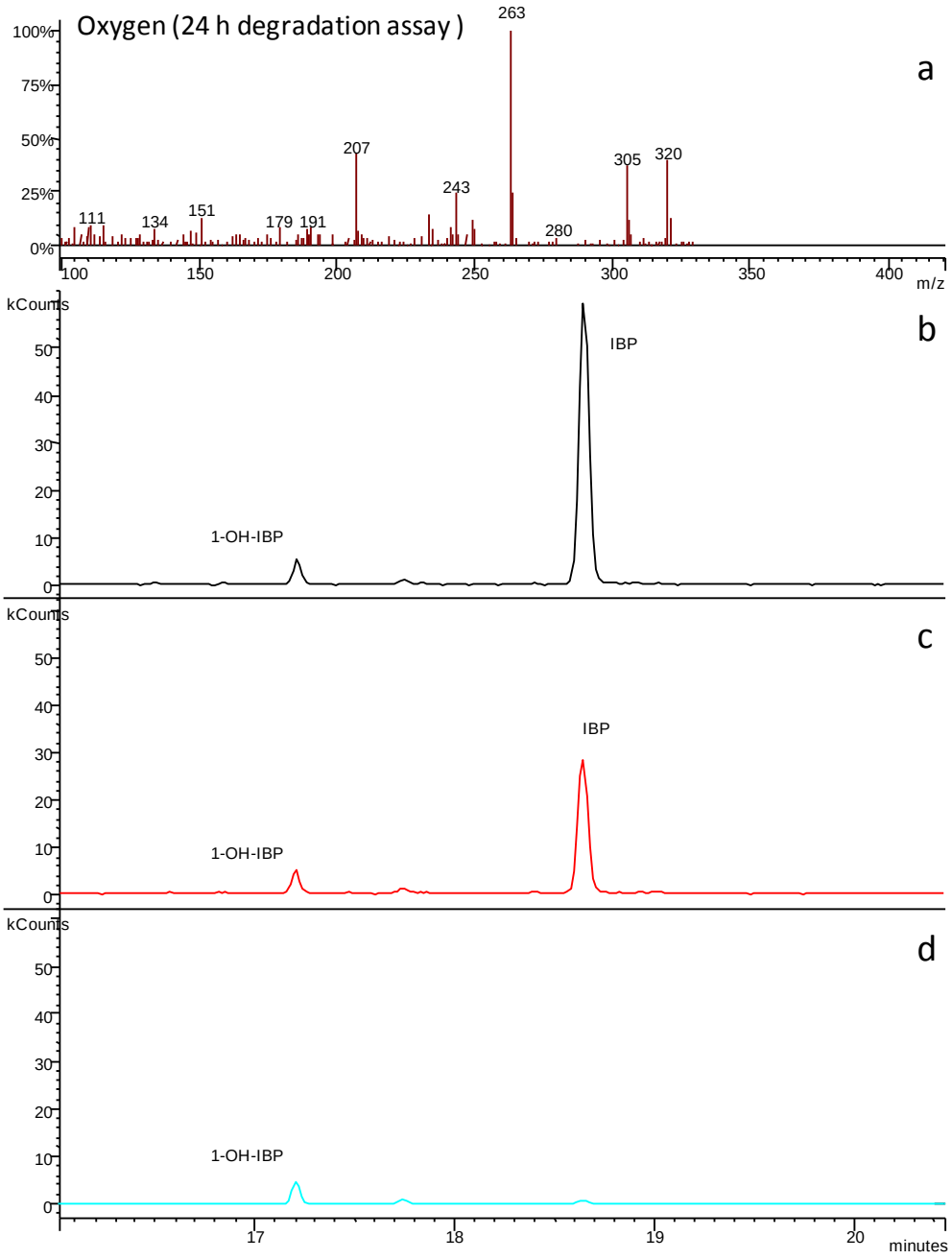


Figure 6-14. Degradation products of IBP during a fed-batch experiment in a STR with oxygen pulses (day 13). Symbols: (a) tentative mass spectrum of 1-OH-IBP; SIM chromatogram m/z: 263+305+320 corresponding to: (b) 0 hours; (c) 4 hours; (d) 18 hours.

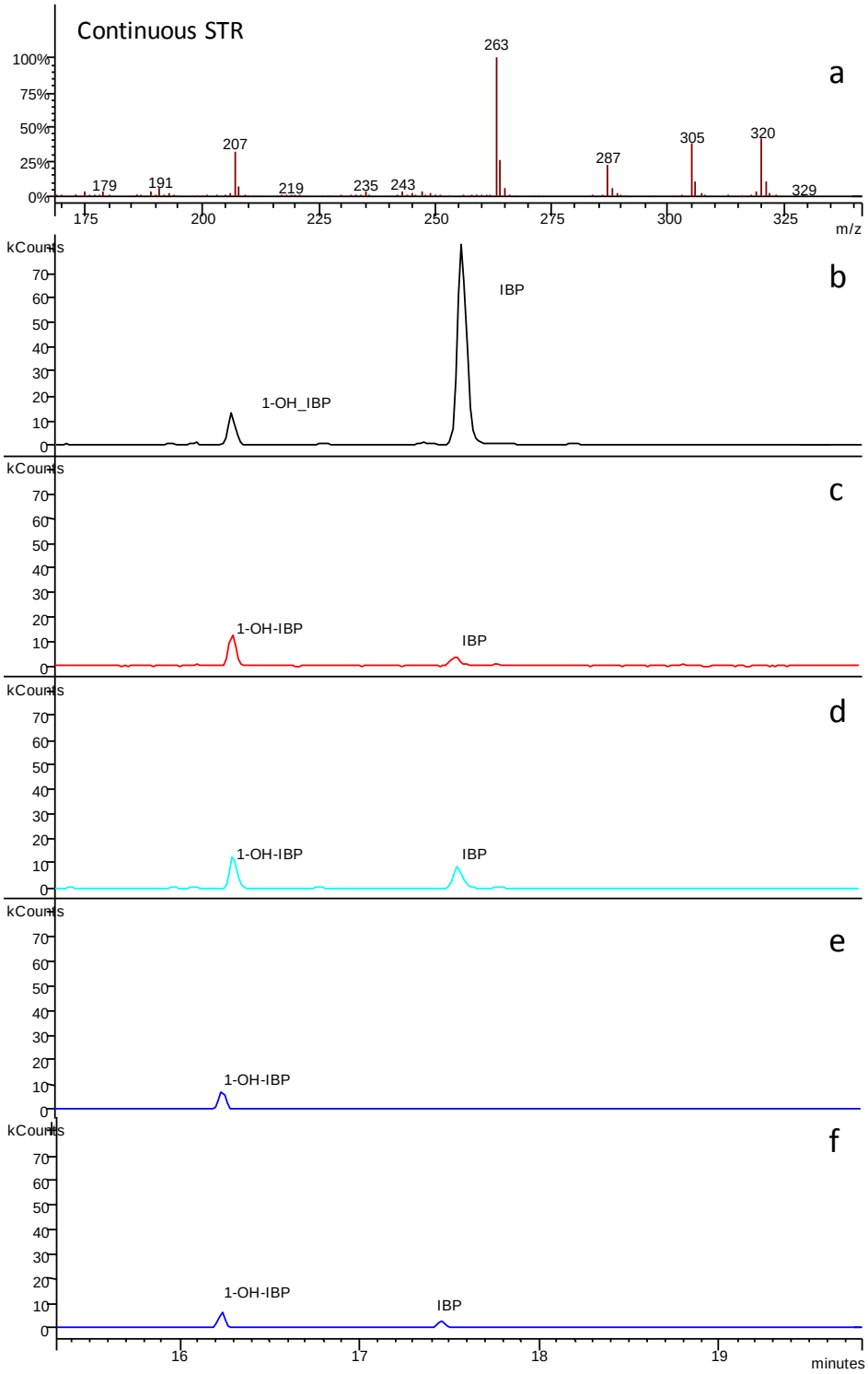


Figure 6-15. Degradation products of IBP during a continuous experiment in a STR. Symbols: (a) tentative mass spectrum of 1-OH-IBP; SIM chromatogram m/z: 263+305+320 corresponding to: (b) day 1; (c) day 19; (d) day 33; (e) day 50; (f) day 50 after a final extraction with acetonitrile.

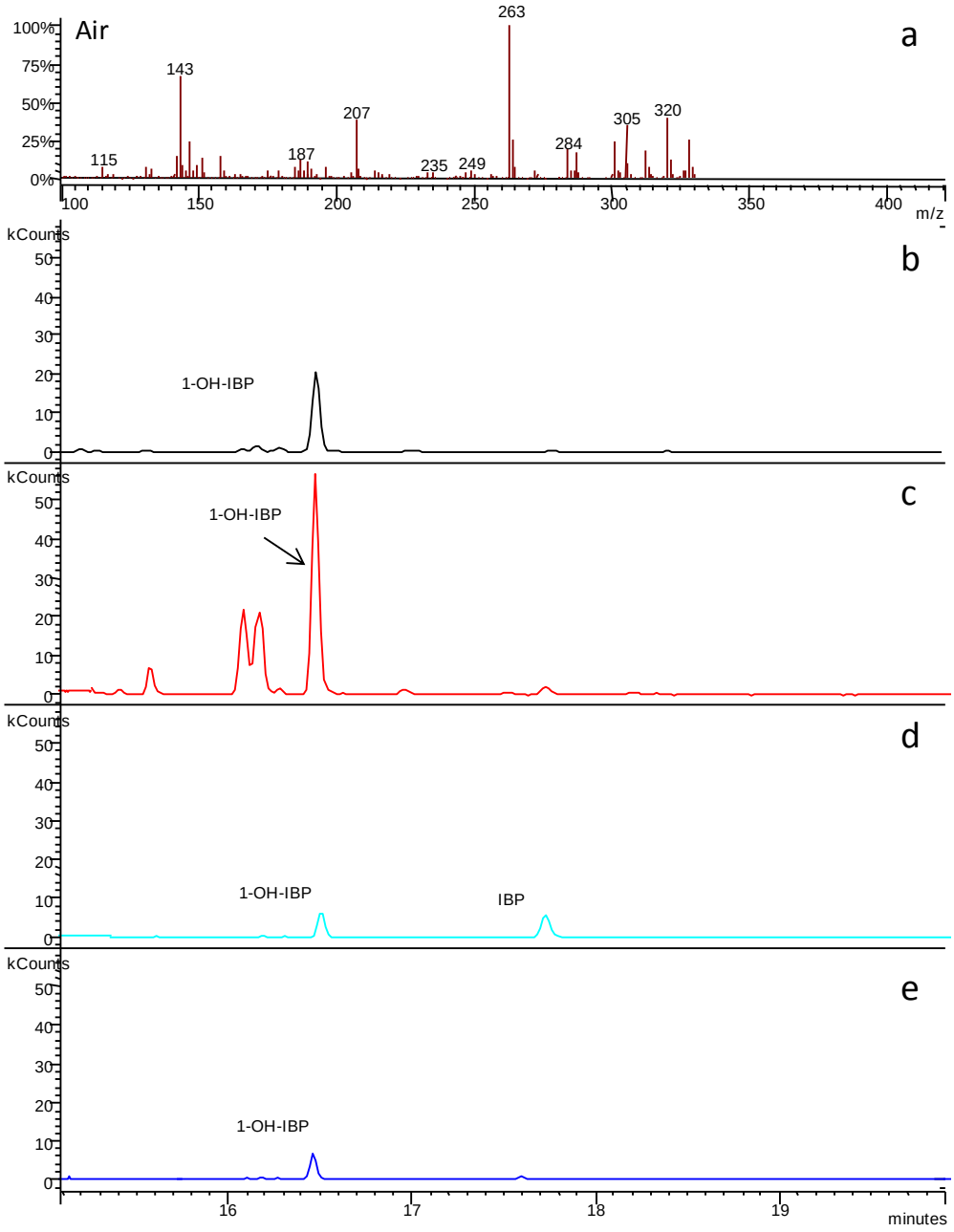


Figure 6-16. Degradation products of IBP during a continuous experiment in a fixed-bed reactor with air. Symbols: (a) tentative mass spectrum of 1-OH-IBP; SIM chromatogram m/z: 263+305+320 corresponding to: (b) day 20; (c) day 40; (d) day 68; (e) day 100.

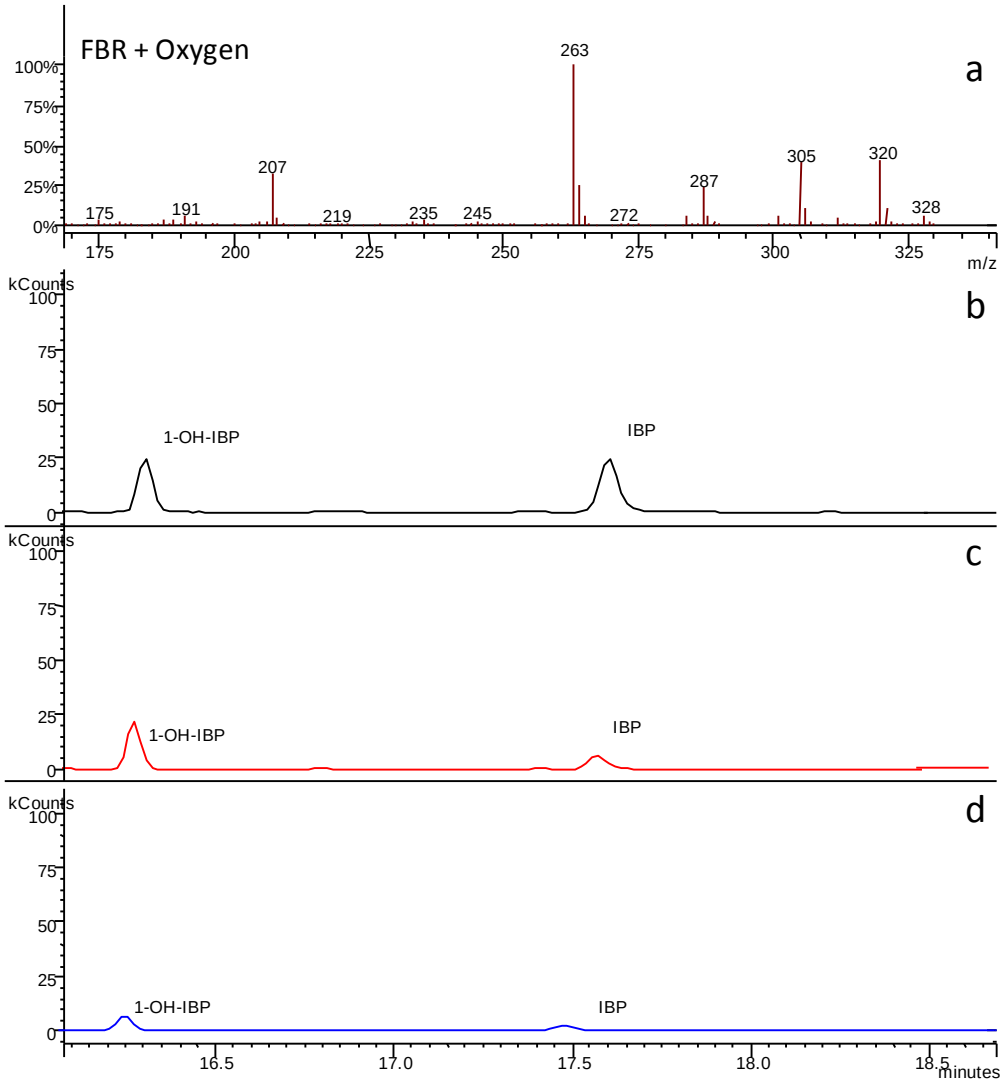


Figure 6-17. Degradation products of IBP during a continuous experiment in a fixed-bed reactor with oxygen pulses. Symbols: (a) tentative mass spectrum of 4-OH-IBP; SIM chromatogram m/z: 263+305+320 corresponding to: (b) day 20; (c) day 40; (d) day 100.

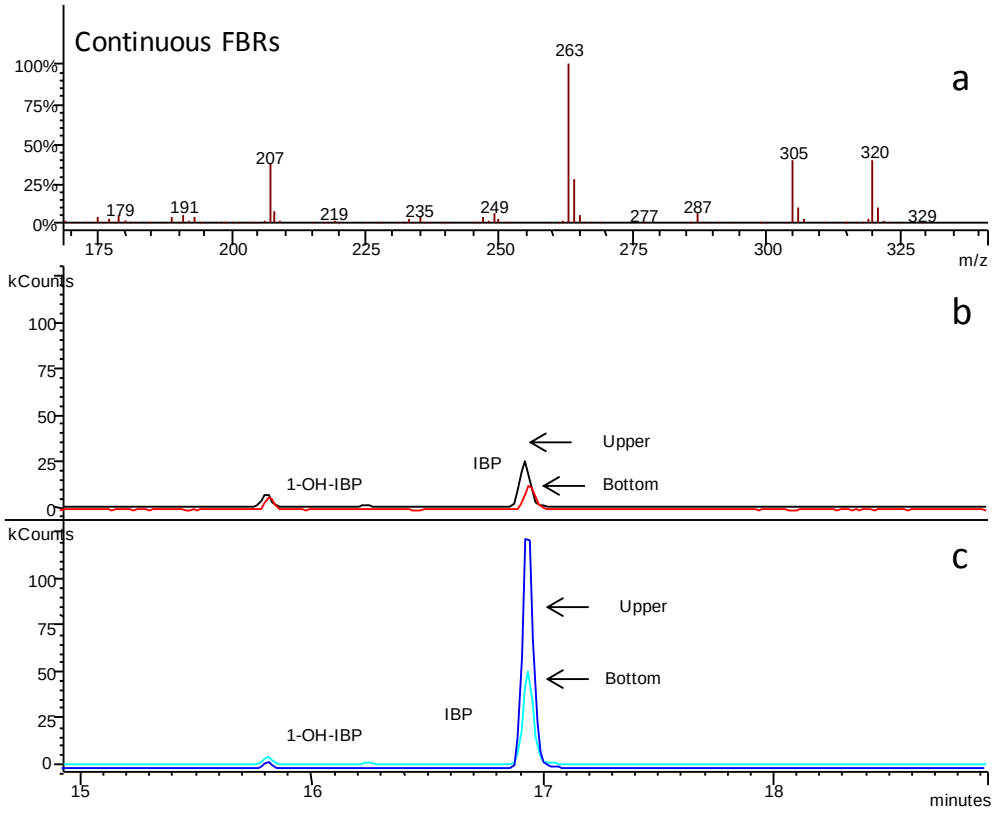


Figure 6-18. Degradation products of IBP during a continuous experiment in a fixed-bed reactor (day 100 after a final extraction with acetonitrile). Symbols: (a) tentative mass spectrum of 1-OH-IBP; SIM chromatogram m/z: 263+305+320 corresponding to: (b) oxygen pulses; (c) continuous air flow; (▬ , ▬) upper zone of the column; (▬ , ▬) bottom of the column.

6.3.4. Determination of the major degradation product of naproxen: 6-*O*-desmethyl-naproxen.

After the analysis of the considered GC-MS chromatograms, the presence of a peak which MS corresponds to a major quantification ion of m/z: 313, observed in all the analyzed samples (Figures 6-20 to 6-26). This peak might correspond to the major degradation product of NPX: 6-*O*-desmethyl-naproxen (6-*O*-DM-NPX) (Aresta et al., 2006). From the chemical structure of the derivatized compound (m/z: 444) a fragmentation pattern is described in Figure 6-19. The losses of the tert-butyl-sylil groups as well as both oxygen atoms are required to form a compound with the same major quantification ion as the one detected in the analyzed samples.

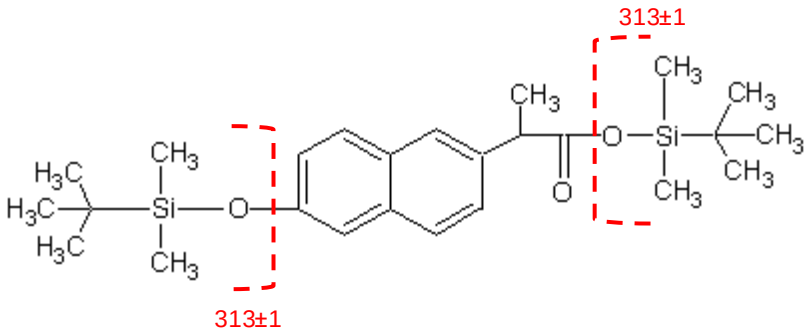


Figure 6-19. Degradation product 6-O-DM-NPX after the possible fragmentation.

In all the analyzed samples, a SIM chromatogram of m/z: 313 was observed. A peak with this major quantification ion was observed during the fed-batch experiment in the STR with air and oxygen pulses. It is important to note that peaks with high signal, similar to that corresponding to the parent compound, were detected since the beginning of the experiment (Figures 6-20 and 6-21). This means that the degradation product could be present in the stock solution; this will be discussed in section 6.4.

The initial presence of these peaks was also detected during the 24 hours time-course experiment in the oxygen pulsed reactor. In addition, the relative area of these peaks was maintained throughout the experiment. Besides, the degradation of NPX is remarkable in Figure 6-22. Similar results were observed in a STR operating in a continuous regime where the presence of 6-O-DM-NPX was detected. Additionally, the degradation of the parent compound was observed. After extraction with acetonitrile, a peak corresponding to the tentative metabolite showed a higher signal than the sample taken at day 50, before the extraction (Figure 6-23).

Both compounds were also detected in the fixed-bed reactor with air and oxygen addition (Figures 6-24 and 6-25). At the end of the experiments performed in the fixed-bed reactor, the extraction with acetonitrile of the upper and bottom zone of the column showed higher amounts of the NPX degradation product on the support of the bioreactor (Figure 6-26).

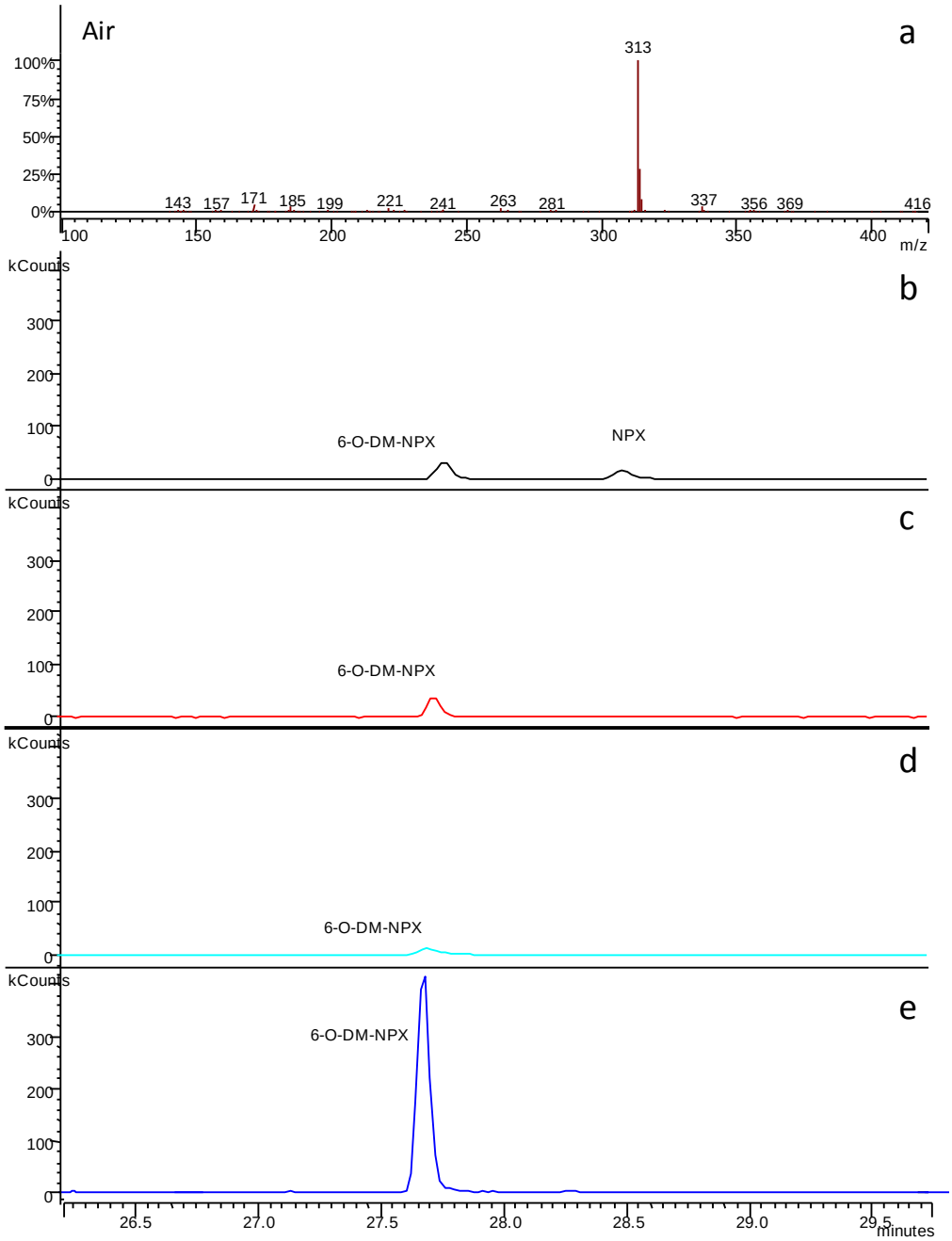


Figure 6-20. Degradation products of NPX during a fed-batch experiment in a STR with continuous air flow. Symbols: (a) tentative mass spectrum of 6-O-DM-NPX; SIM chromatogram m/z: 287+313 corresponding to: (b) day 0; (c) day 10; (d) day 20; (e) day 30.

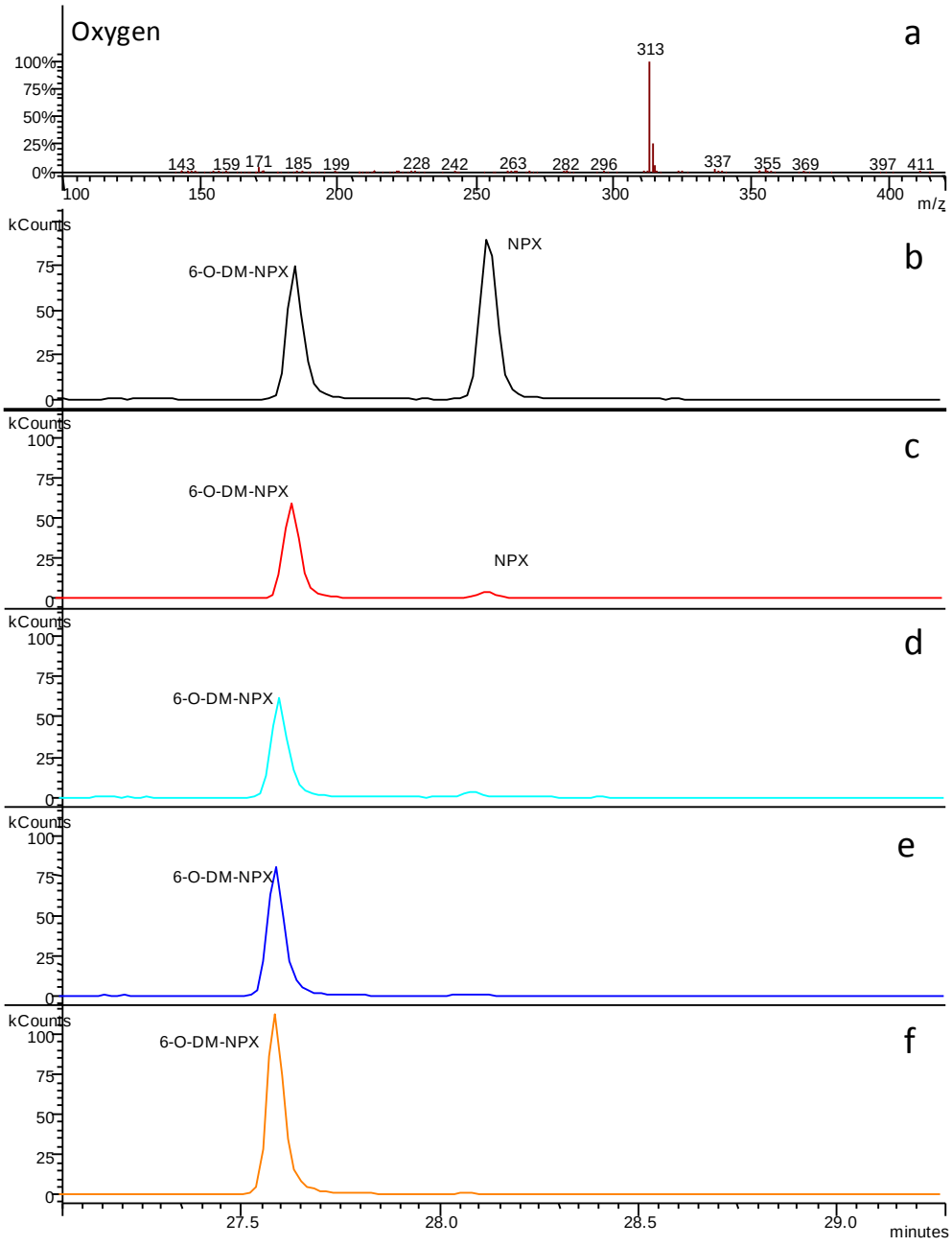


Figure 6-21. Degradation products of NPX during a fed-batch experiment in a STR with oxygen pulses. Symbols: (a) tentative mass spectrum of 6-O-DM-NPX; SIM chromatogram m/z: 287+313 corresponding to: (b) day 0; (c) day 10; (d) day 20; (e) day 30; (f) day 30 after an extraction with acetonitrile.

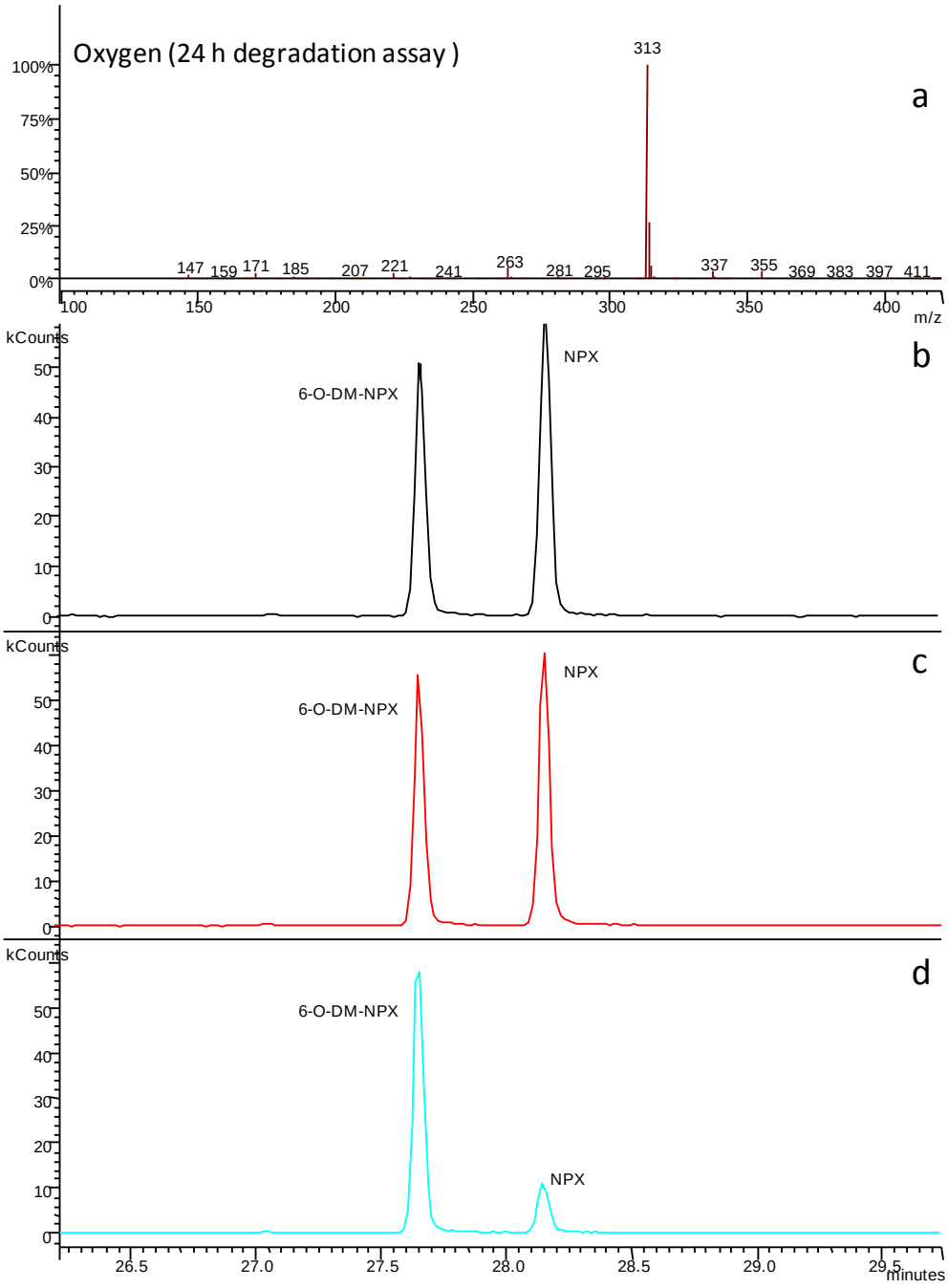


Figure 6-22. Degradation products of NPX during a fed-batch experiment in a STR with oxygen pulses (day 13). Symbols: (a) tentative mass spectrum of 6-O-DM-NPX; SIM chromatogram m/z: 287+313 corresponding to: (b) 0 hours; (c) 4 hours; (d) 18 hours.

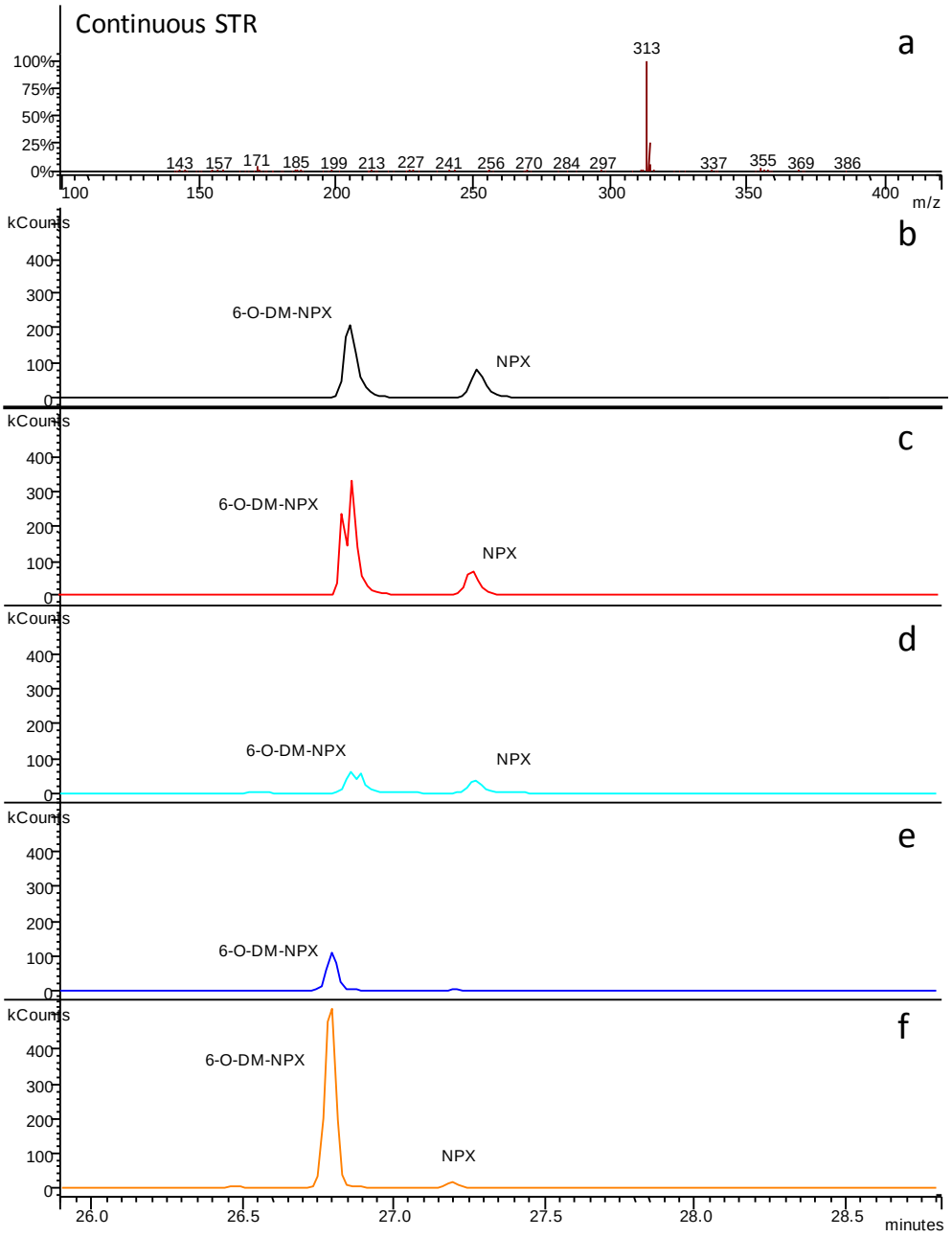


Figure 6-23. Degradation products of NPX during a continuous experiment in a STR. Symbols: (a) tentative mass spectrum of 6-O-DM-NPX; SIM chromatogram m/z: 287+313 corresponding to: (b) day 1; (c) day 19; (d) day 33; (e) day 50; (f) day 50 after a final extraction with acetonitrile.

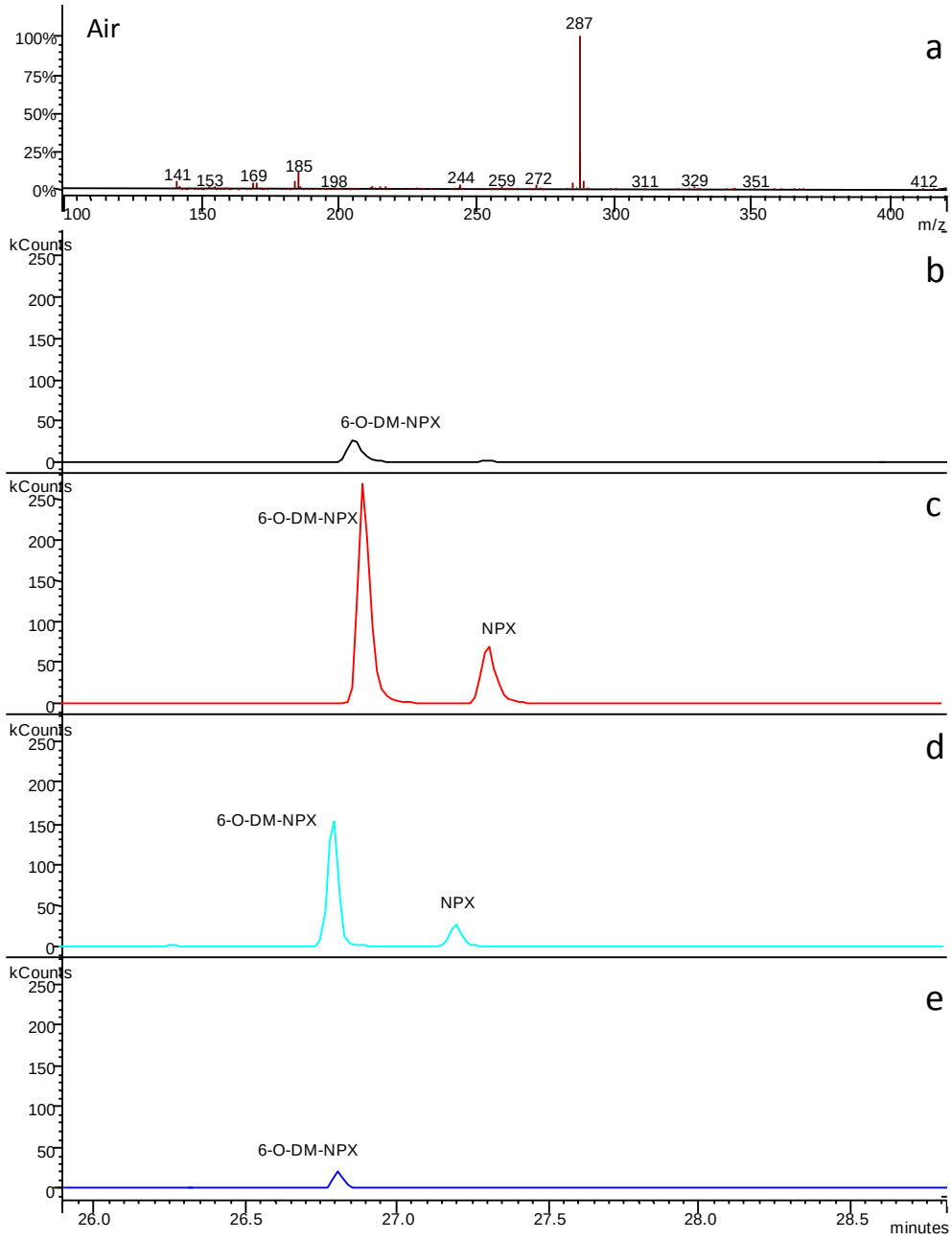


Figure 6-24. Degradation products of NPX during a continuous experiment in a fixed-bed reactor with continuous air flow. Symbols: (a) tentative mass spectrum of 6-O-DM-NPX; SIM chromatogram m/z: 287+313 corresponding to:(b) day 20; (c) day 40; (d) day 68; (e) day 100.

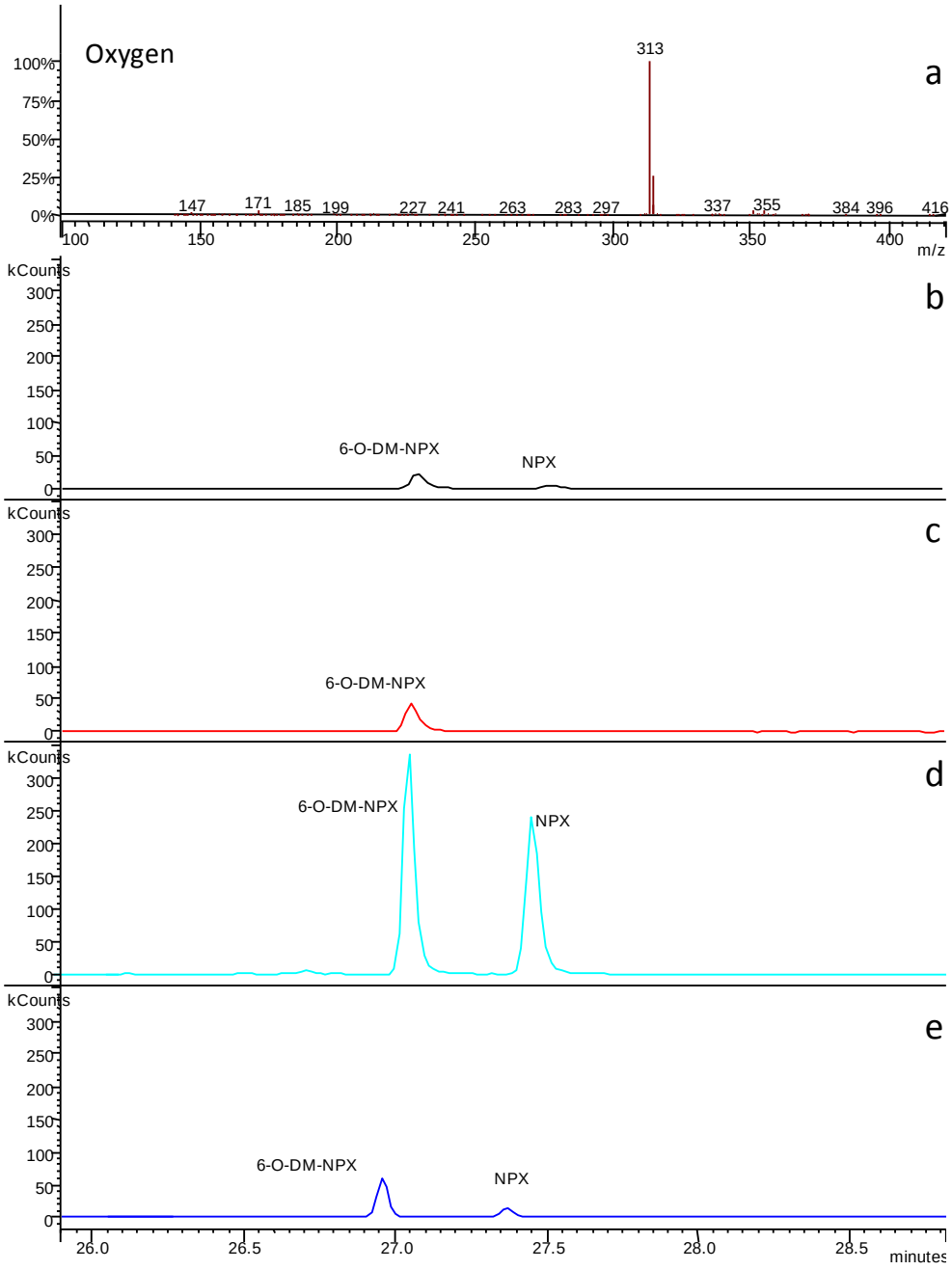


Figure 6-25. Degradation products of NPX during a continuous experiment in a fixed-bed reactor with oxygen pulses. Symbols: (a) tentative mass spectrum of 6-O-DM-NPX; SIM chromatogram m/z: 287+313 corresponding to: (b) day 20; (c) day 40; (d) day 68; (e) day 100.

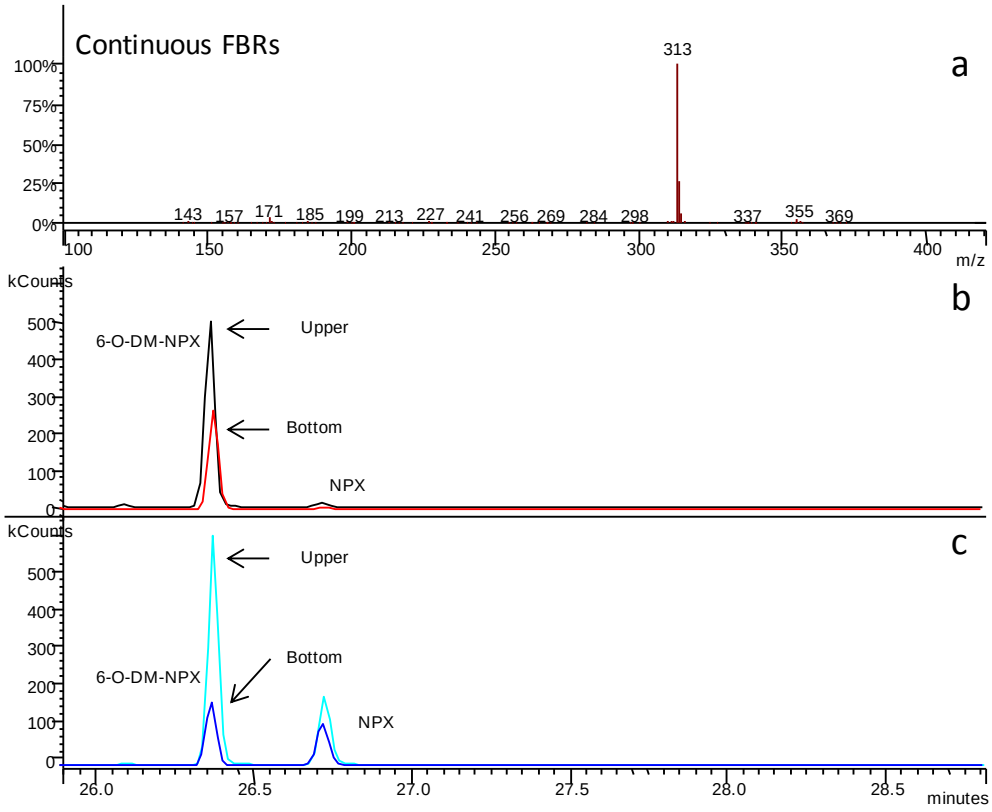


Figure 6-26. Degradation products of NPX during a continuous experiment in a fixed-bed reactor (day 100 after a final extraction with acetonitrile). Symbols: (a) tentative mass spectrum of 6-O-DM-NPX; SIM chromatogram m/z: 287+313 corresponding to: (b) oxygen pulses; (c) continuous air flow; (▬ , ▬) upper zone of the column; (▬ , ▬) bottom of the column.

As explained before, the estimation of the concentration of degradation compounds was carried out from the relative area of the peaks corresponding to the degradation products and the relative area of the parental compound at time zero (T_0) is shown in Table 6-4.

Table 6-4. Quantification of the degradation products by their relative area

Type of bioreactor	Bioreactor operational conditions	Days	Relative area (T_f/T_0)		
			4-OH-DCF	1-OH-IBP	6-O-DM-NPX
STR	Fed-batch with continuous air flow	0	1.8	0.13	1.63
		10	2.8	0.11	1.67
		20	n.d.	0.26	0.94
		30	19.7	1.15	17.12
	Fed-batch with oxygen pulses	0	0.07	0.09	0.81
		10	0.03	0.06	0.67
		20	n.d.	0.06	0.68
		30	0.07	0.09	0.91
		ACN	0.69	0.09	1.27
	24 h degradation experiment	0 h	0.29	0.07	0.80
		4 h	0.16	0.07	0.86
		18 h	0.33	0.07	0.095
	Fed-batch to continuous Fed-batch operation				
	Day 0	0 h	n.d.	0.23	1.19
		24 h	0.21	0.17	2.02
	Day 20	0 h	10.58	0.14	4.39
		24 h	23.28	0.53	7.97
	Continuous operation	29	2.76	0.35	0.02
		40	1.92	0.45	0.01
		52	0.66	0.31	5.00
		59	0.38	0.49	4.09
		69	2.40	0.39	4.79
	Continuous feeding	0	0.00	0.22	2.87
		19	9.22	0.12	1.20
		33	3.93	0.12	0.50
		50	0.46	0.06	0.12
		ACN	8.24	0.06	6.27
FBR	Immobilized fungus with a continuous air flow	20	n.d.	n.d.	n.d.
		40	n.d.	2.03	3.39
		68	n.d.	0.95	1.70
		100	n.d.	0.43	0.21
		ACN	0.02	0.07	n.d.
	Immobilized fungus with oxygen pulses	20	n.d.	0.81	n.d.
		40	n.d.	0.69	n.d.
		68	n.d.	1.68	1.30
		100	n.d.	0.02	0.11
		ACN	0.27	0.45	3.13

n.d. (not detected); ACN (sample corresponding to the final extraction with acetonitrile at the end of the experiment).

6.4. Discussion

The identification of anti-inflammatory metabolites via fungal transformation is a novel research line and quite few investigations are available in literature (Marco-Urrea et al., 2009; Hata et al., 2010; Marco-Urrea et al., 2010a; Marco-Urrea et al., 2010b). As detailed previously, the main degradation product of DCF is 4-hydroxy-diclofenac, for IBP is 1-hydroxy-ibuprofen and for NPX is 6-O-desmethyl-naproxen (Weigel et al., 2004; Aresta et al., 2006; Stülten et al., 2008a and 2008b; Scheurell et al., 2009; Ziylan and Ince, 2011). These compounds have been detected in the aquatic environment, even in higher concentrations than the parent compound (Khan and Ongerth, 2004; Weigel et al., 2004; Stülten et al., 2008a and 2008b). Acidic compounds, such as DCF, IBP and NPX (pK_a 4.2 – 4.9) are highly hydrophilic; therefore, they remain in the aqueous phase during a CAS process. However, in the specific case of IBP and its carboxylated metabolites, these compounds are easily removed in STPs whereas the hydroxylated compounds from IBP are commonly found in STPs effluents and consequently in the aquatic environment (Weigel et al., 2004).

The tentative identification of the main metabolites of the three anti-inflammatory drugs is not a straightforward study due to the fact that in many cases both the parent compound and its metabolite have similar MS. Moreover, the use of standards is required in order to compare the MS and the retention times by GC-MS analysis. Previous to the GC-MS analysis, the three anti-inflammatory drugs require to be derivatizated in order to convert the original compound into one with much higher stability and volatility (Rodríguez et al., 2003; Zhang et al., 2010). After derivatization of DCF, IBP and NPX, the formation of tert-butyl-sylil derivative occurred due to the reaction with the hydroxyl groups from the pharmaceutical molecules.

Diclofenac is metabolized into the cell by cytochrome P450 system, resulting in the formation of the major metabolite which is 4'-OH-DCF (Stülten et al., 2008a and 2008b; Scheurell et al., 2009; Hata et al., 2010; Marco-Urrea et al., 2010a; Ziylan and Ince, 2011). Both compounds have been synthesized by filamentous fungi (Webster et al., 1998); and recently, the production of 4'-OH-DCF using an extracellular peroxygenase of a basidiomycete (*Agrocybe aegerita*) was described by Kinne et al., (2009). These compounds could be classified as hardly biodegradable in the STPs due to the presence of two chlorine atoms and N-H functional group that could inhibit the rate of growth of sewage bacteria (Ziylan and Ince, 2011). Considering the results obtained in this chapter, the presence of 4-OH-DCF was demonstrated in all the degradation experiments. What seems to be evident is the effect of operating conditions on the intensity of its peak. For

instance, during fed-batch operation in an aerated STR, the presence of this degradation product was much higher than in the oxygen bioreactor. This fact could be related the positive effect of high levels of oxygen on removal efficiency. Also, the degradation of this compound during a continuous operation showed an increase of the relative area of 4-OH-DCF.

Ibuprofen has been reported to be largely transformed to its OH and CX derivatives, which may later be converted to the parent compound after a hydrolysis process (Ziylan and Ince, 2011). In samples from a river near to Hamburg in Germany, OH-IBP was the major compound, while in seawater samples CX-IBP was the dominant compound. These could be due to the fact that the carboxylated metabolite can be formed from the hydroxylated compound; also CX-IBP is more stable at low temperatures than IBP and OH-IBP. In this chapter the 1-OH-IBP was selected as the main metabolite of the degradation of IBP, since formation of the hydroxylated metabolites is enhanced under aerobic conditions, whereas the carboxylated metabolites are formed under anaerobic conditions (Zwiener et al., 2002). The results obtained in this chapter shows the presence of the degradation product 1-OH-IBP in all the analyzed samples. This metabolite as well as IBP was highly removed throughout the experiments. The high removal efficiency of these compounds may be related to the presence of only one aromatic ring, which possibly makes the molecules easily biodegradable (IBP has a K_{biol} from 9 up to 35 L/g·SS·d).

The main NPX degradation product, 6-O-desmethyl-naproxen, has been found after a fungal transformation with *T. versicolor* (Marco-Urrea et al., 2010b). These authors suggest that the enzyme laccase catalyzes the removal of a hydrogen atom from the OH group of the NPX structure using molecular oxygen as a primary electron acceptor to form free radicals. In the results obtained after the analysis of the GC-MS chromatograms, the presence of a peak with a MS corresponding to the tentative degradation product (6-O-DM-NPX) was found. The major quantification ion suggested from the fragmentation pattern of this compound is m/z: 313±1; also, a peak with a quantification ion of m/z: 285±1 was observed but with a low intensity. If the fragmented structure of the 6-O-DM-NPX loses its carboxylic group, the major quantification ion would be m/z: 285, which could correspond to the same degradation product as the peak with the quantification ion of m/z: 313±1. Therefore, both peaks could correspond to the degradation product 6-O-DM-NPX. Despite this, the GC-MS chromatograms of the degradation of NPX were included in this chapter since they provide information about the degradation of NPX as well as the accumulation and/or maintenance of the intensity of the tentative metabolite.

6.5. Conclusions

A tentative identification of the degradation products of DCF, IBP and NPX was conducted in this chapter. These compounds were highly degraded during the degradation experiments corresponding to chapters 4 and 5. From the derivatized structure and with the information about the main metabolites of these compounds found in literature, three major degradation compounds were considered: 4-hydroxy-diclofenac, 1-hydroxy-ibuprofen and 6-O-desmethyl-naproxen, corresponding to DCF, IBP and NPX, respectively. A fragmentation pattern of the derivatized structure of the degradation products was also suggested in this chapter. After analysis of GC-MS chromatograms of the selected experiments, the presence of a peak corresponding with the fragmented structure of 4-OH-DCF was detected in all the analyzed samples. Moreoever, it was shown that high oxygen levels may enhance the degradation of this compound and its parental compound. Ibuprofen and its main metabolite (1-OH-IBP) were also detected in all the samples corresponding to the degradation experiments in a STR and a FBR. Both compounds showed low values of relative area; this means that low or even negligible amounts of the metabolite were found. NPX degradation product (6-O-DM-NPX) appeared not only at the end, but also at the beginning of the experiment (in a lower extent). In addition, a secondary peak with a quantification ion of m/z: 285 was also found after an analysis of the GC-MS chromatograms corresponding to the calibration pattern and the analyzed samples. This peak showed a lower intensity than the one with the quantification ion of m/z: 313. The loss of the carboxylic group from the fragmented molecule of 6-O-DM-NPX gives the same quantification ion than that detected with the secondary peak. Therefore, it seems that both peaks correspond with the tentative degradation product. An accurate identification of the degradation products of the three anti-inflammatory drugs requires the use of the standards that are not commercially available.

General conclusions

The results obtained in this doctoral thesis demonstrate the ability of the ligninolytic fungi for removal of emerging pollutants such as pharmaceutical compounds. Three different strains of white-rot fungi (WRF) were used with the goal of the elimination of eight pharmaceutical compounds belonging to different therapeutic groups. Therefore, different culture conditions (static, pellets and immobilized fungus) in batch experiments were studied. In addition, it was examined the removal of these compounds in two types of bioreactors (stirred tank and fixed bed reactors) using different operational conditions: batch, fed-batch and continuous, with a stable operation for long periods of time (from 30 days to 100 days); also, the effect of two aeration systems (air and oxygen) were analyzed. Finally, the major degradation products of the three anti-inflammatory compounds (DCF, IBP and NPX) were likely identified in the chromatograms obtained by gas chromatography-mass spectrometry (GC-MS)

The main conclusions obtained in this doctoral thesis are presented below.

i) The three WRF used (anamorph of *Bjerkandera* sp. R1, *Bjerkandera adusta* and *Phanerochaete chrysosporium*) are able to grow in plates without inhibition effects under concentrations of all pharmaceuticals up to 1 mg/L.

ii) Acetonitrile (1:1) permits a complete extraction of pharmaceuticals from biomass and/or fungal mycelium. This solvent does not interfere during the analysis of these compounds by high performance liquid chromatography with a diode array detector (HPLC-DAD) or gas chromatography and mass spectrometry (GC-MS).

iii) The WRF used are able to remove (biotransform) partially or completely the eight pharmaceutical compounds selected (CTL, FLX, SMX, DCF, IBP, NPX, CBZ, DZP) either grown in static, free pellets or immobilized fungus using polyurethane foam. High removal efficiencies even at low levels of enzymatic activity were achieved.

iv) From the three cultivation techniques used, the highest degradation efficiency corresponded to free pellets cultures, especially for compounds such as DCF, IBP, NPX, SMX, CTL and CBZ; while only partial removal were achieved for FLX and DZP.

v) The lowest degradation percentages were obtained by the fungus *B. adusta*, while the anamorph fungus and *P. chrysosporium* achieved similar values during batch experiments.

vi) The three anti-inflammatory used can be degraded in 24 h (even lower -4 h- for IBP), by the anamorph of *Bjerkandera* sp. R1. Therefore, a hydraulic residence time (HRT) of 24 h was established for a continuous operation in stirred tank reactors (STRs) and fixed bed reactors.

vii) Both fungi, anamorph of *Bjerkandera* sp. R1 and *P. chrysosporium*, were able to effectively remove DCF, IBP, NPX, CBZ and DZP in a batch operation in a STR with pellets. However, the former fungus showed an overgrowth which may cause clogging problems within the bioreactor, while P. chrysosporium showed a more controlled growth even under a continuous air flow as aeration system.

viii) During the operation of a STR in fed-batch mode, the three anti-inflammatory compounds were completely eliminated, while CBZ and DZP were only partially removed in a bioreactor with pellets of P. chrysosporium.

ix) Similar degradation efficiencies of DCF, IBP, NPX, CBZ and DZP by pellets of *P. chrysosporium* were obtained in a continuous STR under air or oxygen; however, pulses of oxygen showed a positive effect over the pellets morphology, allowing also for a controlled growth.

x) Continuous operation of a STR with pellets of *P. chrysosporium* was stable for periods of time up to 70 days.

xi) Culture age can exert an influence over the elimination of the pharmaceuticals considered, since these compounds were slightly degraded during the first days of operation of a STR in fed-batch mode, while after three weeks of operation a complete and rapid removal were achieved.

xii) When STR starts the operation with a continuous feeding, elimination of five drugs (DCF, IBP, NPX, CBZ and DZP) was lower, suggesting than a first stage which favours biomass growth in the reactor can improve degradation yield.

xiii) Operation of a fixed-bed reactor with *P. chrysosporium* immobilized on polyurethane foam was maintained stable for 100 days, demonstrating that the use of a support favours the stability of the operation. This system allowed an effective degradation of the three anti-inflammatory compounds (DCF, IBP and NPX) under both aeration conditions (air and oxygen pulses).

xiv) The procedure of gas supply affects in opposite way the efficiency of degradation of DZP and CBZ. Therefore, a high level of dissolved oxygen improves the elimination of DZP, whereas CBZ degradation is higher in conditions of continuous airflow.

xv) Oxygen can cause a darkening process when applied pulses of oxygen, which could be related to precipitation of manganese oxides on the support surface.

xvi) Immobilization of fungus in polyurethane foam promotes the stability of the operation of the STR; however, elimination of the five considered pharmaceuticals was lower than in the fixed-bed reactor.

xvii) The major degradation products (4-hydroxy-diclofenac, ibuprofen 1-hydroxy-, 6-O-desmethyl-naproxen) of the three anti-inflammatory used (DCF, IBP, NPX) seem to be detected in the chromatograms obtained by GC-MS. However, it would be desirable to use the patterns of parent compounds to confirm these results.

Conclusiones generales

Los resultados obtenidos en esta tesis doctoral demuestran la capacidad de los hongos ligninolíticos para la eliminación de contaminantes emergentes como son los compuestos farmacéuticos. Se utilizaron tres cepas diferentes de hongos ligninolíticos (WRF) para evaluar la eliminación de ocho compuestos farmacéuticos pertenecientes a diferentes grupos terapéuticos. Se estudiaron diferentes condiciones de cultivo (estático, pellets y hongo inmovilizado) en ensayos en batch. Además se analizó la capacidad de eliminación de estos compuestos en dos tipos de biorreactores (reactores de tanque agitado y de lecho fijo) utilizando diferentes modos de operación: discontinuo, fed-batch y continuo, con operación estable durante períodos prolongados de tiempo (desde 30 días hasta 100 días). También se consideró el efecto de dos sistemas de aireación (aire y oxígeno). Finalmente se llevó a cabo una identificación tentativa de los principales productos de degradación de los tres compuestos anti-inflamatorios (DCF, IBP y NPX) a partir de los cromatogramas obtenidos por cromatografía de gases-espectrometría de masas (GC-MS).

Las principales conclusiones obtenidas en esta tesis doctoral se presentan a continuación:

i) Las tres cepas de hongos utilizadas (anamorfo de *Bjerkandera* sp. R1, *Bjerkandera adusta* y *Phanerochaete chrysosporium*) pueden crecer en placa sin efectos de inhibición en concentraciones de fármacos de hasta 1 mg/L.

ii) Se demostró que el uso de acetonitrilo (en una relación 1:1) permite efectuar eficazmente la extracción de los fármacos de la biomasa. Este compuesto, además, no interfiere en el análisis de estos compuestos por cromatografía líquida de alta resolución con un detector array de diodos (HPLC-DAD) o cromatografía de gases y espectrometría de masas (GC-MS).

iii) Los tres hongos utilizados son capaces de eliminar (biotransformar) parcial o totalmente los ocho compuestos farmacéuticos considerados (CTL, FLX, SMX, DCF, IBP, NPX, CBZ, DZP) ya sea en cultivos estáticos, como pellets libres o micelio inmovilizado en espuma de poliuretano. Se logró alta eficiencia de eliminación incluso a niveles bajos de actividad enzimática.

iv) De las tres técnicas de cultivo utilizadas, los mejores resultados se obtuvieron con cultivos de pellets, especialmente para DCF, IBP, NPX, SMX, CTL y CBZ; mientras que sólo se consiguió una eliminación parcial de FLX y DZP.

v) El hongo *B. adusta* consiguió las menores eficacias, mientras que el anamorfo de Bjerkandera y *P. chrysosporium* alcanzaron valores similares durante los experimentos en discontinuo.

vi) Los tres anti-inflamatorios considerados pueden eliminarse en un corto período de tiempo: 24 h (incluso en 4 h para IBP), por el hongo anamorfo de *Bjerkandera* sp. R1. Por ello, se estableció un tiempo de residencia hidráulico (HRT) de 24 h para la operación de los reactores de tanque agitado (RTAs) y reactores de lecho fijo.

vii) Se demostró que tanto el hongo anamorfo de *Bjerkandera* sp. R1 como *P. chrysosporium* son capaces de eliminar de forma eficaz los siguientes compuestos: DCF, IBP, NPX, CBZ y DZP en operación discontinua en un RTA con pellets. Sin embargo se observó que el primero tiene un crecimiento excesivo que puede ocasionar problemas de taponamiento dentro de los biorreactores, mientras que *P. chrysosporium* tiene un crecimiento más controlado incluso bajo un flujo de aire en continuo como sistema de aireación.

viii) Durante la operación de un RTA en modo fed-batch, se observó que los tres compuestos anti-inflamatorios se eliminaron de forma completa mientras que CBZ y DZP sólo parcialmente en un reactor con pellets de *P. chrysosporium*.

ix) La eficacia de eliminación de DCF, IBP, NPX, CBZ y DZP en un reactor con pellets de *P. chrysosporium* con flujo de aire en continuo o bien con pulsos de oxígeno fueron similares; sin embargo, la pulsación de oxígeno presentó un efecto positivo sobre la morfología de los pellets ya que permitió controlar su crecimiento.

x) La operación en continuo de un RTA con pellets de *P. chrysosporium* se mantuvo estable por períodos de tiempo de hasta 70 días.

xi) La edad del cultivo puede influir sobre la eliminación de los fármacos considerados, ya que estos compuestos fueron ligeramente degradados durante los primeros días de operación del RTA en modo fed-batch, mientras que se consiguió eliminarlos completa y rápidamente a partir de tres semanas de operación.

xii) Cuando el RTA comenzó a operar con alimentación en continuo, la eliminación de cinco fármacos (DCF, IBP, NPX, CBZ y DZP) fue menor, lo cual parece indicar que una primera fase en la que se favorezca el crecimiento de la biomasa en todo el reactor puede favorecer el rendimiento final de degradación.

xiii) Se mantuvo la operación de un reactor de lecho fijo con *P. chrysosporium* inmovilizado en espuma de poliuretano de forma estable durante 100 días, demostrando así que el uso de un soporte favorece la estabilidad de la operación. Este sistema permitió

la degradación eficaz de los tres anti-inflamatorios (DCF, IBP y NPX) bajo ambas condiciones de aireación (aire y pulsos de oxígeno).

xiv) El procedimiento de suministro de gas afectó de forma opuesta la eficiencia de degradación de DZP y CBZ. Por ello, un alto nivel de oxígeno disuelto mejora la eliminación de DZP, mientras que la degradación de CBZ es mayor en condiciones de flujo de aire en continuo.

xv) El oxígeno puede generar un proceso de oscurecimiento del soporte cuando se aplica en pulsos, lo cual podría estar relacionado con la precipitación de óxidos de manganeso sobre la superficie del soporte.

xvi) Al utilizar un RTA con hongo inmovilizado en espuma de poliuretano, se favorece la estabilidad de la operación del biorreactor; sin embargo, la eliminación de los cinco fármacos considerados fue menor que en el reactor de lecho fijo.

xvii) En los cromatogramas obtenidos por GC-MS parece detectarse los principales productos de degradación (4-hidroxi-diclofenaco, 1-hidroxi-ibuprofeno, 6-O-desmetil-naproxeno) de los tres anti-inflamatorios utilizados (DCF, IBP, NPX). Sin embargo, sería deseable utilizar los patrones de los compuestos originales para confirmar estos resultados.

Conclusións xerais

Os resultados obtidos nesta tese de doutoramento demostran a capacidade dos fungos ligninolíticos para a eliminación de contaminantes emerxentes como son os compostos farmacéuticos. Utilizáronse tres cepas diferentes de fungos ligninolíticos (WRF) para analizar a eliminación de oito compostos farmacéuticos pertencentes a diferentes grupos terapéuticos. Estudáronse diferentes condicións de cultivo (estático, pellets e fungo inmobilizado) en ensaios en batch. Ademais analizouse a capacidade de eliminación destes compostos en dous tipos de biorreactores (reactores de tanque axitado e de leito fixo) empregando diferentes modos de operación: descontinuo, fed-batch e continuo con operación estable durante períodos prolongados (desde 30 días ata 100 días). Tamén se considerou o efecto de dous sistemas de aireación (aire e osíxeno). Finalmente levouse a cabo unha identificación tentativa dos principais produtos de degradación dos tres compostos anti-inflamatorios (DCF, IBP e NPX) a partir dos cromatogramas obtidos por cromatografía de gases-espectrometría de masas (GC-MS).

As principais conclusións obtidas nesta tese de doutoramento preséntanse a continuación:

i) As tres cepas de fungos utilizadas (anamorfo de *Bjerkandera* sp. R1, *Bjerkandera adusta* e *Phanerochaete chrysosporium*) poden crecer en placa sen efectos de inhibición en concentracións de fármacos de ata 1 mg/L.

ii) Demostrouse que o uso de acetonitrilo (nunha relación 1:1) permite efectuar eficazmente a extracción dos fármacos da biomasa. Este disolvente ademais non interfere na análise destes compostos por cromatografía líquida de alta resolución cun detector *array* de diodos (HPLC-DAD) ou cromatografía de gases e espectrometría de masas (GC-MS).

iii) Os tres fungos utilizados son capaces de eliminar (biotransformar) parcial ou totalmente os oito compostos farmacéuticos considerados (CTL, FLX, SMX, DCF, IBP, NPX, CBZ, DZP) xa sexa en cultivos en estático, coma pellets libres ou micelio inmobilizado en espuma de poliuretano. Acadouse unha alta eficiencia de eliminación mesmo a niveis baixos de actividade encimática.

iv) Das tres técnicas de cultivo empregadas, os mellores resultados obtivéronse con cultivos de pellets especialmente para compostos como DCF, IBP, NPX, SMX, CTL e CBZ; mentres que só se conseguiu unha eliminación parcial de FLX e DZP.

v) O fungo *B. adusta* conseguiu as menores eficacias, mentres que o anamorfo de *Bjerkandera* e *P. chrysosporium* acadaron valores similares durante os experimentos en descontinuo.

vi) Os tres anti-inflamatorios considerados podense eliminar nun curto período de tempo: 24 h (mesmo en 4 h para IBP), polo fungo anamorfo de *Bjerkandera* sp. R1. Por iso, se estableceu un tempo de residencia hidráulico (HRT) de 24 h para a operación dos reactores de tanque axitado (RTAs) e reactores de leito fixo (FBRs).

vii) Demostrouse que tanto o fungo anamorfo de *Bjerkandera* sp. R1 coma *P. chrysosporium* son capaces de eliminar de forma eficaz os seguintes compostos: DCF, IBP, NPX, CBZ e DZP en operación descontinua nun RTA con pellets. Sen embargo, observouse que o primeiro ten un crecemento excesivo que pode ocasionar problemas de taponamento dentro dos biorreactores, mentres que *P. chrysosporium* ten un crecemento máis controlado mesmo baixo un fluxo de aire en continuo como sistema de aireación.

viii) Durante a operación dun RTA en modo fed-batch, observouse que os tres compostos anti-inflamatorios foron eliminados completamente, mentres que CBZ e DZP só parcialmente nun reactor con pellets de *P. chrysosporium*.

ix) As eficacias de eliminación de DCF, IBP, NPX, CBZ e DZP nun reactor con pellets de *P. chrysosporium* con fluxo de aire en continuo ou ben con pulsos de osíxeno foron similares; con todo, a pulsación de osíxeno amosou un efecto positivo sobre a morfoloxía dos pellets xa que permitiu controlar o seu crecemento.

x) A operación en continuo dun RTA con pellets de *P. chrysosporium* mantívose estable por períodos de tempo de ata 70 días.

xi) A idade do cultivo pode influír sobre a eliminación dos fármacos considerados, xa que estes compostos foron lixeiramente degradados durante os primeiros días de operación do RTA en modo fed-batch, mentres que se conseguiu eliminalos completa e rapidamente a partir das tres semanas de operación.

xii) Cando o RTA comezou a operar con alimentación en continuo, a eliminación de cinco fármacos (DCF, IBP, NPX, CBZ e DZP) foi menor, que parece indicar que unha primeira fase na que se favoreza o crecemento da biomasa en todo o reactor pode favorecer o rendemento final de degradación.

xiii) Mantívose a operación dun reactor de leito fixo con *P. chrysosporium* inmobilizado en espuma de poliuretano de forma estable durante 100 días, demostrando así que o uso dun soporte favorece a estabilidade da operación. Este sistema permitiu a degradación eficaz dos tres anti-inflamatorios (DCF, IBP e NPX) baixo ambas condicións de aireación (aire e pulsos de osíxeno).

xiv) O procedemento de subministración de gas afectou de forma oposta a eficiencia de degradación de DZP e CBZ. Por iso, un alto nivel de osíxeno disolto mellora a eliminación de DZP, mentres que a degradación de CBZ é maior en condicións de fluxo de aire en continuo.

xv) O osíxeno pode xerar un proceso de oscurecemento do soporte cando se aplica en pulsos, o cal podería estar relacionado coa precipitación de óxidos de manganeso sobre a superficie do soporte.

xvi) O emprego dun RTA con fungo inmobilizado en espuma de poliuretano, favorece a estabilidade da operación do biorreactor; con todo, a eliminación dos cinco fármacos considerados foi menor que no reactor de leito fixo.

xvii) Nos cromatogramas obtidos por GC-MS parece detectarse a posible presenza dos principais produtos de degradación (4-hidroxi-diclofenaco, 1-hidroxi-ibuprofeno, 6-O-desmetil-naproxeno) dos tres anti-inflamatorios utilizados (DCF, IBP, NPX). Con todo, sería desexable utilizar os patróns dos compostos orixinais para confirmar estes resultados.

References

Adler AC, Bruchet A, Carballa M, Clara M, Joss A, Löffler D, McArdell CS, Miksch KI, Omil F, Tuhkanen T, Ternes TA. (2006). Chapter 2. Consumption and occurrence. Published in Human pharmaceuticals, hormones and fragrances: The challenge of micropollutants in urban water management. Edited by Ternes TA and Joss A. ISBN: 1843390930. IWA Publishing, London, U.K.

Alexy R, Kümpel T, Kümmerer K. (2004). Assessment of degradation of 18 antibiotics in the Closed Bottle Test. Chemosphere 57:505-512.

Andreozzi R, Marotta R, Nicklas P (2003). Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50, 1319-1330.

Aresta A, Carbonara T, Palmisano F, Zambonin CG. (2006). Profilling urinary metabolites of naproxen by liquid chromatography-electrospray mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 41:1312-1316.

Ashger M, Bhatti HN, Ashraf M, Legge RL. (2008). Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 19:771-783

Blanchette RA, Abad AR, Farrell RL, Leathers TD. (1989). Detection of lignin peroxidase and xylanase by immunocytochemical labelling in wood decayed by basidiomycetes. Applied and Environmental Microbiology 55(6):1457-1465.

Blanchette RA. (1984). Manganese accumulation in wood decayed by white rot fungi. Ecology and Epidemiology 74(6):725-730.

Boehmer U, Suhardi SH, Bley T. (2006). Decolorizing reactive textile dyes with white rot fungi by temporary immersion cultivation. Engineering Life Science 6(4) 417-420.

Bound JP, Voulvoulis N (2005). Household disposal of pharmaceuticals as a pathway for aquatic contamination in the United Kingdom. Environmental Health Perspectives 113(12):1705-1711.

Brauer H. (1988). Development and efficiency of a new generation of bioreactors. Part 2: Description of new bioreactors. Bioprocess Engineering 3:11-21.

Brown JN, Paxéus N, Förlin L, Larsson DGJ. (2007). Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. Environmental Toxicology and Pharmacology 24:267-274.

Cabana H, Jones JP, Agathos SN. (2007). Removal of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: A review. Engineering Life Science 7(5): 429-256.

Calisto V, Esteves VI. (2009). Psychiatric pharmaceuticals in the environment. Chemosphere 10:1257-1274.

Camarero S, Ibarra D, Martínez MJ, Martínez AT. (2005). Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Applied and Environmental Microbiology 71(4):1775-1784.

Cañas AI, Camarero S. (2010). Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. Biotechnology Advances 28:694-705.

Carballa M, Omil F, Lema JM, Llompart M, García-Jares C, Rodríguez I, Gómez M, Ternes TA (2004). Behaviour of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Research 38, 2918–2926.

Carballa M, Omil F, Lema JM. (2005). Removal of cosmetic ingredients and pharmaceuticals in sewage primary treatment. Water Research 39:4790-4796.

Carballa M, Omil F, Lema JM. (2008). Comparison of predicted and measured concentrations of selected pharmaceuticals, fragrances and hormones in Spanish sewage. Chemosphere 72:1118-1123.

Carballa M, Omil F, Ternes TA, Lema JM. (2007). Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. Water Research 41:2139-2150.

Carlile MJ, Watinon SC, Gooday GW (2001). Saprotrophs and Ecosystems. In: The Fungi (Second Editon). Academic Press, 588 pp.

Carlsson C, Johansson AK, Alvan G, Bergman K, Kühler T. (2006). Are pharmaceuticals potent environmental pollutants? Part I: Environmental risk assessments of selected active pharmaceutical ingredients. Science of the Total Environment 364:67-87.

Castiglioni S, Bagnati R, Fanelli R, Pomati F, Calamari D, Zuccato E. (2006). Removal of pharmaceuticals in sewage treatment plants in Italy. Environmental Science & Technology 40:357-363.

Chander M, Arora DS. (2007). Evaluation of some white rot fungi for their potential to decolourise industrial dyes. Dyes and Pigments 72:192-198.

Chen B, Wang Y, Hu D. (2010). Biosorption and biodegradation of polycyclic aromatic hydrocarbons in aqueous solutions by a consortium of white-rot fungi. Journal of Hazardous Materials 179:845-851.

Clara M, Strenn B, Gans O, Martinez E, Kreuzinger N, Kroiss H. (2005). Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Research 39:4797-4807.

Cleuvers M. (2004). Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicology and Environmental Safety 59:309-315.

Crane M, Watts C, Boucard T. (2006). Chronic exposure environmental risks from exposure to human pharmaceuticals. Science of the Total Environment 367:23-41.

Cunningham VL, Perino C, D'Aco VJ, Hartmann A, Bechter R. (2010). Human health risk assessment of carbamazepine in surface waters of North America and Europe. Regulatory Toxicoloty and Pharmacology 56:343-351.

Dantas RF, Contreras S, Sans C, Esplugas S. (2008). Sulfamethoxazole abatement by means of ozonation. Journal of Hazardous Materials 150:790-794.

Daughton CG, Ternes TA. (1999). Pharmaceuticals and personal care products in the environment: agents of subtle change? Environmental Health Perspectives 107(6):907-938.

De Lange HJ, Noordown W, Murk AJ, Lüring M, Peeters ETHM. (2006). Behavioural responses of *Gammarus pulex* (Custacea, Anphipoda) to low concentration of pharmaceuticals. Aquatic Toxicology 78:209-216.

Ding J, Cong J, Zhou J, Gao S. (2008). Polycyclic aromatic hydrocarbon biodegradation and extracellular enzyme secretion in agitated and stationary cultures of *Phanerochaete chrysosporium*. Journal of Environmental Sciences 20:88-93.

Dosoretz CG, Chen HC, Grethlein HE. (1990). Effect of environmental conditions on extracellular protease activity in ligninolytic cultures of *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 56(2):395-400.

Drillia P, Dokianakis SN, Fountoulakis MS, Kornaros M, Stamatelatou K, Lyberatos G. (2005). On the occasional biodegradation of pharmaceuticals in the activated sludge process: the example of the antibiotic sulfamethoxazole. Journal of Hazardous Materials 122:259-265.

Ehlers GA, Rose PD. (2005). Immobilized white-rot fungal biodegradation of phenol and chlorinated phenol in trickling packed-bed bioreactors by employing sequencing batch operation. Bioresource Technology 96:1264-1275.

Eibes G, Debernardi G, Feijoo G, Moreira MT, Lema JM. (2011). Oxidation of pharmaceutically active compounds by a ligninolytic fungal peroxidase. Biodegradation 22:539-550.

Eibes G, Lú-Chau T, Feijoo G, Moreira MT, Lema JM. (2005). Complete degradation of anthracene by manganese peroxidase in organic solvent mixtures. Enzyme and Microbiology Technology 37:365–372.

Eichlerová I, Homolka L, Nerud F. (2007). Decolorization of high concentrations of synthetic dyes by the white rot fungus *Bjerkandera adusta* strain CCBAS 232. Dyes and Pigments 75:38-44.

EMEA (2005). Note for guidance on environmental risk assessment of medicinal products for human use, CMPC/SWP/4447/draft. The European agency for the evaluation of medicinal products (EMEA), London.

Esplugas S, Bila D, Krause LGT, Dezoti M. (2007). Ozonation and advanced oxidation technologies to remove endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) in water effluents. Journal of Hazardous Materials 149:631-642.

FDA (2005). RxList - The internet drug index for prescriptions drugs, medications and pill identifier. U.S. Food and Drug Administration. See link: http://www.rxlist.com/script/main/art.asp?articlekey=79509

Feijoo G, Moreira MT, Álvarez P, Lú-Chau TA, Lema JM. (2008). Evaluation of the enzyme manganese peroxidase in an industrial sequence for the lignin oxidation and bleaching of Eucalyptus kraft pulp. Journal of Applied Polymer Science 109:1319-1327.

Feijoo G, Rothschild N, Dosoretz C, Lema JM. (1995). Effect of addition of extracellular culture fluid on ligninolytic enzyme formation in *Phanerochaete chrysosporium*. Journal of Biotechnology 40:21-29.

Fent K, Weston AA, Caminada D. (2006). Review: Ecotoxicology of human pharmaceuticals. Aquatic Toxicology 76:122–159.

Field JA, De Jong E, Feijoo CG, De Bont JAM. (1992). Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Applied and Environmental Microbiology 58(7):2219-2226.

Flaherty CM, Dodson SI. (2005). Effects of pharmaceuticals in *Daphnia* survival, growth, and reproduction. Chemosphere 61:200-207.

Fogler HS. (2006). Elements of chemical reaction engineering. 4th edition. Pearson Education Inc., publishing as Prentice Hall. USA. ISBN 0130473944

Fong PP, Philbert CM, Roberts BJ. (2003). Putative serotonin reuptake inhibitor-induced spawning and parturition in freshwater bivalves is inhibited by mammalian 5-HT2 receptor antagonists. Journal of Experimental Zoology 298A:67–72.

Gagnon C, Lajeunesse A, Cejka P, Gagné F, Hausler R. (2008). Degradation of selected acidic and neutral pharmaceutical products in a primary-treated wastewater by disinfection processes. Ozone Science Engineering 30: 387-392.

Glenn JK, Morgan MA, Mayfield MB, Kuwahara M, Gold MH. (1983). An extracellular H_2O_2 requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycetes *Phanerochaete chrysosporium*. Biochemical and Biophysical Research Communications 114:1077-1083.

González-Alonso S, Catalá M, Romo MR, Rodríguez GJL, de Miguel GA, Valcárcel Y. (2010). Pollution by psychoactive pharmaceuticals in the Rivers of Madrid metropolitan area (Spain). Environment International 36:195–201

Green GA. (2001). Understanding NSAIDs: From aspirin to COX-2. Clinical Cornerstone 3(5):50-59.

Gros M, Petrovíc M, Ginebreda A, Barceló D. (2010). Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environmental International 36:15-26.

Gusse AC, Miller PD, Volk TJ. (2006). White rot fungi demonstrate first biodegradation of phenolic resin. Environmental Science and Technology 40:4196-4199.

Hammel KE, Cullen D. (2008). Role of fungal peroxidases in biological ligninolysis. Current Opinion in Plant Biology 11:349-355.

Hata T, Kawai S, Okamura H, Nishida T. (2010a). Removal of diclofenac and mefenamic acid by the white rot fungus *Phanerochaete sordida* YK-624 and identification of their metabolites after fungal transformation. Biodegradation 21:681-689.

Hata T, Shintate H, Kawai S, Okamura H, Nishida T. (2010b). Removal of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole. Journal of Hazardous Materials 181:1175-1178.

Heberer T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters 131:5-17.

Higuchi T. (2004). Microbial degradation of lignin: Role of lignin peroxidase, manganese peroxidase and laccase. A review. Proceedings of the Japan Academy, Ser. B, Physical and Biological Sciences 80:204-214.

Himmelblau DM. (1996). Basic principles and calculations in Chemical Engineering. 6th edition. Prentice-Hall, Inc, Englewood Cliffs, NJ, USA. 628 pp.

Hirano T, Honda Y, Watanabe T, Kuwahara M. (2000). Degradation of bisphenol A by the lignin-degrading enzyme, manganese peroxidase, produced by white rot fungi basidiomycete, *Pleurotus ostreatus*. Bioscience Biotechnology and Biochemistry 64(9):1958-1962.

Hofmann J, Freier U, Wecks M, Hohmann S. (2007). Degradation of diclofenac in water by heterogeneous catalytic oxidation with H_2O_2. Applied Catalysis B: Environmental 70:447-451.

Hofrichter M. (2002). Review: lignin conversion by manganese peroxidase (MnP). Enzyme and Microbial Technology 30, 454–466.

Holmberg A, Fogel J, Albertsson E, Fick J, Brown JN, Paxéus N, Förlin L, Johnsson JI, Larsson DGJ. (2011). Does waterborne citalopram affect the aggressive and sexual behaviour of rainbow trout and guppy? Journal of Hazardous Materials 187:596-599.

Huang DL, Zeng GM, Feng CL, Hu S, Zhao MH, Lai C, Zhang Y, Jiang XY, Liu HL. (2010). Mycelial growth and solid-state fermentation of lignocellulosic waste by white-rot fungus *Phanerochaete chrysosporium* under lead stress. Chemosphere 81:1091-1097.

Huber M, Gobel A, Joss A, Hermann N, Löffler D, McArdell CS, Ried A, Siegrist H, Ternes TA, Von Gunten U. (2005). Oxidation of pharmaceuticals during ozonation of municipal wastewater effluents: A pilot study. Environmental Science and Technology 39:4290-4299.

Ikehata K, Naghashkar N, El-Din MG. (2006). Degradation of aqueous pharmaceuticals by ozonation and advanced oxidation processes: A review. Ozone: Science and Engineering 28(6):353-414.

Isidori M, Lavorgna M, Nardelli A, Parrella A, Previtera L, Rubino M. (2005). Ecotoxicity of naproxen and its phototransformation products. Science of Total Environment 348:93 -101.

Jiménez-Tobón GA, Penninckx MJ, Lejeune R. (1997). The relationship between pellet size and production of Mn(II) peroxidase by *Phanerochaete chrysosporium* in submerged culture. Enzyme and Microbial Technology 21:537-542.

Jjemba PK. (2002). The potential impact of veterinary and human therapeutic agents in manure and biosolids grown on arable land: a review. Agriculture, Ecosystems and Environment 93:267-278.

Jjemba PK. (2006). Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. Ecotoxicology and Environmental Safety 63:113-130.

Johannes C, Majcherczyk A, Hüttermann A. (1998). Oxidation of acenaphthene and acenaphtylene by laccase of *Trametes versicolor* in a laccase-mediator system. Journal of Biotechnology 61:151-156.

Joss A, Carballa M, Kreuzinger N, Siegrist H, Zabczynski S. (2006a). Chapter 6: Wastewater treatment in Human Pharmaceuticals, hormones and Fragrances: the challenge of micropollutants in urban water management. Ternes TA and Joss A (Eds). IWA publishing, UK.

Joss A, Zabczynski S, Göbel A, Hoffmann B, Löffer D, McArdell CS, Ternes TA, Thomsen A, Siegrist H. (2006b). Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme? Water Research 40:1686-1696.

Kaushik P, Maulik A. (2009). Fungal dye decolorization: Recent advances and future potential. Environmental International 35:127-141.

Kenealy WR, Dietrich DM. (2004). Growth and fermentation responses of *Phanerochaete chrysosporium* to O_2 limitation. Enzyme and Microbial Technology 34:490-498.

Kerem Z, Friesem D, Hadar Y. (1992). Lignocellulose degradation during solid state fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 58(4):1121-1127

Kersten P, Cullen D. (2007). Extracellular oxidative systems of lignin-degrading Basidiomycete *Phanerochaete chrysosporium*. Fungal Genetics and Biology 44:77-87.

Khan SJ, Ongerth JE. (2004). Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. Chemosphere 54:355–367.

Kim I, Tanaka H. (2009). Photodegradation characteristics of PPCPs in water with UV treatment. Environment International 35:793-802.

Kinne M, Poraj-Kobielska M, Aranda E, Ullrich R, Hammel KE, Scheibne K, Hofrichter M. (2009). Regioselective preparation of 5-hydroxypropanolol and 4'-hydroxidifenac with a fungal peroxygenase. Bioorganic and Medicinal Chemistry Letters 19:3085-3087.

Klavarioti M, Mantzavinos D, Kassinos D. (2009). Removal of residual pharmaceuticals from aqueous systems by advanced oxidation processes. Environment International 35:402-417.

Kosjek T, Heath E, Kompare B. (2007). Removal of pharmaceutical residues in a pilot wastewater treatment plant. Analytical and Bioanalytical Chemistry 387:1379-1387.

Kümmerer K. (2009a). Antibiotics in the aquatic environment – A review – Part I. Chemosphere 75:417-434.

Kümmerer K. (2009b). Antibiotics in the aquatic environment – A review – Part II. Chemosphere 75:435-441.

Kümmerer K. (2009c). The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. Journal of Environmental Management 90:2354-2366.

Kummerer K. (2007). Sustainable from the very beginning: rational design of molecules by life cycle engineering as an important approach for green pharmacy and chemistry. Green Chemistry 9:899-907.

Kuwahara M, Glenn JK, Morgan MA, Gold MH. (1984). Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS letters 169:247-250.

Kwon JW, Ambrust K. (2005). Degradation of citalopram by simulated sunlight. Environmental Toxicology and Chemistry 24(7):1618-1623.

Kwon JW, Armbrust K. (2006). Laboratory persistence and fate of fluoxetine in aquatic environments. Environmental Toxicology and Chemistry 25(10):2561-2568.

Larsen TA, Lienert J, Joss A, Siegrist H. (2004). How to avoid pharmaceuticals in the aquatic environment. Journal of Biotechnology 113:295-304.

Larsson DGJ, de Pedro C, Páxeus N. (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. Journal of Hazardous Materials 148:751–755.

Lee SM, Koo BW, Choi JW, Choi DH, An BS, Jeung EB, Choi IG. (2005). Degradation of bisphenol A by white rot fungi, *Stereum hirsutum* and *Heterobasidium insulare*, and reduction of its estrogenic activity. Biological & Pharmaceutical Bulletin 28(2):201-207.

Levenspiel O. (1999). Chemical reaction engineering. Third edition. John Wiley & Sons (Ed). USA.

Lindqvist N, Tuhkanen T, Kronberg L. (2005). Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. Water Research 39:2219-2228.

Linko S, Haapala R, Zhu YH. (1996). Enzyme production with immobilized filamentous fungi. Progress in Biotechnology 11:592-599.

Lishman L, Smyth SA, Sarafin K, Kleywegt S, Toito J, Peart T, Lee B, Servos M, Beland M, Seto P. (2006). Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Science of the Total Environment 367:544-558.

Lister A, Regan C, Van Zwol J, Van Der Kraak G. (2009). Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation. Aquatic Toxicology 95:320-329.

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

López C, Moreira MT, Feijoo G, Lema JM. (2004). Dye decolorization by manganese peroxidase in an enzymatic membrane bioreactor. Biotechnology Progress 20:74-81.

Marco-Urrea E, Pérez-Trujillo M, Blánquez P, Vicent T, Caminal G. (2010b). Biodegradation of the analgesic naproxen by *Trametes versicolor* and identification of intermediates using HPLC-DAD-MS and NMR. Bioresource Technology 101:2159-2166.

Marco-Urrea E, Pérez-Trujillo M, Cruz-Morató C, Caminal G, Vicent T. (2010a). Degradation of the drug sodium diclofenac by *Trametes versicolor* pellets and identification of some intermediates by NMR. Journal of Hazardous Materials 176:836-842.

Marco-Urrea E, Pérez-Trujillo M, Vicent T, Caminal G. (2009). Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. Chemosphere 74:765-772.

Martínez AT. (2002). Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme and Microbial Technology 30:425-444.

Martínez AT, Speranza M, Ruiz-Dueñas FJ, Ferreira P, Camarero S, Guillén F, Martínez MJ, Gutiérrez A, Del Río JC. (2005). Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. International Microbiology 8:195- 204.

Méndez-Arriaga F, Esplugas S, Giménez J. (2010). Degradation of the emerging contaminant ibuprofen in water by photo-Fenton. Water Research 44:589-595.

Mester T, Field JA. (1998). Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. The Journal of Biological Chemistry 273(25):15412-15417.

Mester T, Tien M. (2000). Oxidation mechanism of liginolytic enzymes involved in the degradation of environmental pollutants. International Biodeterioration and Biodegradation 46:51-59.

Mielgo I, Moreira MT, Feijoo G, Lema JM. (2002). Biodegradation of a polymeric dye in a pulsed bed bioreactor by immobilized *Phanerochaete chrysosporium*. Water Research 36:1896-1901.

Miller, GL. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31(3):426-428.

Mishra A, Kumar S, Pandey AK. (2011). Laccase production and simultaneous decolorization of synthetic dyes in unique inexpensive medium by new isolates of white rot fungus. International Biodeterioration & Biodegradation 65:487-493.

Miura D, Tanaka H, Wariishi H. (2004). Metabolomic differential display analysis of the white-rot basidiomycete *Phanerochaete chrysosporium* grown under air and 100% oxygen. FEMS Microbiology Letters 234:111-116.

Mohammadi A, Nasernejad B. (2009). Enzymatic degradation of anthracene by the white rot fungi *Phanerochaete chrysosporium* immobilized on sugarcane bagasse. Journal of Hazardous Materials 161:534-537.

Moldes D, Rodríguez-Couto S, Cameselle C, Sanromán MA. (2003). Study of the degradation of dyes by MnP of *Phanerochaete chrysosporium* produced in a fixed bed reactor. Chemosphere 51:295-303.

Moldovan Z, Schmutzer G, Tusa F, Calinb R, Alder AC. (2007). An overview of pharmaceuticals and personal care products contamination along the river Somes watershed, Romaniaw. Journal of Environmental Monitoring 9:986-993.

Moreira MT, Feijoo G, Lema JM. (2000a). Manganese peroxidase production by *Bjerkandera* sp. BOS55: Regulation of enzymatic production. Bioprocess Engineering 23:657-661.

Moreira MT, Feijoo G, Lema JM. (2000b). Manganese peroxidase production by *Bjerkandera* sp. BOS55: Operation in stirred tank reactors. Bioprocess Engineering 23:663-667.

Moreira MT, Feijoo G, Palma C, Lema JM. (1997a). Continuous production of manganese peroxidase by *Phanerochaete chrysosporium* immobilized on polyurethane foam in a pulsed-bed bioreactor. Biotechnology and Bioengineering 56:130-137.

Moreira MT, Feijoo G, Sierra-Alvarez R, Lema JM, Field JA. (1997b). Biobleaching of oxygen delignified kraft pulp by several white rot fungal strains. Journal of Biotechnology 53:237-251.

Moreira MT, Palma C, Feijoo G, Lema JM. (1998). Strategies for the continuous production of ligninolytic enzymes in fixed and fluidised bed bioreactors. Journal of Biotechnology 66:27-39.

Moreira MT, Sanromán A, Feijoo G, Lema JM. (1996). Control of pellet morphology of filamentous fungi in fluidized bed bioreactors by means of a pulsing flow. Application to *Aspergillus niger* and *Phanerochaete chrysosporium*. Enzyme Microbial Technology 19:261-266.

Mückter H. (2006). Chapter 5. Human and animal toxicology of some water-borne pharmaceuticals. Published in Human pharmaceuticals, hormones and fragrances: The challenge of micropollutants in urban water management. Edited by Ternes TA and Joss. ISBN: 1843390930. IWA Publishing, London, U.K.

Muñoz C, Guillén F, Martínez AT, Martínez MJ. (1997) Laccase isoenzymes of *Pleurotus eryngii*: Characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. Applied and Environmental Microbiology 63:2166–2174.

Nikolaou A, Meric S, Fatta D. (2007). Occurrence patterns of pharmaceuticals in water and wastewater environments. Analytical and Bioanalytical Chemistry 387:1225-1234.

Oaks JL, Gilbert M, Virani MZ, Watson RT, Meteyer CU, Rideout BA, Shivaprasad HL, Ahmed S, Jamshed Iqbal Chaudhry M, Arshad M, Mahmood S, Ali A, Ahemed Khan A. (2004). Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427:630-633.

Paterson G, Metcalfe CD. (2009). Uptake and depuration of the anti-depressant fluoxetine by the Japanese medaka (*Oryzias latipes*). Chemosphere 74:125-130.

Pérez J, Muñoz-Dorado J, de la Rubia T, Martínez J. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. International Microbiology 5:3-63.

Pérez S, Barceló D. (2007). Application of advanced MS techniques to analysis and identification of human and microbial metabolites of pharmaceuticals in the aquatic environment. Trends in Analytical Chemistry 26(6):494-514.

Péry ARR, Gust M, Vollat M, Mons R, Ramil M, Fink G, Ternes T, Garric J. (2008). Fluoxetine effects assessment on the life cycle of aquatic invertebrates. Chemosphere 73:300-304.

Quintero JC, Lú-Chau TA, Moreira MT, Feijoo G, Lema JM. (2007). Bioremediation of HCH present in soil by the white rot fungi *Bjerkandera adusta* in a slurry batch reactor. International Biodeterioration and Biodegradation 60:319-326.

Reddersen K and Heberer T. (2003). Multi-compound methods for the detection of pharmaceutical residues in various waters applying solid phase extraction (SPE) and gas chromatography with mass spectrometric (GC-MS) detection. Journal of Separation Science 26:1443-1450.

Redshaw CH, Cooke M, Talbot HM, McGrath S, Rowland S. (2008). Low biodegradability of fluoxetine HCl, diazepam and their human metabolites in sewage sludge-amended soil. Journal of Soils and Sediments 8:217-230.

Reif R, Suárez S, Omil F and Lema JM (2008). Fate of pharmaceuticals and cosmetic ingredients during the operation of a MBR treating sewage. Desalination 221, 511-517.

Reyes-Contreras C, Matamoros V, Ruíz J, Soto M, Bayona JM. (2011). Evaluation of PPCPs removal in a combined anaerobic digester-constructed wetland pilot plant treating urban wastewater. Chemosphere 84:1200-1207.

Roca E, Sanromán A, Nuñéz MJ, Lema JM. (1994). A pulsing device for packed-bed bioreactors: I. Hydrodynamic behaviour. Bioprocess Engineering 10:61-73.

Rodríguez I, Quintana JB, Carpinteiro J, Carro AM, Lorenzo RA, Cela R. (2003). Determination of acidic drugs in sewage water by gas chromatography-mass spectrometry as *tert.*-butyldimethylsilyl derivatives. Journal of Chromatography A 985:265-274.

Rodríguez-Couto S. (2011). Production of laccase and decolouration of the textile Remazol Brilliant Blue R in temporary immersion bioreactors. Journal of Hazardous Materials (in press). doi:10.1016/j.jhazmat2011.07.098.

Rodríguez-Rodríguez CE, Marco-Urrea E, Caminal G. (2010a). Degradation of naproxen and carbamazepine in spiked sludge by slurry and solid-phase *Trametes versicolor* systems. Bioresource Technology 101:2259-2266.

Rodríguez-Rodríguez CE, Marco-Urrea E, Caminal G. (2010b). Naproxen degradation test to monitor *Trametes versicolor* activity in solid-state bioremediation processes. Journal of Hazardous Materials 179:1152-1155.

Rogalski J, Szczodrak J, Janusz G. (2006). Manganese peroxidase production in submerged cultures by free and immobilized mycelia of *Nematoloma frowardii*. Bioresource Technology 97:469-476.

Rothschild N, Levkowitz A, Hadar Y, Dosoretz CG. (1999). Manganese deficiency can replace high oxygen levels needed for lignin peroxidase formation by *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 65(2): 483-488.

Rubilar O, Diez MC, Gianfreda L. (2008). Transformation of chlorinated phenolic compounds by white rot fungi. Critical Reviews in Environmental Science and Technology 38:227-268.

Rubilar O, Feijoo G, Diez C, Lu-Chau TA, Moreira MT, Lema JM. (2007). Biodegradation of pentachlorophenol in soil slurry cultures by *Bjerkandera adusta* and *Anthracophyllum discolor*. Industrial and Engineering Chemistry Research 46:6744-6751.

Sánchez C. (2009). Lignocellulosic residues: Biodegradation and bioconversion by fungi. Biotechnology Advances 27:185-194.

Saparrat MCN, Mocchiutti P, Liggieri CS, Aulicino MB, Caffini NO, Balatti PA, Martínez MJ. (2008). Ligninolytic enzyme ability and potential biotechnology applications of the

white rot fungus *Grammothele subargentea* LPSC no. 436 strain. Process Biochemistry 43:368:375.

Scheurell M, Franke S, Shah RM, Hühnerfuss H. (2009). Occurrence of diclofenac and its metabolites in surface water and effluent samples from Karachi, Pakistan. Chemosphere 77:870-876.

Schwarze FWMR, Engles J and Mattheck C (2000). Fungal strategies of Wood Decay in Trees. Springer, 183 pp.

Schwarze FWMR. (2007). Wood decay under the microscope. Fungal Biology Reviews 21(4):133-170.

Sedighi M, Karimi A, Vahabzadeh F. (2009). Involvement of ligninolytic enzymes of *Phanerochaete chrysosporium* in treating the textile effluent containing Astrazon Red FBL in a packed bed bioreactor. Journal of Hazardous Materials 169:88-93.

Sei K, Takeda T, Soda SO, Fujita M, Ike M. (2008). Removal characteristics of endocrine-disrupting chemicals by lacasse from white rot fungi. Journal of Environmental Science and Health, Part A 43(1):53-60.

Serrano D, Suárez S, Lema JM, Omil F. (2011). Removal of persistent pharmaceutical micropollutants from sewage by addition of PAC in a sequential membrane bioreactor. Water Research (Article in press). doi: 10.1016j/.watres2011.07.037.

Sette M, Wechselberger R, Crestini C. (2011). Elucidation of lignin structure by quantitative 2D NMR. Chemistry - A European Journal 17:9529-9535.

Sinsabaug RL, Liptak MA. (1997). Enzymatic conversion of plant biomass. Pp 347-357. In: The Mycota, Vol. IV: Environmental and Microbial Relationships. Wicklow/Söderström Eds., Spinger.

Sipma J, Osuna B, Collado N, Monclús H, Ferrero G, Comas J, Rodríguez-Roda I. (2010). Comparison of removal of pharmaceuticals in MBR and activated sludge systems. Desalination 250:653-659.

Skoumal M, Rodríguez RM, Cabot PL, Centellas F, Garrido JA, Arias C, Brillas E. (2008). Electro-Fenton, UVA photoelectro-Fenton and solar photoelectro-Fenton degradation of the drug ibuprofen in acid aqueous medium using platinum and boron-doped diamond anodes. Electrochimica Acta 74:2077-2085.

Soares A, Guieysse B, Mattiason B. (2006). Influence of agitation on the removal of nonylphenol by the white-rot fungi *Trametes versicolor* and *Bjerkandera* sp.BOL13. Biotechnology Letters 28:139-143.

Soares A, Jonasson K, Terrazas E, Guieysse B, Mattiasson B. (2005). The ability of white-rot fungi to degrade the endocrine-disrupting compound nonylphenol. Applied Microbiology and Biotechnology 66:719-725.

Soo-Min L, Bon-Wook K, Joon-Weon C, Don-Ha C, Beum-Soo A, Eui-Bae J, In-Gyu C. (2005). Degradation of bisphenol A by white rot fungi *Stereum hirsutum* and *Heterobasidium insulare*, and reduction of its estrogenic activity. Biological and Pharmaceutical Bulletin 28(2):201-207.

Stülten D, Lamshöft M, Zühlke S, Spiteller M. (2008a). Isolation and characterization of a new urinary metabolite of diclofenac applying LC-NMR-MS and high-resolution mass analyses. Journal of Pharmaceutical and Biomedical Analysis 47:371-376.

Stülten D, Zülke S, Lamshöft M, Spiteller M. (2008b). Occurrence of diclofenac and selected metabolites in sewage effluents. Science of Total Environment 405:310-316.

Stumpf M, Ternes TA, Wilken RD, Rodrigues SV, Baumann W. (1999). Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. The Science of the Total Environment 225:135-141.

Suárez S, Carballa M, Omil F, Lema JM. (2008). How are pharmaceutical and personal care products (PPCPs) removed from urban wastewater? Reviews in Environmental Science and Biotechnology 7:125-138.

Suarez S, Lema JM, Omil F. (2009). Pre-treatment of hospital wastewater by coagulation-flocculation and flotation. Bioresource Technology 100: 2138-2146.

Suárez S, Ramil M, Omil F, Lema JM. (2005). Removal of pharmaceutically active compounds in nitrifying-denitrifying plants. Water Science & Technology 52(8):9-14.

Taboada-Puig R, Lú-Chau T, Moreira MT, Feijoo G, Martínez MJ, Lema JM. (2010). A new strain of *Bjerkandera* sp. production, purification and characterization of versatile peroxidase. World Journal of Microbiology and Biotechnology 27(7):1735-1742.

Tamagawa Y, Hirai H, Kawai S, Nishida T. (2005). Removal of estrogenic activity of endocrine-disrupting genistein by ligninolytic enzymes from white rot fungi. FEMS Microbiology Letters 244: 93-98.

Tamagawa Y, Hirai H, Kawai S, Nishida T. (2006b). Removal of estrogenic activity of 4-tert-octylphenol by ligninolytic enzymes from white rot fungi. Environmental Toxicology 22:281-286.

Tamagawa Y, Yamaki R, Hirai H, Kawai S, Nishida T. (2006a). Removal of estrogenic activity of natural steroidal hormone estrone by ligninolytic enzymes from white rot fungi. Chemosphere 65: 97-101.

Ternes TA, Giger W, Joss A. (2006). Chapter 1. Introduction. Published in Human pharmaceuticals, hormones and fragrances: The challenge of micropollutants in urban water management. Edited by Ternes TA and Joss A. ISBN: 1843390930. IWA Publishing, London, U.K.

Ternes TA, Stüber J, Herrmann N, McDowell D, Ried A, Kampmann M, Teiser B. (2003). Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? Water Research 37:1976–1982.

Ternes TA. (1998). Occurence of drugs in German sewage treatment plants and rivers. Water Research 32(11):3245-3260.

The Merck Index. (2006). The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals (14th edition). Edited by O'Neil MJ, Smith A, Heckelman PE and Budavari S. Whitehouse Station (NJ): Merck & Co., Inc., 2564 pp.

Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. In: Wood, WA and Klogg, ST (ed) Methods in Enzymology-Biomass, part b, lignin, pectin, and chitin. Academic Press Inc, San Diego, CA, USA. Vol 161:238-249.

Tien M, Kirk TK. (1983). Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* burds. Science 221:661-663.

Tsutsumi Y, Haneda T, Nishida T. (2001). Removal of estrogenic activities of bisphenol A by oxidative enzymes from lignin-degrading basidiomycetes. Chemosphere 42:271-276.

Valentin L, Feijoo G, Moreira MT, Lema JM. (2006). Biodegradation of polycyclic aromatic hydrocarbon in forest and salt marsh soils by white rot fungi. International Biodeterioration and Biodegradation 58:15-21.

Valentín L, Lu-Chau TA, López C, Feijoo G, Moreira MT, Lema JM. (2007). Biodegradation of dibenzothiophene, fluoranthene, pyrene and chrysene in a soil slurry reactor by the white-rot fungus *Bjerkandera* sp. BOS55. Process Biochemistry 42: 641–648.

Vogna D, Marotta R, Andreozzi R, Napolitano A, d'Ischia M. (2004). Kinetic and chemical assessment of the UV/ H_2O_2 treatment of antiepileptic drug carbamazepine. Chemosphere 54:497-505.

Vogna D, Marotta R, Napoletano A, Andreozzi R, d'Ischia M. (2004). Advanced oxidation of the pharmaceutical drug diclofenac with UV/H_2O_2 and ozone. Water Research 38:414–422.

Von-Gunten U, Janex-Habibi ML, Ternes TA, Weber L. (2006). Chapter 7: removal of PPCP during drinking water treatment. Published in Human pharmaceuticals, hormones and fragrances: The challenge of micropollutants in urban water management. Edited by Ternes TA and Joss A. ISBN: 1843390930. IWA Publishing, London, U.K.

Webster R, Pacey M, Winchester T, John P, Jezequel S. (1998). Microbial oxidative metabolism of diclofenac: production of 4´-hydroxydiclofenac using *Epiccocum nigrum* IMI354292. Applied Microbiology and Biotechnology 49:371-376.

Weigel S, Berger U, Jensen E, Kallenborn R, Thoresen H, Hühnerfuss H. (2004). Determination of selected pharmaceuticals and caffeine in sewage and seawater from TromsØ/Norway with emphasis on ibuprofen and its metabolites. Chemosphere 56:583-592.

Wesenberg D, Kyriakides I, Agathos S. (2003). White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnology Advances 22:161-187.

Wong DWS. (2008). Structure and action mechanism of ligninolytic enzymes. Applied Biochemistry and Biotechnology 157(2):174-209.

Xiao P, Mori T, Kamei I, Kiyota H, Takagi K, Kondo R. (2011). Novel metabolic pathways of organochlorines pesticides dieldrin and aldrin by the white rot fungi of the genus *Phlebia*. Chemosphere (in press). doi:10.1016/j.chemosphere.2011.06.028.

Xiong X, Wen X, Bai Y, Qian Y. (2008). Effects of culture conditions on ligninolytic enzymes and protease production by *Phanerochaete chrysosporium* in air. Journal of Environmental Sciences 20:94-100.

Yang X, Flowers RC, Weinberg HS, Singer PC. (2011). Occurrence and removal of pharmaceuticals and personal care products (PPCPs) in an advanced wastewater reclamation plant. Water Research (in press) doi:10.1016/j.watres.2011.07.026.

Yeo S, Park N, Song HG, Choi HT (2007). Generation of a transformant showing higher manganese peroxidase (Mnp) activity by overexpression of MnP gene in *Trametes versicolor*. The Journal of Microbiology 45(3):213-218.

Yu G, Wen X, Li R, Qian Y. (2006). In vitro degradation of a reactive azo dye by crude ligninolytic enzymes from nonimmersed liquid culture of Phanerochaete chrysosporium. Process Biochemistry 41:1987-1993.

Yu TH, Yu-Chen AL, Shaik KL, Cheng-Fang L, Ping-Yi Y. (2009). Removal of antibiotics and non-steroidal anti-inflammatory drugs by extended sludge age biological process. Chemosphere 77:175-181.

Zeddel A, Majcherczyk A, Huttermann A. (1993). Degradation of polychlorinated-biphenyls by white-rot fungi *Pleurotus-ostreatus* and *Trametes-versicolor* in a solid-state system. Toxicological and Environmental Chemistry 40(1-4):255-266.

Zhang S, Zhang Q, Darisaw S, Ehie O, Wang G. (2007). Simultaneous quantification of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pharmaceuticals and personal care products (PPCPs) in Mississippi river water, in New Orleans, Louisiana, USA. Chemosphere 66:1057-1069.

Zhang Y, Geißen SU, Gal C. (2008). Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. Chemosphere 73:1151-1161.

Zhang Y, Geiβen SU. (2010). *In vitro* degradation of carbamazepine and diclofenac by crude lignin peroxidase. Journal of Hazardous Materials 176:1089-192.

Ziylan A, Ince NH. (2011). Review: The occurrence and fate of anti-inflammatory and analgesic pharmaceuticals in sewage and fresh water: Treatability by conventional and non-conventional processes. Journal of Hazardous Materials 187:24-36.

Zuccato E, Castiglioni S, Fanelli R, Reitano G, Bagnati R, Chiabrando C, Pomati F, Rosetti C, Calamari D. (2006). Pharmaceutical in the environment in Italy: causes, occurrence, effects and control. Environmental Science & Pollution Research 13(1):15-21.

Zuehlke S, Duennbier U, Heberer T. (2007). Investigation of the behavior and metabolism of pharmaceutical residues during purification of contaminated ground water used for drinking water supply. Chemosphere 69:1673-1680.

Zwiener C, Seeger S, Glauner T, Frimmel FH. (2002). Metabolites from biodegradation of pharmaceutical residues of ibuprofen in biofilm reactors and batch experiments. Analytical and Bioanalytical Chemistry. 372:569–575.

Acronyms

1,2-OH-IBP	1,2-hydroxy-ibuprofen
1-OH-IBP	1-hydroxy-ibuprofen
2-Cl-1,4-DMB	2-chloro-1,4-dimethoxybenzene acid
2-OH-IBP	2-hydroxy-ibuprofen
3-HAA	3-hydroxyanthralinic acid
3-OH-4-OMeDCF	3-hydroxy-4-methoxy-diclofenac
3-OH-DCF	3-hydroxy-diclofenac
4,5-$(OH)_2$-DCF	4,5-dihydroxy-diclofenac
4-OH-DCF	4-hydroxy-diclofenac
5-OH-DCF	5-hydroxy-diclofenac
6-O-DM-NPX	6-O-desmethyl-naproxen
AAD	Aryl alcohol dehydrogenase
AAO	Aryl alcohol oxidase
ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACN	Acetonitrile
AOP	Advanced oxidation process
ASA	Acetylsalicylic acid
BDL	Below detection limit
BOD	Biological oxygen demand
BPA	Bisphenol A
BRF	Brown-rot fungi
C	Concentration of a compound (µg/L)
C_0	Initial concentration of a compound (µg/L)
C_{air}	Fraction of compound present in the air (µg/m^3 air)
CAS	Conventional activated sludge
CBZ	Carbamazepine
CLOFI	Clorfibric acid
CNS	Central nervous system
$C_{octanol}$	Concentration of a compound in organic phase (n-octanol) (µg/L)
$C_{soluble}$	Dissolved concentration of a compound (µg/L)
C_{sorbed}	Concentration of a compound in the sludge (µg/L)
CSTR	Complete stirred tank reactor
CTL	Citalopram
C_{water}	Fraction of compound present in the water (µg/m^3 water)
CX-IBP	Carboxyl-ibuprofen

DCF	Diclofenac
DDT	Dichlorodiphenyl trichloroethane
DMP	Dimethoxyphenol
DNS	Dinitrosalicylic acid
DZP	Diazepam
EDAR	Estación depuradora de aguas residuales
EDC	Endocrine disruptor chemical
ERA	Environmental risk assessment
FBR	Fixed-bed reactor
FDA	Food and drug administration
FLX	Fluoxetine
FMT	Flexible membrane tube
GABA	γ-aminobutyric acid
GC-MS	Gas chromatography-mass spectrometry
H	Henry's coefficient
HBT	1-hydroxybenzothriazole
HPLC-DAD	High performance liquid chromatography with a diode array detector
HRT	Hydraulic residence time
IBP	Ibuprofen
INM	Indomethacin
IPM	Iopromide
K_a	Dissociation constant
K_{biol}	Pseudo-first order degradation constant
K_d	Solid-liquid distribution coefficient
K_{ow}	Octanol-water partition coefficient
KPF	Ketoprofen
Lac	Laccase
LC_{50}	Lethal concentration 50
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LiP	Lignin peroxidase
LME	Lignin modifying enzyme
MBR	Membrane bioreactor
MFA	Mefenamic acid
MnP	Manganese peroxidase
MS	Mass spectrum
MTBSTFA	N-methyl-N-(tert.-butyldimethylsilyl)-trifluoroacetamide

N	Number of tanks-in-serie
n.a.	Non available
n.d.	Not detected
NPX	Naproxen
NSAID	Non-steroidal anti-inflammatory drug
NT	Total nitrogen
OH-IBP	Hydroxyl-ibuprofen
OTC	Over-the-counter
PAC	Powdered activated carbon
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyls
PCB30	2,4,6-thrichlorophenyl
PCP	Pentachlorophenol
Pk_a	Negative decadal logarithm of the dissociation constant
PO_2	Dissolved oxygen
PRX	Piroxicam
QR	Quinone oxidoreductase
RBBR	Remazol brilliant blue R
ROX	Roxithromycin
RTA	Reactor de tanque agitado
RTD	Residence time distribution
S	Solubility
SAL	Salycilic acid
SF	Stain fungi
SMBR	Sequential membrane bioreactor
SMX	Sulfamethoxazole
SOD	Superoxide dismutase
SPE	Solid phase extraction
SRF	Soft-rot fungi
SRT	Solid retention time
SS	Suspended solids (gSS/L)
SSF	Solid state fermentation
SSRI	Selective serotonin reuptake inhibitor
STP	Sewage treatment plant
STR	Stirred tank reactor
t	Time

T_0	Relative area of the parent compound at the beginning of the experiment
TCS	Triclosan
T_f	Relative area of the corresponding metabolite at the analyzed day of experiment
TMP	Trimethoprime
TOC	Total organic carbon
TSS	Total suspended solids
UDP	UDP-glucuronosyltranferanses
UDPGA	UDP-glucuronic acid
VA	Veratryl alcohol
VP	Versatile peroxidase
WRF	White-rot fungi