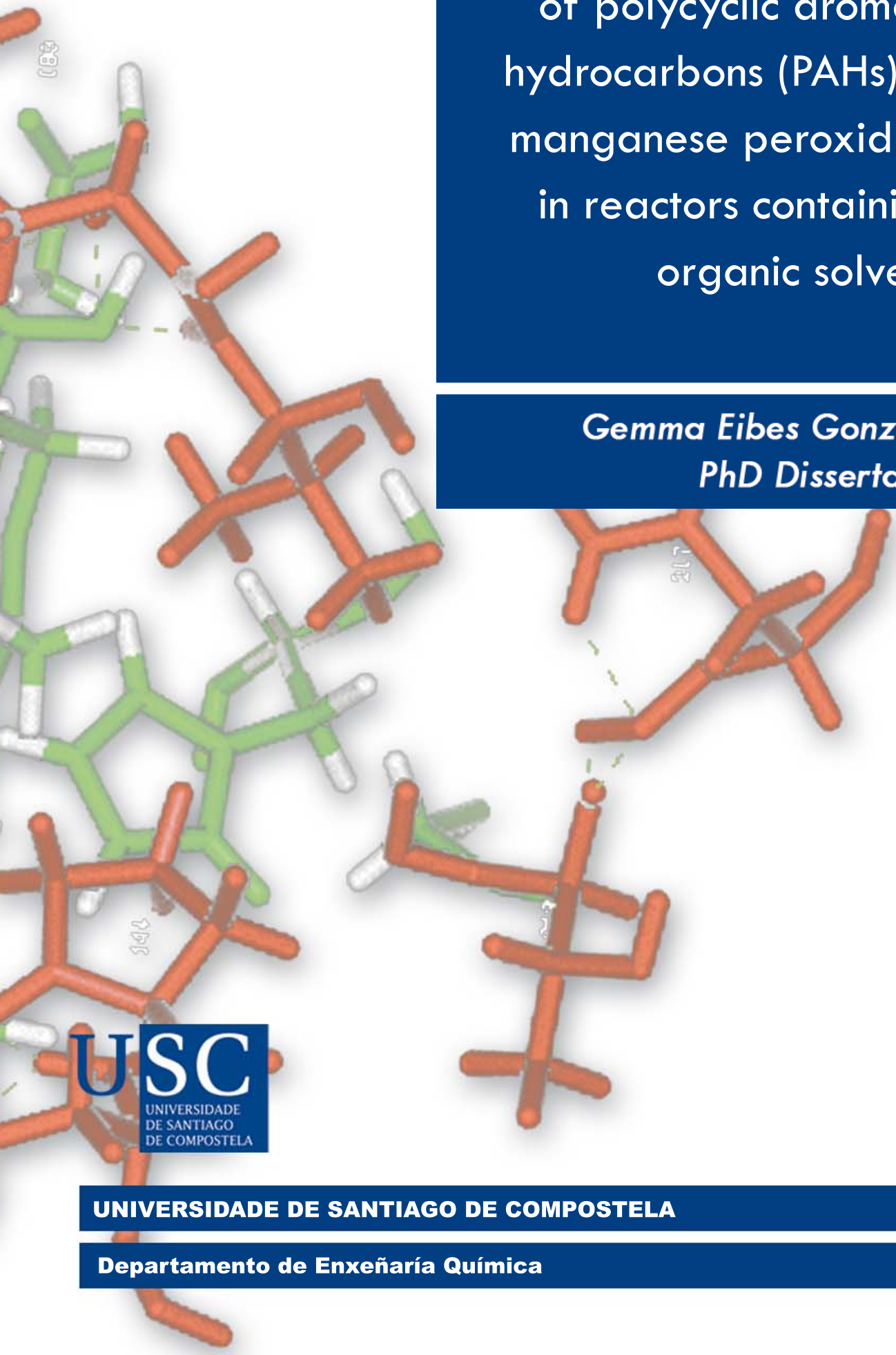
Enzymatic degradation of polycyclic aromatic hydrocarbons (PAHs) by manganese peroxidase in reactors containing organic solvents

Gemma Eibes González

PhD Dissertation



UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Enxeñaría Química

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Departamento de Ingeniería Química

Enzymatic degradation of polycyclic aromatic hydrocarbons (PAHs) by manganese peroxidase in reactors containing organic solvents

Memoria presentada por
Gemma Mª Eibes González
Para optar al grado de Doctor por la
Universidad de Santiago de Compostela

Santiago de Compostela, 26 de marzo de 2007

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA
Departamento de Ingeniería Química

Juan Manuel Lema Rodicio, Catedrático de Ingeniería Química y Mª Teresa Moreira Vilar, Profesora Contratada Doctor de Ingeniería Química de la Universidad de Santiago de Compostela,

Informan:
Que la memoria titulada "Enzymatic degradation of polycyclic aromatic hydrocarbons (PAHs) by manganese peroxidase in reactors containing organic solvents" que, para optar al grado de Doctor en Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Doña Gemma Mª Eibes González, 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, diciembre de 2006.

Juan M. Lema Rodicio Mª Teresa Moreira Vilar

Esta memoria fue presentada el 26 de marzo de 2007 en la Escola Técnica Superior de Enxeñaría de la Universidade de Santiago de Compostela ante el tribunal compuesto por:

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Obtuvo la calificación de *Sobresaliente cum laude*

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"Todo es según el color del cristal con que se mira."
Ramón de Campoamor

"Sorprenderse, extrañarse, es comenzar a entender."
José Ortega y Gasset

"La naturaleza benigna provee de manera que en cualquier parte halles algo que aprender."
Leonardo Da Vinci

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Resumen

Los hidrocarburos aromáticos policíclicos (HAPs) son compuestos orgánicos de origen tanto natural como antropogénico y presentar carácter tóxico y altamente recalcitrante. Debido a su naturaleza hidrófoba suelen presentarse adsorbidos a suelos o sedimentos y, por tanto, su disponibilidad se ve limitada, lo cual dificulta su degradación biológica. Estas características, junto con el poder cancerígeno y mutagénico de alguno de estos compuestos, ha suscitado el interés de la comunidad científica por su eliminación. Frente a otro tipo de tecnologías físicas y químicas comúnmente aplicadas, el tratamiento biológico se ha demostrado que no es sólo una tecnología eficaz sino que además destaca por los bajos costes asociados.

Desde mediados de la década de los 80, se ha demostrado que los hongos de podredumbre blanca tienen capacidad para eliminar contaminantes persistentes del medioambiente, entre ellos los HAPs. Estos hongos se caracterizan por poseer un sistema enzimático extracelular de carácter no específico capaz de degradar la lignina presente en la corteza de los árboles. La lignina presenta una estructura irregular, compleja y totalmente heterogénea, es decir, con una gran variedad de enlaces. El mecanismo que permite iniciar la depolimerización y degradación de la lignina se lleva a cabo mediante un grupo de hemoperoxidasas secretadas por estos hongos de podredumbre blanca en limitación de nutrientes durante el metabolismo secundario. Se han descrito varias clases de enzimas extracelulares, entre ellas se encuentra la enzima manganeso peroxidasa (MnP). Debido a la capacidad de degradación de un compuesto tan irregular y complejo como es la lignina, se ha considerado el uso de estas peroxidasas para la oxidación de compuestos de carácter persistente en el ecosistema, especialmente aquellos de baja solubilidad y de carácter hidrófobo como son los HAPs. Se ha demostrado que las enzimas ligninolíticas que oxidan HAPs dan lugar a la formación de quinonas, que son compuestos más polares y de mayor solubilidad en agua, y por tanto más disponibles para un posible ataque bacteriano posterior. Entre las ventajas de trabajar con reactores enzimáticos en lugar de microorganismos se puede destacar que el tiempo de operación es más corto y que no existen períodos de adaptación, las condiciones de trabajo son menos estrictas (temperatura, pH, etc), existe un mayor control del proceso, no se generan lodos, la composición de los medios es menos compleja y las enzimas no presentan un problema derivado, ya que se degradan fácilmente por la microflora autóctona.

En este trabajo se ha seleccionado antraceno como compuesto poliaromático modelo puesto que su mecanismo de oxidación es muy similar al de otros HAPs más complejos. Aunque su efecto cancerígeno no ha sido demostrado, este HAP es uno de los 16 listados por la US-EPA (Agencia de la protección ambiental de EE.UU.) para su control y seguimiento en el medioambiente. El antraceno tiene además una solubilidad en agua muy baja (0,07 mg/L), por lo que se plantea como modelo de compuesto poco soluble para su biodegradación enzimática mediante la enzima MnP. En la degradación se empleó crudo enzimático de MnP puesto que en una aplicación práctica no se plantea la purificación de la enzima ya que multiplicaría el coste del tratamiento.

El principal problema de estos compuestos es su baja solubilidad en agua, que limita la transferencia de materia y por lo tanto su eliminación enzimática. Para resolver este problema de disponibilidad, se planteó la adición de disolventes orgánicos incrementado así la solubilidad del HAP en el medio acuoso, y por lo tanto reduciendo o eliminando los problemas difusionales. Tradicionalmente, se creía que las enzimas no podían trabajar en presencia de disolventes ya que éstos se utilizaban de forma habitual para la precipitación de las mismas. Hace unos años se descubrió que ciertas enzimas podían trabajar en presencia de disolvente, incluso a elevadas concentraciones, superiores a las descritas como concentraciones tóxicas para los microorganismos. Desde los años 80 ha habido un incremento sustancial en el número de publicaciones que contemplan el uso de enzimas en medios orgánicos. En la presente tesis se estudia el comportamiento de la enzima MnP en dos tipos de medios: i) en un sistema monofásico en mezclas disolvente miscible:agua y ii) en un sistema bifásico, con un disolvente inmiscible.

Disolventes miscibles en agua

La utilización de disolventes miscibles en agua presenta como ventaja que no existen limitaciones difusionales en el medio, puesto que se trata de un sistema monofásico. Otra ventaja de este sistema es que se evita la contaminación por microorganismos en mezclas con contenido en disolvente superior a 5% v/v. Por otro lado presenta una serie de limitaciones, como la recuperación del disolvente para una posible reutilización o para evitar su presencia en el efluente, que sería posible mediante procesos de separación del tipo evaporación u otras técnicas similares. Además, la retención de la enzima en el reactor es importante en la operación en continuo y en este caso habría que considerar un método físico (membranas) o químico (inmovilización) para evitar pérdidas de enzima en el efluente.

La primera etapa para considerar la degradación de antraceno en medios con disolventes miscibles es la selección del disolvente y la concentración que se utilizará del mismo. Este trabajo se desarrolló en el Capítulo 2 de la presente tesis.

En primer lugar se preseleccionaron 4 disolventes por su disponibilidad y coste: dos alcoholes y dos cetonas. Los factores que se tuvieron en cuenta para selección final del disolvente más adecuado fueron: solubilidad de antraceno en las mezclas con distintas cantidades de agua:disolvente a las temperaturas de trabajo y estabilidad de la enzima en esas mezclas. El disolvente que produjo una mayor solubilización de antraceno fue etil-metil-cetona, pero a concentraciones superiores a 30% (v/v) se producía una separación de fases. El metanol fue el disolvente que disolvió en menor medida antraceno y en general ambos alcoholes fueron peores que las cetonas en términos de incremento de solubilidad de antraceno. La inactivación de la enzima se estudió para dos crudos enzimáticos de diferentes hongos de podredumbre blanca: MnP de crudo enzimático de *Bjerkandera* sp. BOS55 y de *Phanerochaete chrysosporium.* Los disolventes provocaron un efecto similar en la estabilidad de ambas enzimas, pero se observó que el crudo de *P. chrysosporium* se desactivó en mayor medida. El disolvente que provocó una mayor inactivación de la enzima en incubaciones fue el metanol. De entre los 4 disolventes estudiados se seleccionó acetona a la concentración 36% (v:v) por su alto poder solubilizante (incrementa la solubilidad del antraceno 143 veces) y por su baja interacción con el crudo enzimático de *B.* sp. A esa concentración de acetona, la enzima se mantenía estable en incubaciones de 24 h. Además, altas concentraciones de acetona (90% v/v) producían una leve inactivación de la enzima, al contrario de lo que se podría presuponer. El crudo enzimático de *B.* sp es el que se utilizó para los posteriores experimentos de degradación debido a sus características más favorables. Experimentos de toxicidad anaerobia mostraron que concentraciones de acetona superiores al 6% daban lugar a una clara inhibición del lodo, siendo totalmente tóxica en concentraciones cercanas al 10% (v/v). Por lo tanto es necesaria una dilución del efluente del tratamiento enzimático hasta obtener, al menos, concentraciones de acetona del 5% (v/v) para que el disolvente no sea significativamente tóxico en poblaciones aerobias y anaerobias.

Una vez seleccionado el disolvente y la enzima se llevó a cabo la optimización del proceso de degradación de antraceno en reactores en discontinuo (Capítulo 3). Se evaluó el efecto de parámetros que afectan al ciclo catalítico (tales como H_2O_2, ácido orgánico, Mn^{2+}) y parámetros ambientales (tales como temperatura, presencia de oxígeno y luz). En el caso de los parámetros relacionados con el ciclo catalítico se vio que el peróxido de hidrógeno y el ácido orgánico tenían un efecto doble. Por un lado concentraciones altas favorecían una degradación mayor, pero por otro lado, producían una pérdida de actividad mayor. El coste mayor de los reactores enzimáticos suele estar asociado al coste de la enzima. Por este motivo es muy importante mantener la estabilidad del catalizador para lograr la viabilidad de la operación del reactor enzimático. Se definió la eficacia como la relación de cantidad de substrato eliminado por unidad de enzima inactivada. De los

parámetros ambientales, la temperatura fue el que tuvo una mayor influencia en la eficacia, puesto que temperaturas altas daban lugar a una inactivación rápida de la enzima. Los experimentos en discontinuo permitieron optimizar el proceso, obteniendo una degradación total de 5 mg/L de antraceno tras 6 h de operación con las siguientes condiciones: 5 μmol/L·min de H_2O_2, 20 mM de malonato sódico, 20 μM de Mn^{2+}, a temperatura ambiente, atmósfera de oxígeno y con luz.

El sistema de degradación en discontinuo se aplicó para otros HAPs de carácter más recalcitrante y se describió el mecanismo de degradación de los mismos, utilizando técnicas de cromatografía de gases asociada a espectrometría de masas (Capítulo 4). Se obtuvieron resultados positivos en la degradación de dibenzotiofeno y pireno cuyos potenciales de ionización son superiores a los de antraceno (8.1, 7.5 y 7.4 respectivamente). Tras 24 h de reacción, el dibenzotiofeno fue eliminado completamente, mientras que la oxidación de pireno fue del 60%. Asimismo se evaluó la cinética de degradación de los compuestos como pseudo-primer orden con respecto al substrato, y se determinaron las constantes cinéticas para distintas cantidades de enzima inicial. Se vio que las cinéticas de degradación (antraceno > dibenzotiofeno > pireno) seguían un orden distinto al del carácter recalcitrante de los compuestos, que viene dado por sus potenciales de ionización (antraceno < pireno < dibenzotiofeno). Finalmente, se determinó el mecanismo de degradación de los tres HAPs degradados por MnP tomando muestras a distintos tiempos de la reacción. Todos los compuestos intermedios se detectaron en concentraciones traza, excepto antraquinona, que fue el compuesto mayoritario de la degradación de antraceno. A partir de los productos determinados, se concluyó que en la degradación de antraceno y dibenzotiofeno se produce una rotura del anillo aromático, lo cual no había sido descrito utilizando crudo enzimático de MnP y en ausencia de mediadores. Además se dedujo que en el mecanismo oxidativo podrían estar implicados radicales $^{\bullet}OH$ debido a la presencia de ciertos compuestos intermedios en la degradación de antraceno y pireno. Por otro lado se llevó a cabo la oxidación biomimética de los HAPs directamente con Mn^{3+} generado químicamente utilizando acetato de manganeso (III). Los experimentos biomiméticos se realizaron en las mismas condiciones que los experimentos *in vitro* pero evaluando dos concentraciones de Mn^{3+}. Se vio que el orden de la cinética de degradación corresponde al obtenido con los experimentos enzimáticos, pero la eliminación fue muy inferior (aún cuando la concentración de Mn^{3+} utilizada fue 50 veces superior) y en el caso de pireno no se vio oxidación en ninguno de los experimentos realizados.

A partir de los resultados obtenidos en los ensayos discontinuos se seleccionaron los parámetros operacionales más adecuados para la degradación de antraceno en continuo, pero previamente se estudiaron distintas estrategias de operación: fed batch y semi-continuo (Capítulo 5). Se comenzó estudiando la

degradación en reactores en discontinuo pero con adición de enzima en fed-batch, de modo que se mantuviera una actividad enzimática en el reactor entre 100 y 200 U/L. Se observó que los datos experimentales no se ajustaban a una cinética de primer orden con respecto al substrato ya que se advirtió una estabilidad de la velocidad de degradación durante las primeras horas. Este hecho se atribuyó a un efecto autocatalítico de los productos de reacción, principalmente quinonas. Se aplicó una ecuación de primer orden y autocatalítica, con lo que se obtuvo un ajuste satisfactorio. Además, se modeló la desactivación enzimática como una cinética de primer orden con respecto al enzima, observando dos etapas en todos los experimentos en discontinuo: la primera correspondiente al inicio de la reacción con constantes de inactivación elevadas, y una segunda etapa en que la inactivación de la enzima era menor. A continuación, se realizaron tanto un experimento en semi-continuo como otro en continuo, ambos con un tiempo de residencia de 12 h. Se comprobó que la actividad enzimática dentro del reactor era determinante en la degradación de antraceno: a mayor actividad, menor concentración de antraceno en el reactor, lo cual derivaba en una mayor degradación. De este modo, analizando distintas velocidades de adición de enzima y determinando la degradación obtenida, se estableció un término nuevo en la ecuación cinética dependiente de la actividad enzimática en el reactor. Esta función se ajustó a una ecuación sigmoidal, de modo que el efecto de la enzima es notable para valores por debajo de 100 U/L, pero por encima de este valor, su efecto se va atenuando. El reactor en continuo se operó por más de 100 h, obteniendo una eliminación del 90% en la última etapa de operación.

Disolventes inmiscibles en agua.

Los reactores bifásicos constan de una fase orgánica inmiscible en agua en la que se encuentra el contaminante en la concentración deseada. En la fase acuosa se encuentra la enzima así como los cosustratos y cofactores necesarios para completar el ciclo catalítico. El disolvente sirve como depósito de antraceno, y se transfiere el mismo a la fase acuosa mediante un equilibrio termodinámico. Allí se produce la catálisis enzimática, oxidando antraceno presente en el medio acuoso. Una de las ventajas de la operación de reactores bifásicos fue que se logró operar con cantidades mayores de contaminante que en el reactor monofásico. Además se mantuvo la enzima dentro del reactor, facilitando la recuperación del disolvente para su reutilización. La operación de reactores bifásicos se desarrolla en el Capítulo 6.

En primer lugar se seleccionó el disolvente más apropiado para la operación del reactor bifásico. Un disolvente adecuado para la operación en reactores bifásicos debe presentar las siguientes características: poco soluble en agua, poco volátil e inerte para el enzima, es decir que no se oxide por la acción del catalizador.

Además, el coeficiente de reparto de antraceno y la interacción del enzima con el disolvente son otros factores clave. En primer lugar, para seleccionar el disolvente más adecuado se evaluó el coeficiente de reparto de antraceno en disolventes inmiscibles en agua de diferente naturaleza: aceites minerales, aceites vegetales, alcoholes, hidrocarburos, etc. Se seleccionaron dos disolventes para un posterior estudio: el de menor coeficiente de reparto (aceite de silicona) y el de un coeficiente intermedio (dodecano). A continuación, se estudió la inactivación de la enzima provocada por el contacto con el disolvente a distintas velocidades de agitación. De ambos disolventes, aceite de silicona fue el que provocó una menor inactivación sobre el enzima, de modo que se selección para los experimentos posteriores.

Se optimizaron los factores implicados en el ciclo catalítico de la enzima (H_2O_2, ácido orgánico y pH). De entre las velocidades de adición de H_2O_2, 5 µmol/L·min fue la seleccionada para alcanzar mayor eficacia. En estos experimentos se vio que el pH aumentaba notablemente por lo que la concentración de malonato sódico se incrementó para favorecer una mayor estabilidad enzimática. Sin embargo sucedió lo opuesto, por lo que se optó por el control de pH a 4,5 mediante la adición de ácido malónico. De este modo la eficacia se aumentó un 53%.

Posteriormente, se estudiaron los factores que afectan a la transferencia de materia: fracción de disolvente y velocidad de agitación. Se realizó un diseño de experimentos para evaluar el efecto de la agitación y la fracción de disolvente, y para ello se consideraron velocidades de agitación entre 200 y 300 rpm (agitaciones menores no producían emulsión, y superiores, del orden de 400 rpm, daban lugar a una inactivación del enzima casi inmediata). El incremento de ambos factores tuvo un efecto positivo sobre la difusión del antraceno a la fase acuosa debido a que se aumentó el área interfacial, pero por otro lado afectó negativamente a la actividad. La eficacia de degradación fue óptima para un 30% de aceite de silicona y 300 rpm: 0,243 mg/U. Experimentos sobre la línea de ascenso no incrementaron la eficacia, debido a que la pérdida de actividad se vio incrementada pero sin mejorar la degradación de antraceno.

Se modeló el comportamiento del reactor bifásico para la oxidación enzimática de antraceno. Inicialmente, se determinaron los coeficientes de transferencia de materia en experimentos a distintas agitaciones (50 a 300 rpm) y fracción de disolvente (10-30%) y en ausencia de enzima. A partir de los resultados se obtuvo una correlación empírica para cada fracción de disolvente y agitación de forma sigmoidal, de modo que los máximos coeficientes de transferencia de materia se hallaban entre 200 y 300 rpm. Una vez conocidos los coeficientes de transferencia de materia, la aplicación de los correspondientes balances a la fase orgánica y la acuosa, permitió obtener los parámetros cinéticos y por lo tanto, se obtuvo el

modelo que ajustó el comportamiento del reactor bifásico para cada condición de fracción de disolvente y velocidad de agitación. La cinética se ajustó a una ecuación de primer orden y autocatalítica con respecto a los productos, tal como se describió en el Capítulo 5. En esta ecuación se evitó la incorporación de un término enzimático debido a que en los experimentos se mantuvo la actividad enzimática por encima de 100 U/L, por lo que la degradación no se vio limitada por la enzima. Las constantes cinéticas se obtuvieron a partir de los experimentos en discontinuo, con lo que se pudo modelar y predecir la concentración de antraceno para distintas condiciones de agitación y de fracción de disolvente.

El trabajo realizado en la presente tesis presenta dos tecnologías de carácter innovador y de amplia aplicación en el campo medioambiental. La utilización de reactores con disolventes miscibles para la degradación de compuestos poco solubles ya había sido presentada por otros autores, si bien la investigación se basaba principalmente en la determinación de los substratos oxidados por la enzima, sin realizar la optimización del proceso. La optimización de la degradación de antraceno mediante MnP logró resultados de degradación superiores a los obtenidos por otros autores. Además esta tecnología se aplicó en la eliminación de otros HAPs de carácter más recalcitrante, obteniéndose resultados positivos. En el caso de los reactores enzimáticos bifásicos se presentó un esquema innovador, puesto que hasta el momento sólo se conocían reactores microbianos bifásicos para la degradación de compuestos poco solubles, y los reactores enzimáticos existentes se centraban en procesos de síntesis de compuestos orgánicos. Las ventajas que presenta este sistema, tales como la posibilidad de reutilización del disolvente y/o del enzima, lo hacen muy atractivo para la aplicación a otros compuestos poco solubles y de carácter recalcitrante.

Resumo

Os hidrocarburos aromáticos policíclicos (HAPs) son contaminantes producidos de forma natural ou antropoxénica, e principalmente son xerados durante a combustión incompleta de combustibles sólidos ou líquidos, ou derivados de actividades industriais. Estes compostos son altamente hidrofóbicos e con baixa solubilidade en auga, polo que se adsorben facilmente en chans e sedimentos. Ademais, o seu carácter recalcitrante impide a súa degradación biolóxica natural.

Unha alternativa non agresiva co medioambiente, podería estar baseada na utilización dos fungos de putrefacción branca, entre outras posiblidades. Estes fungos son coñecidos por degradar unha gran variedade de compostos debido ao seu sitema enzimático complexo. Lignino peroxidasa (LiP) e manganeso peroxidasa (MnP) son enzimas extracelulares producidas polos estes fungos en condicións de metabolismo secundario, en resposta a unha limitación de nutrientes. O sistema ligninolítico é nonselectivo e, consecuentemente, outros sustratos aromáticos tales como HAPs son potencialmente oxidados e biodegradados polos fungos de putrefacción branca. A acción catalítica destas enzimas xera metabolitos máis polares e con maior solubilidade, coma as quinonas, que son máis susceptibles dunha degradación posterior polas bacterias indíxenas presentes en chans e sedimentos. Con todo, unha aplicación máis ampla destas enzimas está limitada porque estas enzimas funcionan correctamente en medio acuoso, donde os compostos non-polares presentan unha solubilidade moi baixa.

Unha solubilidade aumentada en medio acuoso dos poliaromáticos tería efectos beneficiosos na degradation potencial destes compostos. Unha boa alternativa para incrementar a solubilidade dos HAPs en varios ordes de magnitude é a adición de disolventes ou surfactantes. Estes últimos compostos poderían presentar unha baixa solubilización dos HAPs e unha inhibición parcial da actividade ligninolítica. O emprego de disolventes orgánicos podería considerarse como a alternativa máis adecuada. Aínda que a catálise enzimática en disolventes orgánicos se considera unha alternativa prometedora para resolver problemas medioambientais, a maioría dos traballos dispoñibles están relacionados con enzimas hidrolíticas aplicadás á síntese de compostos orgánicos. A utilización de enzimas máis complexas, tales como as enzimas ligninolíticas producidas polos fungos de putrefacción blanca, está todavía pouco desenvolvido.

O obxectivo deste traballo é a evaluación dun sistema baseado na utilización de MnP para a degradación dun HAP modelo, antraceno, nun medio con disolventes orgánicos. Propuxéronse dúas configuracións para a operación en reactores: monofásicos (con disolventes miscibles en auga) e reactores bifásicos (con disolventes inmiscibles). Antraceno, un HAP tricíclico, foi seleccionado debido á súa baixa solubidade (0,07 mg/L) e porque é sustrato das peroxidasas ligninolíticas. A degradación enzimática foi seleccionada como unha alternativa aos procesos bacterianos porque a degradación biolóxica normalmente precisa de maiores períodos de tratamento (de 2 a 4 semanas) e presenta fases de adaptación (por ex. 2 días) ata que comece a degradación. O Capítulo 1 presenta o problema asociado a ambientes contaminados con HAPs así como as tecnoloxías dispoñibles para o seu tratamente, centrádose no uso da enzima MnP en reactores con disolventes orgánicos.

Reactores monofásicos

En primeiro lugar considerouse a adición de diferentes disolventes miscibles en auga (acetona, metil-etil-cetona, metanol e etanol) para incrementar a biodispoñibilidade de antraceno (Capítulo 2). Seleccionouse acetona como disolvente óptimo debido á maior solubilidade de antraceno e á menor pérdida de actividade MnP. Conseguiuse incrementar 140 veces á solubilidade de antraceno en medios cun 36% (v:v) de acetona. Seleccionouse o crudo enzimático procedente de *Bjerkandera* sp BOS55 debido á maior estabilidade en comparación co crudo de *Phanerochaete chrysosporium*.

No Capítulo 3 investigouse a degradación *in vitro* de antraceno para diferentes concentraciones dos cofactors e sustratos principais que afectan ao ciclo catalítico de MnP (Mn^{2+}, H_2O_2 e ácidos orgánicos) así como outros parámetros ambientais (temperatura, atmósfera de aire/osíxeno, fonte de luz). O sistema alcanzou unha degradación casi completa de antraceno (alrededor do 100%) tras 6 horas de operación baixo as condicións óptimas.

No Capítulo 4 evaluouse a acción enzimática de MnP nun medio con acetona para a degradación *in vitro* doutros HAPs. Este sistema foi capaz de eliminar de forma extensa dibenzotiofeno e pireno nun período corto de tempo (24 h) ás condicións que maximizaron o sistema oxidativo de MnP. A cantidade inicial de enzima presente no medio de reacción foi determinada para a cinética do proceso. A orde de degradabilidade, segundo a velocidade de degradación, foi a seguinte: antraceno > dibenzotiofeno > pireno. Os compostos intermedios foron determinados mediante cromatografía de gas - espectroscopía de masas, e propuxéronse os mecanismos de degradación. Antraceno foi degradado a ácido ftálico. A rotura do anel aromático foi tamén observada na degradación de

dibenzotiofeno a ácido 4-metoxibenzoico. A solubilidade en auga dos productos de degradación dos tres compostos é maior que a dos compostos orixinais.

No Capítulo 5 estudouse a cinética da degradación enzimática de antraceno en presencia de acetona para incrementar a súa solubilidade. Evaluáronse diferentes configuracións de reactor, primeiro en fed-batch e logo aplicouse a un reactor semi-continuo e finalmente a un continuo. Considerouse o antraceno como sustrato da reacción enzimática, aínda que o sustrato real da enzima MnP son H_2O_2 e Mn^{2+} pero considérase como etapa limitante da renovación do ciclo catalítico a transformación de antraceno a productos oxidados. Os experimentos en fed-batch, donde MnP engadiuse para manter a actividade enzimática nun determinado rango, mostraron que as velocidades de degradación mantíñanse constantes nas primeiras horas do experimento. Este efecto explicouse por un proceso autocatalítico debido á formación de quinonas como productos de degradación (principalmente antraquinona), que actúan como transportadores de electrones. O modelo proposto, xunto coas cinéticas de inactivación enzimática, aplicouse á predicción do perfil de eliminación de antraceno en un reactor semi-continuo (con adición en continuou de tódolos compostos excepto MnP) e un reactor en continuo. Os resultados obtidos demostraron que a actividade MnP no reactor foi un factor a ter en consideración no modelo do proceso. O reactor en continuou operouse eficazmente durante 104 h obtendo unha eliminación dun 90% de antraceno.

Reactores bifásicos

No Capítulo 6 realizouse un estudo da aplicabilidade de reactores bifásicos para a eliminación de antraceno mediante a enzima MnP. Nos reactores bifásicos o sustrato está distribuido principalmente na fase inmiscible e difunde á fase acuosa donde ahí ou na interfase a enzima cataliza a conversión do sustrato. A selección do disolvente apropiado foi unha etapa clave para minimizar a súa interacción co enzima e para favorecer a transferencia dende a fase orgánica á acuosa. O disolvente seleccionado foi aceite de silicona debido as súas propiedades: coeficiente de reparto non excesivo e baixa interacción co enzima. A optimización do proceso de degradación fíxose tendo en conta os factores que poden afectar directamente o ciclo catalítico de MnP (adición de H_2O_2 e concentración de ácido malónico) e aqueles que afectan a transferencia de materia de antraceno entre as fases orgánicas e acuosas (fracción de disolvente e velocidade de axitación). O obxectivo principal foi maximizar a eficacia, é dicer, a cantidad de antraceno oxidado por unidade de enzima consumida. O reactor bifásico alcanzou unha oxidación casi completa de antraceno a unha velocidade de degradación de 1,8 mg/L·h en 56 h, o que suxire a súa aplicabilidade para a eliminación de compostos de baixa solubilidade en auga.

A continuación propúxose a modelización da operación en reactores bifásicos tendo en conta os dous principais mecanismos involucrados: a transferencia de materia de antraceno e a cinética enzimática. Para modelizar a transferencia de materia dende a fase orgánica realizouse un estudo dos coeficientes de transferencia de materia en ausencia de reacción enzimática. Obtívose unha correlación sigmoidal entre os coeficientes de transferencia e a axitación, alcanzándose os valores máximos a 250 ou 300 rpm, independentemente da fracción de disolvente. A continuación aplicouse unha ecuación cinética, considerada como de primeiro orde con respecto ao sustrato e cun efecto autocatalítico debido aos productos, resultando nun axuste satisfactorio dos datos experimentais procedentes do diseño de experimentos a diferentes velocidades de axitación e fracción de disolvente. A ecuación cinética aplicada foi consistente coa que se aplicou en reactores monofásicos, excepto que o término correspondente á actividade enzimática non foi considerado xa que se mantivo a actividade MnP en valores superiors a 100 U/L.

Summary

Polycyclic aromatic hydrocarbons (PAHs) are pollutants produced via natural and anthropogenic sources, generated during the incomplete combustion of solid and liquid fuels or derived from industrial activities. These compounds are hydrophobic with low water solubility; thus, they are easily adsorbed onto soils and sediments. Besides, their recalcitrant behaviour greatly hampers their naturally biological degradation.

Among other possibilities, an environmentally friendly approach for PAHs degradation could be based on the use of white rot fungi, which are known to degrade a great variety of compounds due to their complex enzymatic system. Lignin peroxidase (LiP) and Manganese peroxidase (MnP) are extracellular peroxidases produced by white rot fungi and the onset of their production is associated to secondary metabolism conditions in response to nutrient depletion. The ligninolytic system is nonselective, consequently, other aromatic substrates, such as PAHs, are potentially oxidized and biodegraded by white rot fungi. The catalytic action of these enzymes generates more polar and water-soluble metabolites, such as quinones, which are more susceptible to further degradation by indigenous bacteria present in soils and sediments. However, a wider application of these enzymes is hindered by the fact that enzymes work properly in aqueous media, where nonpolar compounds present very low solubility.

An increased solubilization of polyaromatics in aqueous media would have beneficial effects on the potential degradation of these compounds. A good approach to enhance PAHs solubility in several orders of magnitude is the addition of cosolvents or surfactants. These latter compounds may present low solubilization of PAHs and partial inhibition of the ligninolytic activity. The use of organic solvents may be considered as the most suitable alternative. Although enzymatic catalysis in organic solvents is considered a promising approach for solving environmental problems, most of the available work is related to hydrolytic enzymes, applied for synthesis of organic compounds. The potential of using more complex enzymes such as ligninolytic enzymes produced by white rot fungi is almost untapped.

The goal of this work is the evaluation of a system based on the use of MnP for the degradation of a PAH model compound, anthracene, in media containing organic solvents. Two different reactor configurations were proposed: monophasic reactors

(with water-miscible organic solvents) and biphasic reactors (immiscible organic solvent). Anthracene, a three-ring PAH, was chosen due to its low aqueous solubility (0.07 mg/L) and this compound has been proved to be substrate of ligninolytic peroxidases. Enzymatic degradation was selected as an alternative to bacterial processes because biological degradation usually requires long periods of treatment (from 2 to 4 weeks) and presents lag phases (e.g. 2 days) till the degradation begins. Chapter 1 presents the problems associated to PAH-contaminated environments, as well as the available technologies for their treatment, focusing in the use of MnP in reactors containing organic solvents.

Monophasic reactors

The addition of different water miscible organic solvents (acetone, methyl-ethyl-ketone, methanol and ethanol) was considered as a previous step to increase anthracene bioavailability (Chapter 2). Due to the maximal solubilisation of anthracene and the minimum loss of MnP activity, acetone was selected as the optimal cosolvent, enabling to enhance 140-fold anthracene solubility for an acetone concentration of 36% (v/v). Crude of MnP from *Bjerkandera* sp BOS55 was selected due to its higher stability in comparison with crude MnP from *Phanerochaete chrysosporium*.

The *in vitro* degradation of anthracene by MnP was investigated for different concentrations of the main cofactors and substrates that affect the catalytic cycle of MnP (Mn^{2+}, H_2O_2 and organic acids) as well as for other environmental parameters (temperature, air/oxygen atmosphere and light source) in Chapter 3. The system attained nearly complete degradation of anthracene, around 100%, after 6 hours of operation under optimal conditions.

The enzymatic action of MnP in media containing acetone was evaluated as a feasible system for the *in vitro* degradation of other PAHs, obtaining evidence of degradation for dibenzothiophene and pyrene (Chapter 4). These compounds were degraded to a large extent after a short period of time (24 h) at conditions maximizing the MnP-oxidative system. The initial amount of enzyme present in the reaction medium was determinant for the kinetics of the process. The order of degradability, in terms of degradation rates was as follows: anthracene > dibenzothiophene > pyrene. The intermediate compounds were determined using gas chromatography-mass spectrometry and degradation mechanisms were proposed. Anthracene was degraded to phthalic acid. A ring cleavage product of dibenzothiophene oxidation, 4-methoxybenzoic acid, was also observed. All degradation products had higher solubilities than their parent compounds.

The kinetics of the enzymatic degradation of anthracene in the presence of acetone for an increased solubility was studied in fed-batch reactors and then

applied to semi-continuous and continuous reactors (Chapter 5). Anthracene was considered as the substrate of the enzymatic reaction, although the real substrates for manganese peroxidase (MnP) are H_2O_2 and Mn^{2+}, but their quantification was not possible. Fed-batch experiments, where MnP was added in order to maintain the activity in a specific range, showed that degradation rates increased with time. This effect could be explained by a catalytic-process due to the formation of the degradation products, such as anthraquinone, which can act as electron carriers. The proposed model, together with the MnP decay kinetics, was applied to predict the time course of anthracene and MnP in a semi-continuous (with continuous addition of all compounds except MnP) and continuous reactor. Results showed that MnP activity in the reactor was a factor to consider in the model of the process. The continuous reactor was efficiently operated for 104 h, obtaining 90% of anthracene degradation in its last stage of operation.

Biphasic reactors

A study was conducted to determine the potential of a two-phase partitioning bioreactor (TPPB) for the treatment of anthracene by MnP (Chapter 6). In biphasic reactors, the substrate is located mostly in the immiscible phase and diffuses to the aqueous phase. The enzyme catalyzes the substrate conversion at the interface and/or in the aqueous phase. The selection of the appropriate solvent was a key step in order to minimize its interaction with the enzyme and to favor the substrate transfer from the organic to the aqueous phase. Silicone oil was selected due to its favorable properties (non-excesive partition coefficient and low interaction with the enzyme). The optimization of the oxidation process was conducted taking into account the factors which may directly affect MnP catalytic cycle (the concentration of H_2O_2, pH and malonic acid) and those that affect mass transfer of anthracene between organic and aqueous phases (fraction solvent and agitation speed). The main objective was carried out in terms of improved efficiency, i.e., maximizing the anthracene oxidized per unit of enzyme used. The TPPB reached nearly complete oxidation of anthracene at a conversion rate of 1.8 mg/L·h in 56 h, which suggests the application of enzymatic TPPBs for the removal of poorly soluble compounds.

The next step consisted on modeling the operation in a biphasic reactor taking into account the two main mechanisms involved: mass transfer of anthracene and enzymatic kinetics. In order to model transfer of anthracene from the organic phase a study of the mass transfer coefficients was conducted in absence of enzymatic reaction. A sigmoid correlation of the coefficients with agitation was obtained and maximum values were obtained at 250 or 300 rpm, regardless the solvent fraction. Next, a kinetic equation which considered first order with respect to substrate and an autocatalytic effect of the products was applied, resulting in satisfactory fitting of the data obtained from discontinuous experiments of the experimental design (at

different agitation rates and fractions of solvent). The kinetic equation was consistent with that applied in monophasic reactors, except that the enzymatic activity term was avoided by maintaining the enzymatic activity superior than 100 U/L.

Chapter 1

General introduction

Summary

The presence of recalcitrant compounds in wastewaters and soils is an important environmental problem. Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with low water solubility, high hydrophobicity and environmental persistence. These characteristics greatly hamper their degradation by endogenous bacteria. The oxidative enzymes from white-rot fungi have been successfully used for the *in vitro* degradation of PAHs. Manganese peroxidase (MnP), one of the extracellular peroxidases produced by white-rots, promotes the oxidation of Mn^{2+} to Mn^{3+}, acting as a low-molecular mass, strong diffusing oxidizer that attacks organic molecules non-specifically at locations remote from the enzyme active site. The *in vitro* degradation of poorly soluble compounds such as PAHs by MnP requires the addition of a compound to increase PAH solubility and facilitate the action of the enzyme. The addition of miscible and immiscible organic solvents is proposed as feasible alternatives to increase PAH solubilization and to reduce mass transfer limitations in enzymatic reactors.

Outline

1.1. Polycyclic aromatic hydrocarbons

1.1.1. Physical and chemical properties

1.1.2. Toxicity and health concerns

1.1.3. PAHs origin and release to the environment

1.2. PAHs removal

1.2.1. Physical and chemical treatments

1.2.2. Biological treatment. White rot fungi

1.3. PAHs availability for bioremediation

1.3.1. Surfactants

1.3.2. Solvents

1.4. Enzymatic reactors

1.5. Ligninolytic enzymes

1.6. *In vitro* degradation of recalcitrant compounds by ligninolytic peroxidases

1.7. Objectives

1.8. References

1.1. Polycyclic aromatic hydrocarbons

Recalcitrant compounds are a major hazard for the environment and in many cases they constitute risk to human and animal health. Special attention has been focused on pollutants with low aqueous solubility and high hydrophobicity because they are highly persistent. Among other poorly-soluble compounds, a type of pollutants facing particular attention nowadays is polycyclic aromatic hydrocarbons (PAHs). Because of the increased consumption of fossil fuels, their occurrence in the environment has steadily increased since last 100 to 150 years (Cerniglia 1992).

1.1.1. Physical and chemical properties

PAHs are chemical compounds that consist of fused aromatic rings (Fig. 1-1). The "hydrocarbon" term refers to its carbon and hydrogen composition. "Polycyclic" indicates that these molecules consist of several rings, and "aromatic" refers to the chemical bonds between carbon atoms. When an alkyl or another radical is linked to the ring, they are called "PAH derivatives", and "heterocyclic aromatic compounds" when any carbon atom in the ring is replaced by nitrogen, oxygen, or sulphur.

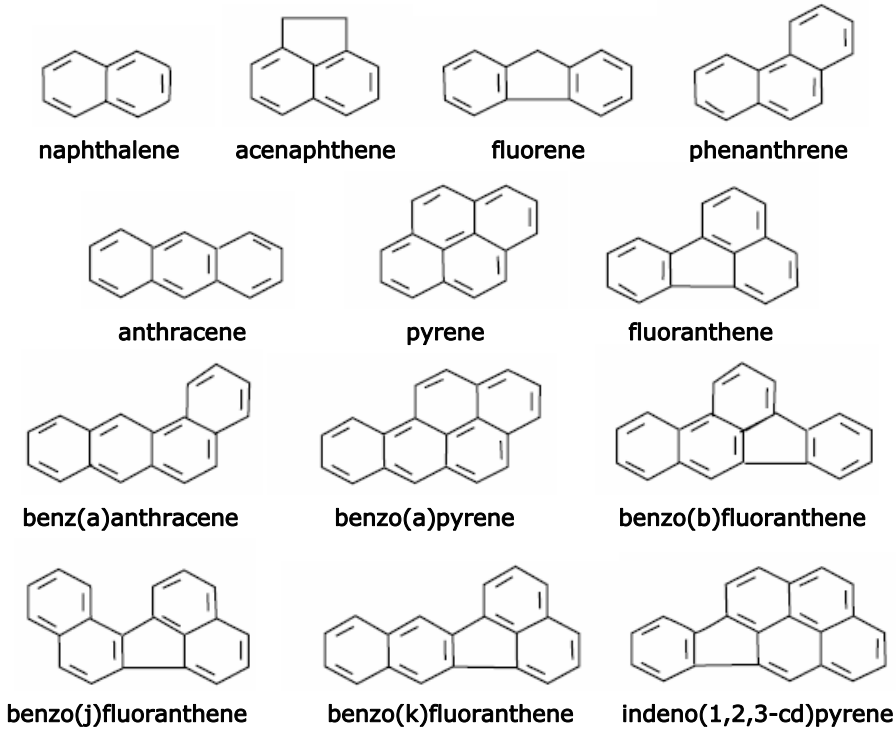

Figure 1-1. Chemical structures of representative PAHs

PAHs containing up to 4 fused benzene rings are known as light PAHs and those containing more than 4 are known as heavy PAHs. The latter have low aqueous solubility and vapor pressure, and they are more stable and toxic than the light ones (Table 1-1). PAH octanol-water coefficients, K_{OW}, a measure of hydrophobicity, are relatively high, which indicates potential for adsorption on solid particles and accumulation in organisms (Slooff et al. 1989).

Table 1-1. Physical properties of representative PAHs

Compound		Molecular weight	log K_{OW}	Water solubility (mg/L)	Melting point (ºC)	Vapor pressure (mPa)
Naphthalene	1	128.16	3.37	31.7	80.5	11960
Acenaphthene	1	154.21	3.92	3.42	95	594
Fluorene	1	166	4.18	1.98	116.5	94.7
Phenanthrene	1	178.24	4.57	1.29	101	20
Anthracene	1	178.24	4.54	0.07	216	2.3
Pyrene	1	202.26	5.18	0.135	156	0.6
Fluoranthene	1	202.26	5.22	0.26	111	1.2
Benz(a)anthracene	1	228	5.91	0.011	162	$2.8 \cdot 10^{-2}$
Benz(a)pyrene	1,2	252.32	5.91	0.0038	179	$7 \cdot 10^{-4}$
Benzo(b)fluoranthene	2	252.32	5.80	0.0015	168	$6.7 \cdot 10^{-2}$
Benzo(j)fluoranthene	2	252.32	6.12	0.0068	166	$2 \cdot 10^{-3}$
Benzo(k)fluoranthene	2	252.32	6.06	0.0008	217	$5.2\ 10^{-5}$
Indeno(1,2,3-cd)pyrene	2	276	6.50	0.00019	164	$1.3\ 10^{-5}$

1 compounds addressed in the assessment of environment effects
2 compounds addressed in the assessment of human health effects

References: ATDSR 1995; CRC 1987-1988; Mackay and Shiu 1977; Merck 1989; NRCC 1983; Slooff et al. 1989

1.1.2. Toxicity and health concerns

PAHs cause serious deleterious effects to human health as was already evidenced by the physician John Hill in 1761 who indicated the link between use of snuff and nasal cancer (Cerniglia and Heitkamp 1984). Many PAHs display acute carcinogenic,

mutagenic and teratogenic properties and may produce tumors in some organisms at even single doses. Other non-cancer-causing effects include adverse effects on reproduction, development and immunity (Eisler 1987). Their effects have been found in many organisms, including non-human mammals, birds, invertebrates, plants, amphibians, fish and humans. Mammals can absorb PAHs by inhalation, dermal contact or ingestion (Eisler 1987).

Sixteen PAHs are recognized as priority pollutants by US Environmental Protection Agency (EPA) (Table 1-2). Among these, benzo[a]pyrene is known to be one of the most powerful carcinogenic of all PAHs (Juhasz and Naidu 2000).

Table 1-2. Carcinogenetic factors related to benzo[a]pyrene of 16 individual PAHs recognized as environmental pollutants by US EPA (Nisbet and LaGoy 1992)

PAH	Carcinogenetic factor	PAH	Carcinogenetic factor
Naphthalene	0.001	Benz(a)Anthracene	0.1
Acenaphthylene	0.001	Chrysene	0.01
Acenaphthene	0.001	Benzo(b)fluoranthene	0.1
Fluorene	0.001	Benzo(k)fluoranthene	0.1
Phenanthrene	0.001	Benzo(a)pyrene	1
Anthracene	0.01	Indeno(1,2,3-cd)pyrene	0.1
Fluoranthene	0.001	Dibenz(ah)Anthracene	5
Pyrene	0.001	Benzo(ghi)perylene	0.01

1.1.3. PAHs origin and release to the environment

There are two main PAH sources: natural and anthropogenic (Fig. 1-2). In nature, one of their origins is related to pyrolysis of wood and biomass at high temperature. Another natural process occurs during the formation of fossil fuels such as coal and crude oil deposits as a result of diagenesis (that is, low temperature heating of organic material at 100-150 °C over a significant period of time) (Blumer 1976).

The anthropogenic source is becoming more significant with increasing industrialization. Examples of the most important anthropogenic sources are the industrial processes described in Table 1-3. Some PAHs are used in medicines, dyes, plastics, or pesticides. These pure PAHs are usually colorless, white, or pale yellow-green solids (Mackay et al. 1992). PAHs are generally found in a mixture such as soot, creosote, coal tar, crude oil, and roofing tar. For example, creosotes

and coal tar, coke by-products, contain significant quantities of PAHs (eg creosote contains up to 85% PAHs). PAH-contaminated sites are also commonly associated with accidental spills, leaks from storage tanks as well as wood treatment activities involving creosote use (Wilson and Jones 1993).

Figure 1-2. Pictures of some natural and anthropogenic PAH sources

The distribution and magnitude of certain emissions of PAHs are related to human population density (residential heating, transportation); however, others depend on power availability (aluminum smelters) or presence of natural resources (open air fires and agricultural burning, sawmill residue incinerators, tepee burners). Factors such as type and quantity of fuel, temperature and combustion duration and oxygen availability determine PAH formation (NRCC 1983). Soils can be polluted in levels between 1 µg/kg and 300 g/kg PAHs, depending on contamination source, e.g. coal gasification sites have the highest levels stated (Bamforth and Singleton 2005). Background levels of PAHs in air are reported to be 0.02-1.2 mg/m^3 in rural areas and 0.15-19.2 mg/m^3 in urban areas. Background levels of PAHs in drinking water range from 4-24 ng/L (ATDSR 1995).

Table 1-3. Potential sources for natural and anthropogenic PAHs

Natural	Anthropogenic			
	Domestic processes	Industrial processes		
Volcanoes	Tobacco	Power plants	Pulp mills	Coke ovens
Decaying organic matter	Charbroiled meat	Primary aluminum producers	Petroleum catalytic cracking	Hot-mix asphalt plants
Petroleum and coal deposits	Automobile exhaust fumes	Industrial boilers	Carbon black manufacture	Ferrous foundries
Forest and brush fires	Domestic heating	Electric-arc furnaces	Wood preservation	Asphalt roofing manufacture
		Municipal incinerators		

The possible routes of entering PAHs into the environment can be described as follows:

Air: PAH presence in air can be related to volcanoes, forest fires, burning coal, and automobile exhaust gases. Moreover, some PAHs can readily evaporate from soil or surface waters. PAHs in air can also be present attached to dust particles.

Soil: PAHs are likely to be adsorbed onto soil particles and sediments. PAHs are released into soil and water when plants polluted with PAHs die, are decomposed or burned.

Water: Discharges from industrial and wastewater treatment plants are the main sources of PAHs in water. Certain PAHs are leached from the soil to groundwater. They can also enter water directly from rain precipitation.

Others: PAHs dissolved in water can be uptaken by plants or animals.

PAHs release is controlled by laws, regulations and agreements designed to protect environment and human health. The European environmental law is defined by the Parliament and Council Regulation No 166/2006 of 18 January 2006, concerning the establishment of a European Pollutant Release and Transfer Register, amending Council Directives 91/689/EEC and 96/61/EC.

1.2. PAHs removal

In general, the higher the molecular weight of the PAH molecule is, the higher hydrophobicity, toxicity and persistence of the molecule. The "ageing" of the contaminant in the soil/sediment may also limit PAH biodegradability due to the theory of chemicals becoming sequestered into inaccessible microsites within the soil matrix (Hatzinger and Alexander 1995; White and Alexander 1996). Moreover, PAH association with co-pollutants such as metals is another factor that may increase their persistence in the environment (Bamforth and Singleton 2005).

1.2.1. Physical and chemical treatments

Physical treatments are used for effective decontamination of PAHs from polluted sites. Activated carbons are extensively used to remove PAHs from exhaust gases (Cudahy and Helsel 2000; Mastral et al. 2003). Moreover, since PAHs in aqueous media tend to be adsorbed onto particulate matter, removal of suspended solids containing adsorbed PAH are used for water and wastewater treatment. Depending on the complexity of the aqueous system, different capacities may be observed in PAHs adsorption (Walters and Luthy 1984). Membrane-based technology in the field of wastewater treatment has developed as a tertiary treatment to obtain a high-quality effluent. Nevertheless, even though technical feasibility is very well recognized, their implementation is limited because of the high investment and operational costs involved (Alonso et al. 2001).

Chemical oxidation for PAHs removal is usually associated to physical treatment. If the compound is present in the soil matrix, wash-out with an organic solvent is necessary prior to chemical oxidation process. On the contrary, if PAH is present in the wastewater, solvent extraction or adsorption could be required for concentration of the effluent.

The recalcitrant behavior of PAH for natural degradation requires a more powerful chemical approach to achieve remediation. Table 1-4 shows the oxidation potential of some chemical reagents. Fluorine is the strongest oxidative agent but it is not appropriate for water treatment. Efficient methods to degrade polycyclic aromatic hydrocarbons are the so-called advanced oxidation processes (AOPs) (Higgins and Halmann 1996). They consist of ozone, hydrogen peroxide, UV treatments and combination of these (Goi and Trapido 2004; Ledakowicz et al. 1999; Ledakowicz et al. 2001; Miller and Olejnik 2004). Hydroxyl radicals produced by several methods such as Fenton reaction (Martens and Frankenberger 1995; Nadarajah et al. 2002), hydrogen peroxide/UV reaction (Mokrini et al. 1997) and ultrasonic cavitation (Wheat and Tumeo 1997), have been shown to oxidize aromatics and selected PAHs. Ozone is a very powerful oxidant that can oxidize PAH at constant rates greater than 620 M^{-1} s^{-1} (Butkovic et al. 1983). It can be applied

for PAH remediation in subsurface areas (Masten and Davies 1997) and those dissolved in water (Kornmuller and Wiesmann 1999). Organic compounds treated with ozone are transformed to oxygenated intermediates which are more soluble and, thus, more biodegradable. Soils and sediments contaminated with practically insoluble PAHs may be open to *in situ* and *ex situ* remediation by means of permanganate oxidation reaction (Brown et al. 2003). While PAHs are likely not to be completely mineralized by permanganate oxidation, their structure is altered by polar functional groups providing increase of aqueous solubility and availability for natural biotic mineralization.

Table 1-4. Oxidation potential of the most powerful chemical agents

Oxidant	Oxidation Potential, V
Fluorine	3.0
Hydroxyl radical	2.8
Oxygen atom	2.4
Ozone	2.1
Hydrogen peroxide	1.8
Potassium permanganate	1.7
Chlorine dioxide	1.5
Chlorine	1.4

1.2.2. Bioremediation

Bioremediation can be defined as any process that uses microorganisms, green plants or their enzymes to return polluted sites to their original condition. Biodegradation of recalcitrant compounds is an environmentally friendly and, even, economically viable technology. The most common techniques in soil remediation such as soil incineration or land-filling are now less satisfactory and cost-effective than they used to. Therefore, bioremediation is gaining wider endorsement as a feasible treatment for soil remediation and polluted wastewater treatment.

Polluted soils, sediments and groundwaters can decontaminate by *in situ* and *ex situ* methods considering surfactant-enhanced solubility, nutrient addition and bioaugmentation (Hughes et al. 1997). Table 1-5 shows different technologies used for bioremediation. It is worth going into the use of white rot fungi for bioremediation because they can degrade pollutants that cannot be removed by prokaryotes (or by chemical means), offering the possibility to expand the substrate range of existing biodegradation treatments (Pointing 2001).

Table 1-5. Bioremediation strategies from Vidali (2001)

Technology	Examples	Benefits	Limitations	Factors to consider
In situ	*In situ* bioremediation Biosparging Bioventing Bioaugmentation	Most cost efficient Non-invasive Relatively passive Natural attenuation processes Treats soil and water	Environmental constraints Extended treatment time Monitoring difficulties	Biodegradative abilities of indigenous microorganisms Presence of metals and other inorganics Environmental parameters Biodegradability of pollutants Chemical solubility Geological factors Distribution of pollutants
Ex situ	Landfarming Composting Biopiles	Cost efficient Low cost Can be done on site	Space requirements Extended treatment time Need to control abiotic loss Mass transfer problem Bioavailability limitation	See above
Bioreactors	Slurry reactors Aqueous reactors	Rapid degradation kinetics Optimized environmental parameters Enhances mass transfer Effective use of inoculants and surfactants	Soil excavation is required Relatively high capital cost Relatively high operating cost	See above Bioaugmentation Toxicity of amendments Toxic concentrations of contaminants

Bioremediation with white rot fungi

White rot fungi differ from other microorganisms in their ability to mineralize all components of lignin (a heterogeneous polyphenolic polymer) to carbon dioxide and water. The name white-rot derives from the appearance of wood attacked by these fungi, in which lignin removal results in a bleached appearance. The ligninolytic enzymes of white-rot fungi have broad substrate specificity and have been involved in transformation and mineralization of organopollutants with structural similarities to lignin, specially those present in sensitive ecosystems such as soils and natural water courses (Field et al. 1993; Romero et al. 2006).

White-rot fungi secrete one or more of four extracellular enzymes that are essential for lignin degradation. The four ligninolytic oxidative enzymes comprise: three glycosylated heme-containing peroxidases, lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and versatile peroxidase (VP) which presents both dependent and independent-Mn activity (Martínez 2002; Orth and Tien 1995) and a copper-containing phenoloxidase, laccase (Lac) (Reinhammer 1984). Other enzymes are involved in lignin breakdown but they are unable to degrade lignin themselves. Glyoxal oxidase and superoxide dismutase produce H_2O_2 required by ligninolytic peroxidases to complete the catalytic cycle. Other enzymes are involved in feedback mechanisms and participate in lignocellulose degradation pathways. These comprise glucose oxidase, aryl alcohol oxidase, cellobiose, quinone oxidoreductase and cellobiose dehydrogenase (Leonowicz et al. 1999).

There have been many experiments performed in the last few years to evaluate degradation capability of white rot fungi (Pointing 2001; Verdin et al. 2004). In 1985 Bumpus and coworkers demonstrated the potential of *Phanerochaete chrysosporium* to degrade recalcitrant compounds (Bumpus et al. 1985). In subsequent years, research was focused on the ability of different white rot fungi to degrade light and heavy PAHs and the correlation with ligninolytic enzyme production. To date, most survey of PAH degradation have been carried out in fungal cultures with spiked media at lab and bench scale (Bogan and Lamar 1996; Field et al. 1995; Field et al. 1992; Sack and Gunther 1993). Only very few studies test their biodegradative capabilities on real polluted soil (Canet et al. 2001; Eggen and Majcherczyk 1998) or *in situ* technologies (Davis et al. 1993). Bioremediation at lab scale involves processing of solid material (soil, sediment, sludge) or water through an engineered containment system. A slurry bioreactor may be defined as a vessel which contains high proportion of soil in water to create a slurry phase. The reactor is inoculated with microorganisms capable to degrade target contaminants. These conditions are designed to increase the bioremediation rate of soil-bound and water-soluble pollutants (Vidali 2001). Slurry bioreactors are usually more manageable and hence more controllable and predictable than *in situ* or in solid-

phase systems. However, little attention has been given to the use of white-rot fungi in this kind of bioreactors, although their good growth in soil and lignocellulosic material suggests that they have potential in composting of solid waste (Valentin et al. 2006; Zheng and Obbard 2000).

Although the works carried out in PAHs degradation by white-rot fungi have proved the removal of most organopollutants from the soil in laboratory conditions, a common feature in the reported studies has been the low or unpredictable level of transformation and mineralization compared to submerged liquid cultures (Boyle et al. 1998). The low bioavailability of PAHs is often considered the major rate-limiting factor in the biodegradation of these compounds. Therefore, special attention requires the enhancement of PAHs availability by means of surfactants or solvents.

1.3. Availability of PAHs in bioremediation

1.3.1. Surfactants

A possible way to enhance bioavailability of hydrophobic organic compounds is the application of surfactants, which comprise hydrophilic and hydrophobic fractions. An important characteristic of surfactants is the fact that aggregates of 10 to 200 molecules, called micelles, are formed above the critical micelle concentration.

Two mechanisms explain the increased bioavailability of organic compounds in presence of surfactants: i) solubility of the pollutant is increased because of the hydrophobic organic fractions in micelles (Edwards et al. 1991); and ii) transport of the pollutant from the solid to the aqueous phase is favored, probably due to reduction of surface tension of pore water in soil particles, interaction of the surfactant with solid interfaces or interaction of the pollutant with single surfactant molecules (Volkering et al. 1995).

In many works, it has been shown that non-ionic surfactants stimulate PAH degradation by increased bioavailability (Tiehm 1994; Volkering et al. 1995; Zheng and Obbard 2001). For example, surfactants such as Tween 80 and polyoxyethylene 10 lauryl ether (PLE) increased anthracene, pyrene and benzo(a)pyrene oxidation rate by 2 to 5-fold (Kotterman et al. 1998a). However, contradictory results are found in literature, since some authors have found that surfactants inhibit biodegradation (Grimberg et al. 1995; Laha and Luthy 1991; Laha and Luthy 1992). One hypothesis is that microorganisms do not have access to PAHs in the micellar phase. Another proposal is that surfactants may be toxic or used by microorganisms as carbon source. For the reasons mentioned above, careful study is needed before using surfactants for biological soil treatment.

1.3.2. Solvents

The use of organic solvents is another alternative to enhance availability of hydrophobic substances. Solubility of these compounds in organic solvents is usually orders of magnitude higher than aqueous solubility. Their use may be interesting for soil treatment because regeneration of the solvent after extraction is possible. However, the use of solvents has potential disadvantages, such as inherent complexity, cost increase, solvent recycling, little experience and potential toxicity. Many organic solvents are toxic to living organisms because of their devastating effects on biological membranes (Heipieper et al. 1994). This factor correlates inversely with the hydrophobic character of the solvent, expressed by the logarithm of the partition coefficient between octanol and water (log K_{OW} value) (Inoue and Horikoshi 1989). Solvents with log K_{OW} between 1 and 5 such as toluene, are highly toxic to whole cells (Heipieper et al. 1994).

Two possibilities arise when using organic solvents, which determine the technology and the characteristics of the system:

i) Single-phase systems
ii) Biphasic systems

Single-phase systems are based on the use of water-miscible co-solvents to increase solubility of poorly soluble substrates. This type of system can considerably reduce mass-transfer limitations with faster reaction rates. These systems have been used for PAH degradation by bacteria and white-rot fungi. Arithmetic increments of miscible solvents in water increase PAH solubility in a logarithmic mode (Morris et al. 1988). However, the amount of solvent to be used is limited by its toxicity on the microorganism. As an example, acetone or ethanol concentrations higher than 20% had an inhibitory effect on the growth and action of the white-rot fungus *Bjerkandera* sp BOS55 (Field et al. 1995). In that work, additions of acetone or ethanol at the proportions 11%-21% (v/v) increased anthracene degradation rate by a factor of 2-3 compared to fungal cultures receiving 1%-3% solvent. The degradation of 10 mg/L of anthracene was completed after 4 days of incubation.

Biphasic systems consist of two immiscible phases: organic and aqueous. The organic phase delivers toxic substrates at a sub-inhibitory level in the aqueous phase and permits increased mass transfer of poorly soluble substrates (Déziel et al. 1999; Efroymson and Alexander 1991). The system is self-regulated, as the pollutant delivery to the aqueous phase is only directed by the partitioning ratio between the two phases and the culture consumption rate (Daugulis 1997). PAH degradation in biphasic reactors was carried out with pure or mixed bacterial cultures (Ascón-Cabrera and Lebeault 1995; Guieysse et al. 2001; MacLeod and Daugulis 2003; Muñoz et al. 2003; Villemur et al. 2000), and no references are

available for white-rot fungi. The use of *Sphingomonas aromaticivorans* achieved complete biodegradation of four PAHs with a volumetric consumption rate of 90 mg/L·h in a biphasic reactor (Janikowski et al. 2002).

1.4. Enzymatic reactors

Numerous advantages arise from the use of enzymes against microorganisms for environmental purposes:

i) Enzymes can be active under a wider variety of conditions such as pH, ionic strength or temperature;
ii) Higher pollutant concentrations can be maintained in enzymatic reactors with reduced inhibition problems;
iii) Shorter operational times with no lag period due to microbial growth;
iv) Simpler media composition and lower enzymatic requirements provided that the enzyme can be reused;
v) Easy process control;
vi) No sludge production.

On the contrary, cost of enzyme, its sensitivity to changes in environmental conditions and the requirements of cofactors to complete the catalytic cycle are the main limitations that have to be taken into account to favor the efficiency of the enzymatic process.

The enzyme used as catalyst for degradation of pollutants should exhibit different properties:

i) High oxidation and ionization potentials, in order to degrade recalcitrant compounds;
ii) Unspecific action, which would permit degradation of a broad range of compounds as those present in polluted effluents or soils;
iii) Diffusible enzymes or related mediators are desirable, taking into account that the interaction of the enzyme and the substrate may be constrained to the large size of the enzyme;
iv) Extracellular enzymes are preferred, since their production is easier and cheaper.

All these characteristics are fulfilled by the ligninolytic enzyme referred as MnP. The use of crude enzymes instead of purified preparations is currently a requirement to be applied in environmental engineering because of the high cost related to the enzyme purification procedures (Yu et al. 2006).

The configurations of enzymatic reactors can be classified according to the manner in which the enzyme is retained: i) immobilized onto a support, forming bigger structures that can be retained due to their size or ii) free in solution, being retained by a membrane or iii) retained in an organic phase.

Immobilization of the enzyme onto a support is usually complex and expensive, and increases processing costs. To improve the economical feasibility of immobilized enzyme reactors, a number of requirements should be met: the specific activity of the derivative (units of enzyme per g of support) should be as high as possible; the support or membrane could be applied with a secondary function, such as the separation of substrates or products; and the support should have good mechanical resistance and minimum interaction with the substrates or products. Previous studies have determined a support based on agarose activated with glutaraldehyde groups as suitable for the immobilization of MnP for the degradation of the dye Orange II in a continuous stirred tank reactor (Mielgo et al. 2003b).

The second option corresponds to enzymatic membrane reactors, where the biocatalyst is separated from substrates and/or products by means of a semi-permeable membrane that creates a selective physical/chemical barrier (López et al. 2002; Prazeres and Cabral 2001). Among other possibilities, direct contact membrane system consists on a solid/liquid membrane separation, which employs ultra or microfiltration modules for the retention and possible recirculation of biocatalysts, coupled to a bioreactor where the reaction takes place (López et al. 2004). The main advantages of this configuration are: i) operation with free enzyme, avoiding limitations of mass transfer and, consequently, low kinetic rates; ii) retention of non-biodegradable molecules with high molecular weights; iii) ability of the products of degradation to cross the membrane, being discharged in the effluent; and iv) easy operation.

A third approach can be considered when dealing with poorly soluble pollutants, and an immiscible organic phase is introduced in the reactor, that is, biphasic reactors. In this case, the enzyme is trapped onto the aqueous phase. Chapter 6 will be focused on this kind of enzymatic reactors.

1.5. Ligninolytic enzymes

After discovery of the ligninolytic enzymes of white rot fungi (Glenn and Gold 1983; Tien and Kirk 1983), Bumpus et al. (1985) proposed that these enzymes could be candidates for bioremediation due to their non-specific activity. The most ubiquitous ligninolytic enzymes produced by white-rot fungi are peroxidases (LiP, VP and MnP) and phenol oxidases (Lac), the latter using molecular oxygen for activation.

Peroxidases are hemo-proteins which require presence of hydrogen peroxide to oxidize lignin. Their molecular weights range from 35-47 kDa and their oxidation potentials from 1.45-1.51 V (Mester and Tien 2000; Wesenberg et al. 2003). MnP preferably oxidizes phenolic compounds by means of Mn^{2+} as reducing substrate; meanwhile, LiP is able to oxidize phenolic and non-phenolic substrates.

The catalytic cycle of the ligninolytic peroxidases is similar to other peroxidases and consists in a set of three reactions, being the third reaction (the enzyme returns to the resting state) 10-times slower and rate-limiting (Kuan et al. 1993; Dunford 1991). With excess of hydrogen peroxide, an enzyme intermediate converts into an inactive form of the peroxidase.

LiP has been extensively studied since it was the first discovered ligninolytic peroxidase and was considered as the most important lignin-degrading enzyme (Hatakka 1994). When many different fungi had been studied in detail, it became clear that MnP is the most commonly occurring peroxidase while it was difficult to demonstrate the expression of LiP in several fungi (Hatakka 1994; Orth et al. 1993).

Manganese peroxidase

MnP was first discovered in *P. chrysosporium* (Kuwahara et al. 1984) and produced by a number of white-rot fungi such as *Pleurotus*, *Trametes*, *Phlebia* or *Bjerkandera* species (de Jong et al. 1992; Tien and Kirk 1988). Its molecular weigh ranges from 43-49 kDa, slightly higher to that of LiP (Sundaramoorthy et al. 1994). MnP occurs as a series of isozymes; up to 11 different isoforms have been described in one fungal strain (*Ceriporiopsis subvermispora*) (Lobos et al. 1994). *B.* sp BOS55 produces two different isozymes whereas *P. chrysosporium* produces three (Palma et al. 2000). The isoforms of the different fungi differ mostly in their isoelectric points (pIs), which are usually rather acidic (pH 3–4), though less acidic and neutral isoforms were found in certain fungi (Hatakka 1994; Steffen et al. 2002).

The enzyme is a glycoprotein and contains one iron protoporphyrin IX prosthetic group. In order to stabilize protein structure, it presents 10 cysteine residues forming 5 disulfide bridges and two Ca^{2+} ions which are essential to maintain the three-dimensional structure (Martínez 2002). Mn^{2+} binding site is close to the surface of the protein, consisting of three acidic amino acid residues, Asp-179, Glu-35, and Glu-39 and one heme propionate (Sundaramoorthy et al. 1994). The distal side of the heme cavity, containing His, Arg, Asp and Leu residues, is directly involved in the reaction with hydrogen peroxide and the stabilization of the oxidized stages of the enzyme. The proximal side residues might play some role in the structural arrangement of the heme (Santucci et al. 2000).

The first step required for a successful application is a deep knowledge of the enzyme behavior, regarding the cofactors and cosubstrates involved in the catalytic cycle. MnP has a similar catalytic cycle to other peroxidases involving a 2-electron oxidation; however, MnP is unique in its ability to oxidize Mn^{2+} (Fig. 1-3).

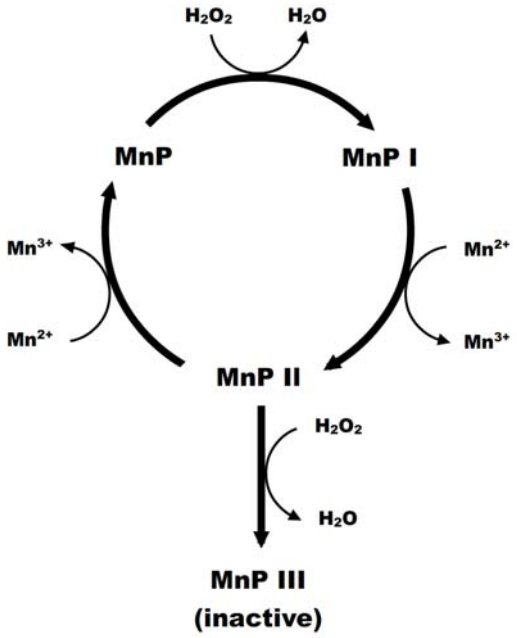

Figure 1-3. Scheme of the catalytic cycle of MnP

The initial oxidation of MnP by H_2O_2 or an organic peroxide conducts to an intermediate compound I which is a Fe^{4+}-oxo-porphyrin-radical complex and one water molecule is expelled. Subsequent reduction proceeds through MnP Compound II (Fe^{4+}-oxo-porphyrin complex). A monochelated 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. Compound I of MnP resembles that of LiP and HRP and can be reduced by both Mn^{2+} and other electron donors such as ferrocyanide and phenolic compounds, whereas compound II is only very slowly reduced by other substrates and requires Mn^{2+} to complete the catalytic cycle (Wariishi et al. 1988). 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.

Mn^{+3} ions are quite unstable in aqueous media. To overcome this drawback they can be stabilized by organic acids (Fig. 1-4), such as oxalic and malonic acid, and the Mn^{+3}-organic acid complex formed acts as a low-molecular mass, strong diffusing oxidizer (1.54 V) that attacks organic molecules non-specifically at locations remote from the enzyme active site (Kishi et al. 1994; Kuan and Tien 1993). Organic acids were also described to play an important role in the interaction

of manganese ions at the active site of the enzyme. They might facilitate Mn^{2+} oxidation and release of Mn^{3+} from the enzyme (Kishi et al. 1994; Wariishi et al. 1992). Additionally, chelators were suggested to reduce the ability of Mn^{3+} to oxidatively decompose H_2O_2 (Aitken and Irvine 1990). The value of this enzyme is supported by its capability to degrade a great variety of complex compounds (Kuan et al. 1993; Martínez 2002).

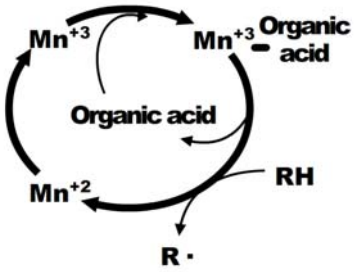

Figure 1-4. Formation of Mn^{3+}-organic acid complex

Despite the MnP/Mn^{2+} couple is not able to oxidize non-phenolic compounds as LiP does, several studies expanded the role of MnP in lignin biodegradation via thiol and lipid-derived free radicals that are able to oxidize a variety of non-phenolic aromatic compounds (Bao et al. 1994; Wariishi et al. 1989). These compounds which act as mediators increasing the oxidative strength of MnP, can be unsaturated fatty acids and their derivatives (e. g. Tween 80, linoleic acid) or organic sulphur compounds (e. g. reduced glutathione, L-cystein) forming particularly reactive peroxyl and thiyl radicals, respectively (Bermek et al. 2002; Jensen et al. 1996; Kapich et al. 1999; Moen and Hammel 1994).

Versatile peroxidase

VP has been discovered in *Pleurotus* and *Bjerkandera* species (Martínez et al. 1996; Mester and Field 1998). VP is able to oxidize both MnP and LiP substrates and therefore can be considered a hybrid between both enzymes. It has high affinity for Mn^{2+}, hydroquinones and dyes, and also oxidizes veratryl alcohol, dimethoxybenzene and lignin dimers. However, its catalytic efficiency in presence of Mn^{2+} is much higher than in presence or other aromatic substrates (Heinfling et al. 1998a). Its optimal pH for oxidation of Mn^{2+} (pH 5) and aromatic compounds and dyes (pH 3) differ, being similar to those of optimal MnP and LiP activity (Ruiz-Dueñas et al. 2001). Moreover, the presence of Mn^{2+} at moderate concentrations (0.1 mM) was demonstrated to severally inhibit oxidation of LiP substrates, (Mester and Field 1998). A non-competitive inhibition was proposed for both substrates, which means that VP has, at least, two binding sites (Heinfling et al. 1998a; Martínez 2002). Although the peroxidase from *B.* sp BOS55 has been described as

VP (Ruiz-Dueñas et al. 2001), the conditions used for the application of the enzyme in the present work are the most favorable for the oxidation of MnP substrates (pH 4.5 and presence of Mn^{2+}), therefore this enzyme would be named as MnP in the subsequent chapters.

A comparison of the general molecular structure of both peroxidases is shown in Fig. 1-4. The helices are represented as cylinders named following CCP nomenclature and the positions of the heme groups, calcium ions, manganous ions, C and N termini are highlighted. The main differences correspond to the length of the C terminal tails, a loop present in MnP and an additional helice for VP. A detailed description of the these differences in both structures is given by Martínez (2002).

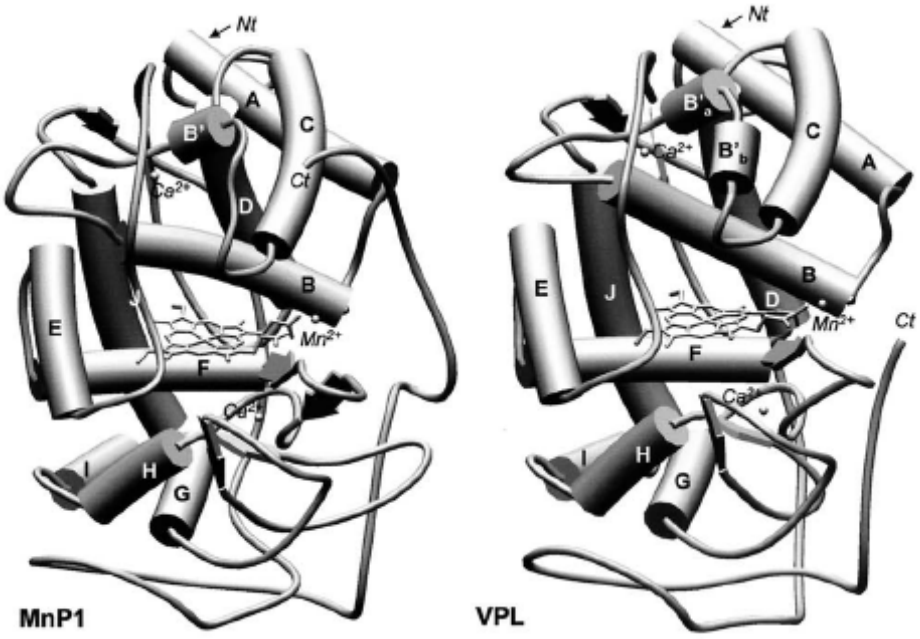

Figure 1-4. Schematic representations of the complete MnP1 and VPL polypeptide chains obtained from Martínez (2002).

1.6. In vitro degradation of poorly soluble compounds by ligninolytic peroxidases

Ligninolytic peroxidases have been traditionally used for the degradation of organopollutants, xenobiotics and industrial contaminants, such as dyes, phenols, PAHs, insecticides or nitroaromatic compounds as well for biopulping and biobleaching in the paper industry (Cohen et al. 2002; Pointing 2001). Some of these applications are summarized in Table 1-6.

Table 1-6. *In vitro* degradation of organopollutants by ligninolytic enzymes (from (Pointing 2001))

Organopollutant	Enzyme	Specie	Reference
TNT	MnP	*Nematoloma forwardii*	Scheibner and Hofrichter 1998
	MnP	*Phlebia radiate*	Van Aken et al. 1999
Organochlorines	LiP and MnP	*P. chrysosporium*	Valli et al. 1992
Polychlorinated biphenyls	Lac	*Trametes versicolor*	Dec and Bollag 1995; Roper et al. 1995
Bleach-plant effluent	MnP	*P. chrysosporium*	Jaspers et al. 1994
	Lac	*T. versicolor*	Archibald et al. 1990
Synthetic dyes	LiP	*P. chrysosporium*	Cripps et al. 1990
	MnP	*B. adusta P. chrysosporium*	Heinfling et al. 1998b
	MnP	*B. sp BOS55*	Mielgo et al. 2003a; López et al. 2004
PAHs	LiP	*P. chrysosporium*	Hammel et al. 1986; Haemmerli 1988; Bumpus 1989
	LiP	*N. forwardii*	Günther et al. 1998
	MnP	*P. chrysosporium*	Bogan and Lamar 1995; Bogan and Lamar 1996; Bogan et al. 1996a; Bogan et al. 1996b
	MnP	*N. forwardii*	Günther et al. 1998
	Lac	*T. versicolor*	Collins et al. 1996; Johannes et al. 1996 Majcherczyk et al. 1998
	Lac	*Coriolopsis gallica*	Pickard et al. 1999

In the specific case of poorly soluble compounds, the *in vitro* degradation requires the presence of a compound which makes more available the substrate to the enzyme. The use of solvents, surfactants and reverse micelles are the most extended systems to reduce mass transfer limitations in enzymatic reactors.

Enzymatic reactors with surfactants

The use of surfactants in microbial bioreactors has been discussed previously. They can also be used in enzymatic reactors in order to improve the solubility of the substrate (Bogan et al. 1996a). In the case of surfactants containing unsaturated fatty acids, such as Tween 80 and Tween 85, they could have a stimulating effect due to lipid peroxidation via formation of peroxyl radicals which would increase the extent of degradation (Steffen et al. 2003). However, Kotterman et al. (1998b) did not find evidence of lipid peroxidation when using a fully saturated lipid surfactant, polyoxyethylene 10 lauryl ether.

Enzymatic reactors in media containing solvents

From a classical point of view, it is difficult to visualize enzymes catalyzing reactions in presence of organic solvents, because their addition has been traditionally performed for enzyme precipitation or denaturation. This simplistic notion is wrong since many enzymes, including lipases, esterases or dehydrogenases function in natural hydrophobic environments (Dordick 1989). It is not surprising, then, that enzymes can be catalytically active in organic solvents systems. In actual fact, enzymatic catalysis in organic solvents has undergone rapid expansion in the last decades, opening a new field of biotechnological applications of proteins (Dordick 1989; Khmelnitsky et al. 1988). The use of organic solvents presents as a major advantage the increased solubilization of hydrophobic pollutants or their degradation products and also it prevents from bacterial contamination.

Although enzymatic catalysis in organic solvents is believed to be a promising approach in a decontamination approach, most of the work reported is related to hydrolytic enzymes (Takamoto et al. 2001; Wehtje and Adlercreutz 1997; Zaks and Klibanov 1988). The potential of using more complex enzymes as the ligninolytic ones, which require specific substrates and cofactors for the catalytic cycle, is almost untapped (Field et al. 1996).

Miscible solvents: The most important criterion in selecting a miscible solvent is its compatibility with maintenance of enzymatic activity. Hydrophilic solvents have a greater tendency to strip bound water from enzyme molecules (Klibanov 2001), therefore it is expected higher enzymatic inactivation than that with immiscible organic solvents. The following works utilizing miscible organic solvents for the *in vitro* degradation of PAHs are provided as examples: Baborova et al. 2006; Bogan

and Lamar 1996; Field et al. 1995; Günther et al. 1998; Sack et al. 1997; Torres et al. 1997; Wang et al. 2003.

Immiscible solvents: The enzyme can be retained in the aqueous phase of a biphasic system, inside reverse micelles or finally used as insoluble catalyst in nearly anhydrous media. In the latter, no aqueous phase is present, and water content of the enzyme, as well as biocatalyst preparation and properties of the organic solvent are the main factors that affect enzymatic catalysis in monophasic anhydrous solvents (Dordick 1989; Khmelnitsky et al. 1988). The application of this technology is focused to favor synthesis of single compounds, increase solubility of a reactant as well as to reduce side reactions. However, there are no references of the environmental application of enzymes in anhydrous solvents.

In biphasic reactors the substrate is located mostly in the immiscible phase and diffuses to the aqueous phase. The enzyme catalyzes conversion of the substrate at the interface and/or in the aqueous phase. The design of a biphasic reactor requires as a critical consideration the solvent selection, which should be non toxic for the enzyme. Moreover, it should present suitable physical and chemical properties (be immiscible, non-volatile, etc.), low cost and easy availability (MacLeod and Daugulis 2003). In the last years biphasic enzymatic reactors have been applied for synthesis of compounds, having the substrates or products low water solubility, as well for resolution of racemic mixtures (Baldascini and Janssen 2005; D'cunha et al. 1994; Hickel et al. 2001; Mandenius et al. 1988; Patel et al. 1992; Sakaki and Itoh 2003). The use of ionic liquid/supercritical carbon dioxide for enzyme-catalyzed transformation is gaining attention (Lozano et al. 2004). However, the application of biphasic reactors for *in vitro* degradation of environmental pollutants is still lacking.

Reverse micelles are spherical aggregates of water and a surfactant dispersed in a nonpolar solvent, which protect the enzyme from the solvent. Structurally water forms a microdroplet surrounded by a monolayer of surfactant molecules arranged with their polar heads towards the water pool and their hydrophobic tails in contact with the bulk nonpolar solvent. This technology belongs to the most promising non-aqueous biocatalytic systems owing to their essential advantages, such as versatility (to date, several enzymes have been shown to retain catalytic activity in reversed micelles), almost complete absence of diffusion limitations, optical transparency and ease of preparation (Carvalho and Cabral 2001; Khmelnitsky et al. 1992). Recovery of products and regeneration of enzyme are the main drawbacks of using reversed micelles. The presence of surfactant makes extraction or distillation procedures extremely difficult due to foaming and emulsion formation. MnP has been entrapped in reversed micelles and catalytic features of the complex were characterized (Michizoe et al. 2003; Okazaki et al. 2001). However its application in enzymatic reactor for the degradation of poorly soluble

compounds has not been carried out. A review from (Fadnavis and Deshpande 2002) discusses various applications of enzymes entrapped in reverse micelles for resolution of amino acids, peptide synthesis, reduction of prochiral ketones, synthesis of glycerides and chiral intermediates useful in production of agrochemicals and pharmaceuticals.

1.5. Objectives

In the present Thesis, the treatment of poorly soluble compounds has been considered, selecting PAHs as models due to their recalcitrant behavior and toxicity. The use of solvents in enzymatic reactors to increase PAH availability and hence their degradation by MnP is the general objective of this work. Two technologies have been selected for removal of PAHs in enzymatic reactors: the first consists in a reactor in media containing water:miscible solvent mixtures (chapter 2 to 5). The optimization of the process is the major goal for this approach. The second technology is a biphasic reactor using MnP as catalyst (chapter 6). This is the first attempt of degradation of PAHs in an enzymatic biphasic reactor.

The specific objectives to achieve the general goal can be described as follows:

i) Selection of an adequate solvent, miscible or immiscible, fulfilling the requirements for its application in a monophasic or biphasic reactor;

ii) Study of the effect of the main catalytic parameters on the system efficiency;

iii) Study of the effect of operational parameters, such as temperature, pH, agitation, on the system efficiency; and

iv) Study of the enzymatic kinetics and development of the process model for a controlled operation of the system.

1.6. References

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Chapter 2

Selection of a miscible organic solvent for the degradation of anthracene by MnP from *Bjerkandera* sp. BOS55 and *Phanerochaete chrysosporium*[1]

Summary

The goal of this study is the selection of the adequate solvent for the degradation of anthracene by MnP in monophasic systems. Four water-miscible organic solvents (acetone, methyl-ethyl-ketone, methanol and ethanol) were considered. Two main characteristics were evaluated: solubility of anthracene and stability of MnP in presence of the organic solvent. MnP from two different white-rot fungi were tested. The enzyme obtained from *Bjerkandera* sp. BOS55 was more stable than MnP from *Phanerochaete chrysosporium*. Considering a compromise solution between maximum solubilization of anthracene and minimum loss of MnP activity, acetone was selected as the best cosolvent, allowing to enhance 140-fold the anthracene solubility with acetone concentration of 36% (v/v), and permitting a high stability of the enzyme in long-term incubations. Furthermore, low concentrations of acetone (below 5%) were not toxic to aerobic and anaerobic cultures.

[1] Part of this chapter has been published as:

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2.1. Introduction

An increased solubilization of polyaromatics in aqueous media would have beneficial effects on the potential degradation of these compounds (Bumpus 1989; Cerniglia and Heitkamp 1984; Kilbane 1997). A good approach to enhance PAHs solubility in several orders of magnitude is the addition of water-miscible cosolvents or surfactants (Field et al. 1995; Lee et al. 2001; Zheng and Obbard 2002). The use of the latter compounds may result into low solubilization of PAHs and partial inhibition of the ligninolytic activity (Kotterman et al. 1998). Organic solvents, in enzymatic catalysis, have been mainly applied for synthesis of organic compounds, and most of the works are related to hydrolytic enzymes (Klibanov 2001; Zaks and Klibanov 1988). Although the use of solvents for decontamination is considered a promising approach, the application of complex enzymes, such as ligninolytic enzymes produced by white rot fungi requiring specific environmental conditions for activation of their catalytic cycle, in media containing organic solvents is almost untapped.

The use of water miscible organic solvents has several advantages when compared with other systems. In monophasic reactors containing hydrophobic solvents, enzymes in nearly dry conditions have to be solubilized by modification with amphipathic compounds, lipids or surfactants (Dordick 1989; Khmelnitsky et al. 1988; Vazquez-Duhalt et al. 1992). In biphasic systems, diffusional resistance for substrates and products across the water-organic solvent interface may be a major problem (Ogino and Ishikawa 2001). Finally, the use of miscible solvents can prevent from bacterial contamination.

The choice of an organic solvent for a given reaction should be based on three factors: i) ecological toxicity of the solvent; ii) effects of the solvent on the reaction (including substrate solubility); and iii) effect of the solvent on biocatalyst stability.

In monophasic systems, the enzymatic activity loss has been mainly attributed to the fact that water molecules in the enzyme are stripped away or replaced with solvent molecules causing deformation and enzyme denaturation (Bell et al. 1995; Gorman and Dordick 1992; Schulze and Klibanov 1991). According to that, hydrophobic solvents affect enzymatic activity in a lower extent. Laane et al. (1987b) found a quantitative correlation between the hydrophobicity of the solvent and the activity retention of the biocatalyst: solvents with high values of log K_{OW} (partition coefficient between water and n-octanol) are more favorable for enzymatic activity of different biocatalysts. Other authors reported similar conclusions: Khmelnitsky et al. (1988) indicated that one solvent has more favorable effect on enzyme activity provided that it is able to preserve the solvophobic interactions, essential for the native structure of the enzyme. Girard

and Legoy (1999) studied the influence of miscible organic solvents on the activity and stability of dextransucrase, obtaining similar results of enzyme inactivation for acetone and ethanol and concluding that a correlation could be derived from the effect of organic solvents and log K_{OW}. However, occasional discrepancies have been reported, and were rationalized by using an additional parameter, water solubility, which is not a direct function of log K_{OW} (Gupta 1992).

The goal of this work is the evaluation of use of MnP for the degradation of anthracene, selected as a model compound, in water-miscible organic solvents. Anthracene, a three-ring PAH, was chosen due to its low aqueous solubility: 0.07 mg/L (Mackay and Shiu 1977). Moreover, this compound has been proved to be degraded by ligninolytic peroxidases (Hammel et al. 1986). The first stage of the process was the selection of the most appropriate cosolvent from a list of four relatively safe, easily available, fairly inexpensive chemicals and presenting relatively low environmental toxicity: acetone, methyl-ethyl-ketone (MEK), methanol and ethanol. The influence of the solvent on the anthracene solubility and its effect on MnP activity were used as criteria for this selection.

2.2. Materials and methods

2.2.1. Enzymes

MnP was obtained from two metabolically distinct white-rot fungi, *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) and *Bjerkandera* sp. BOS55 (ATCC 90940), with different catalytic properties. The latter presents a superior resistance against high H_2O_2 concentrations (Palma et al. 1997). *P. chrysosporium* was cultured in 250-mL Erlenmeyer flasks on N-limited BIII medium (Tien and Kirk 1988). *B.* sp. BOS55 was grown in a 10-L fermenter (Braun-Biotech International) on skimmed cheese whey medium (Moreira et al. 2001). Once the peak production of MnP was detected, fermentation was stopped. Crude enzyme was concentrated by ultrafiltration using a 10-kDa cut-off type YM-10 membrane (Amicon), and then it was centrifuged for 10 min at 20,000 × g.

2.2.2. Chemicals

Anthracene and anthraquinone were obtained from Janssen Chimica (99% purity). Acetone, methanol and ethanol were purchased from Panreac (chemical purity); methyl-ethyl-ketone was supplied by Sigma-Aldrich (99.5% purity).

2.2.3. Anthracene solubility assays

The solubility of anthracene was determined in 20-mL aliquots containing 25 mg anthracene (final concentration of 1.25 mg/L) with different concentrations of solvent ranging from 1% to 100%. The aliquots were placed in 100-mL Erlenmeyer

flasks sealed with Teflon plugs in triplicate, equilibrated for 24 h on a shaker (150 rpm) at 20 or 30ºC (± 0.1ºC). The flasks were weighted after 24 h to check solvent volatilization and no differences were observed. Afterwards, 20-mL assays were filtered through a Millex-LCR13 cartridge (Millipore Corp.), with a pore diameter of 0.45 µm in order to remove non-dissolved anthracene. The filters, specially selected for solvents, do not adsorb anthracene. Samples were analyzed by high-pressure liquid chromatography (HPLC).

2.2.4. Inactivation of MnP by solvent:water mixtures

The inactivation of crude MnP from cultures of *B.* sp. BOS55 and *P. chrysosporium* was evaluated in water: solvent mixtures by monitorization of MnP activity. The assays were carried out at room temperature (22ºC±1ºC) in a total volume of 10 mL containing 10 mM sodium malonate (pH 4.5), solvent concentrations ranging from 0 to 90% (v:v) and crude MnP (100 U/L). Immediately after addition of the enzyme, a sample was withdrawn and MnP activity was spectrophotometrically determined.

2.2.5. MnP stability in solvent:water mixtures during long term incubations

The stability of crude MnP from cultures of *B.* sp. BOS55 and *P. chrysosporium* was evaluated in water: solvent mixtures by MnP activity monitorization at periodic intervals in long term incubations. Different experiments were performed considering three conditions: fixed concentration of the solvents, fixed solubilization of anthracene and variable concentrations of acetone.

The assays at a fixed concentration of the solvents (10% v:v) were carried out at room temperature (22ºC±1ºC) in a total volume of 10 mL containing 10 mM sodium malonate (pH 4.5) and MnP crude (100 U/L). The assays performed at a solvent concentration permitting solubilization of 10 mg/L of anthracene were carried out at identical experimental conditions to those of the previous experiment at two temperatures: 20ºC and 30ºC. The effect of variable concentrations of acetone were carried out at identical experimental conditions for solvent concentrations ranging from 0 to 90% (v:v).

2.2.6. Anaerobic and aerobic toxicity of acetone

Anaerobic toxicity of different mixtures acetone:water was determined by methanogenesis assays. The granular sludge used in this study came from an up-flow anaerobic sludge blanket bioreactor treating winery industry wastewater. The reactor had been operated for at least 2 years with an organic loading rate of 5 kg COD/m^3·d, prior to sludge sampling. The granular sludge had excellent

sedimentation characteristics, with an average diameter of 1.5 mm and a biomass concentration of 60 g/L of volatile suspended solids (VSS) and a specific methanogenic activity of 0.4 g COD_{CH4} /g (VSS) d. The sludge was stored at 4 ºC and washed two times with distilled water to remove residual soluble substrate before being used in the experiments. Methanogenesis assays are based on the production of methane during incubations at 30ºC and 170 rpm. Methanogenic activity measurements were conducted in 250 mL serum flasks. The anaerobic sludge (final assay concentration of 2 g VSS/L) was transferred to serum flasks with 100 mL of basal medium ABM (Table 2-1) and different proportions of acetone (from 1 to 10% v:v). $Na_2S{\cdot}9H_2O$ (100 mg/L) was added to remove dissolved oxygen. Final pH was adjusted to 7.5 ± 0.1. Before start-up, the headspace of the bottles was flushed with N_2/CO_2 (80:20) for 1 min. The flasks were tightly capped with a needle in the plug for gas sampling. Pressure was measured at periodic intervals and gaseous samples were withdrawn to determine the concentration of methane. Biological assays were carried out in duplicate.

Table 2-1. Composition of the anaerobic basal medium

Anaerobic sludge	2 g VSS/L		
$NaHCO_3$	200 mg/L		
VFA (acetic:propionic:butyric 4:1:1)	2 g COD/L		
Macronutrients solution	1 mL/L		
NH_4Cl	170 g/L	$CaCl_2{\cdot}2H_2O$	8 g/L
KH_2PO_4	37 g/L	$MgSO_4{\cdot}4H_2O$	9 g/L
Micronutrients solution	1 mL/L		
$FeCl_3{\cdot}4H_2O$	2 g/L	$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	90 mg/L
$CoCl_2{\cdot}6H_2O$	2 g/L	$Na_2SeO_3{\cdot}5H_2O$	100 mg/L
$MnCl_2{\cdot}4H_2O$	5 g/L	$NiCl_2{\cdot}6H_2O$	50 mg/L
$CuCl_2{\cdot}2H_2O$	30 mg/L	EDTA	1 g/L
$ZnCl_2$	50 mg/L	HCl 36%	1 mL/L
H_3BO_3	50 mg/L	Resazurine	500 mg/L

Aerobic toxicity of a medium containing 5% of acetone (v:v) was evaluated by the monitorization of oxygen consumption during 5 days by aerobic biomass comparing with a control without acetone. For that, aerobic sludge from the wastewater treatment plant of Silvouta (Santiago de Compostela) was used.

Sludge, with a concentration of 3.6 g VSS/L, was previously washed with phosphate buffer (10 mM pH 7) and stored at 4ºC. An Oxitop system (WTW, Germany) with a total volume of 510 mL and a sample volume of 97 mL was used to monitor the pressure inside the closed flasks, its decrease being proportional to oxygen consumption. The experiments were carried out in duplicate at 20°C and the sludge concentration was 0.05 g VSS/L in all the experiments.

2.2.7. Analytical determinations

MnP activity was measured spectrophotometrically by the oxidation of 2,6-dimethoxyphenol (2,6-DMP) to cerulignone, an orange-brown dimer, at 30ºC and 468 nm (Shimadzu UV-160, Kyoto). The reaction mixture (1 mL) contained a final concentration of 50 mM sodium malonate (pH 4.5), 1 mM DMP, 1 mM $NaSO_4$, 0.4 mM H_2O_2 and the sample. The reaction was initiated with the addition of H_2O_2. The molar extinction coefficient is 49600 $M^{-1}cm^{-1}$ (Wariishi et al. 1992). One unit of activity is defined as the amount which releases 1 µmol of the oxidation product per minute.

A HP 1090 HPLC, equipped with a diode array detector, a 4.6×200 mm Spherisorb ODS2 reverse phase column (5 µm; Waters) and a HP ChemStation data processor were used for determining the concentration of anthracene at a wavelength of 254 nm. The injection volume was set at 10 µL and the isocratic eluent (80% acetonitrile:20% water) was pumped at a rate of 1 mL/min. The calibration was performed with concentrations ranging from 0.1 to 10 mg/L of anthracene in acetone.

Pressure of gaseous samples from the anaerobic assays was measured by a differential pressure transducer 0–5 psi (Centrepoint Electronics). Biogas composition (CO_2, CH_4 and N_2) was measured using a Hewlett- Packard chromatograph model 5890 Series II, equipped with a TC detector.

2.3. Results and discussion

2.3.1. Solubility of anthracene in water: solvent mixtures

The solubility of anthracene in four water miscible solvents: acetone, methyl-ethyl-ketone (MEK), ethanol and methanol, was determined at 20 and 30ºC (Fig. 2-1). Identical amounts of anthracene were added to all samples leading to total solubilization of anthracene (1.25 g/L) at 100% solvent except for methanol at 20ºC which dissolved 0.84 g/L. Acetone attained total solubilization of anthracene at concentrations higher than 70% while alcohols attained lower solubilities, only being equivalent at 100% solvent. Methanol attained the lowest anthracene solubilization for all mixtures and temperatures. The addition of MEK provided the

highest anthracene solubility in a concentration range between 10 and 30% (v:v) in comparison with the other solvents. However, higher concentrations of MEK resulted in the formation of two differentiated phases: aqueous and non-aqueous, which impeded utilization of MEK as a water miscible solvent.

Table 2-2 shows the solvent concentrations required for the solubilization of 1, 10 and 100 mg/L of anthracene at 20 and 30ºC. The solubilization at 30ºC was slightly more beneficial for all cosolvents since it implied a reduction in the addition of the organic solvent between 7-12% in comparison with that required for 20ºC (36% acetone to dissolve 10 mg/L anthracene at 20ºC, whereas 33% acetone was required at 30ºC). The concentrations of the organic solvents attaining an anthracene solubilization of 10 mg/L -which represents 140-fold increase of the anthracene solubility in water at 25ºC- were the following: 27% MEK, 36% acetone, 44% ethanol and 55% methanol.

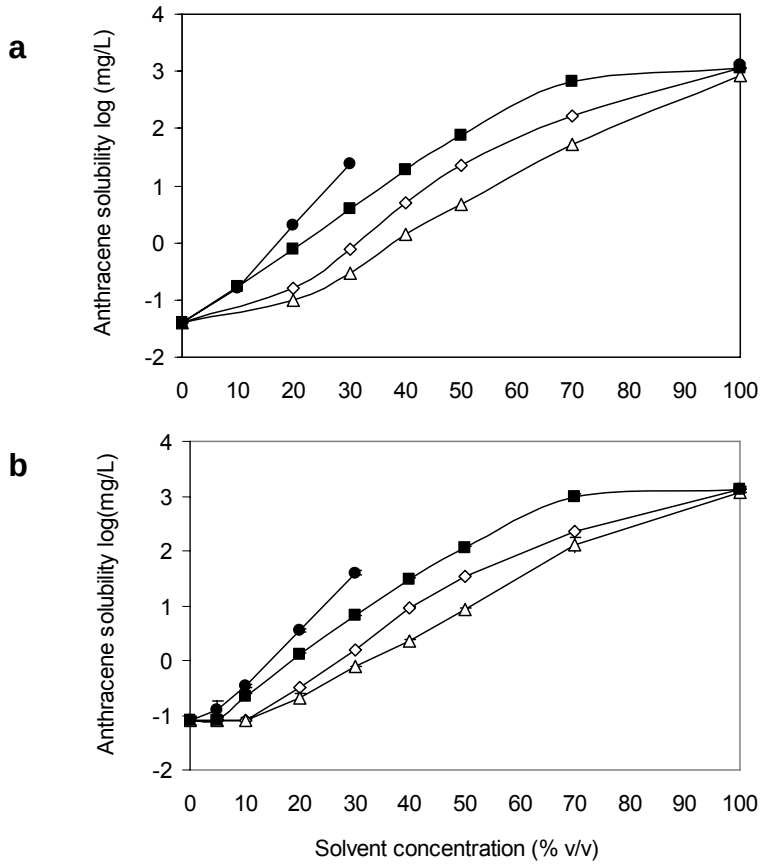

Figure 2-1. Anthracene solubility at 20 (a) and 30ºC (b) in solvent: water mixtures. Symbols: methyl-ethyl-ketone (●), acetone (■), ethanol (◇), methanol (△)

Table 2-2. Solvent concentration required for the solubilization of 1, 10 and 100 mg/L of anthracene

		Solvent concentration (%)		
Solvent	T (ºC)	1 mg/L	10 mg/L	100 mg/L
MEK	20	17	**27***	ND
	30	14	24	ND
Acetone	20	21	**36***	53
	30	19	33	49
Ethanol	20	31	**44***	64
	30	28	41	60
Methanol	20	37	**55***	76
	30	32	51	67

ND: not determined

*Solvent concentrations selected for the following experiments

Several authors studied the solubility of anthracene in organic solvents and in binary mixtures (Hansen et al. 2000; Jouyban et al. 2002; Powell et al. 1997), but there is little information about water:miscible solvent mixtures (Field et al. 1996). In this study MEK: water mixtures gave rise to the dissolution of major amounts of anthracene in the range 1-30% (v: v), followed by acetone, ethanol and, finally, methanol. Our results are in agreement with those of other authors: Cepeda and Diaz (1996) measured the solubility of anthracene in 3 solvents (isopropyl alcohol, MEK and acetonitrile), obtaining the highest solubility with MEK; Field et al. (1996) determined the solubility of anthracene in acetone: water mixtures at 20ºC, obtaining similar results to those presented in this work.

2.3.2. Inactivation of MnP in water:solvent mixtures

The short-term effect of solvent: water mixtures on the activity of crude MnP from *B.* sp. BOS55 and *P. chrysosporium* was evaluated. MnP activity was instantaneously determined after mixing the solvent mixtures with MnP (Fig. 2-2). As observed in the solubility experiments, MEK:water mixtures at fractions higher

than 25% (v:v) formed two different phases. Considering that the aim of this chapter was to study miscible solvents, only MEK:water mixtures with a fraction lower than 25% (v:v) were evaluated.

Regarding the experiments with MnP from *B.* sp. BOS55 (Fig. 2-2a), acetone:water mixtures maintained enzymatic activity at values near 100%, whereas proportions of methanol higher than 50% caused a sharp decay of the initial activity. Ethanol caused a slight decline of MnP activity, being more evident for ethanol proportions higher than 50%. The effect of MEK addition was found to be negligible for the range considered (1-30%).

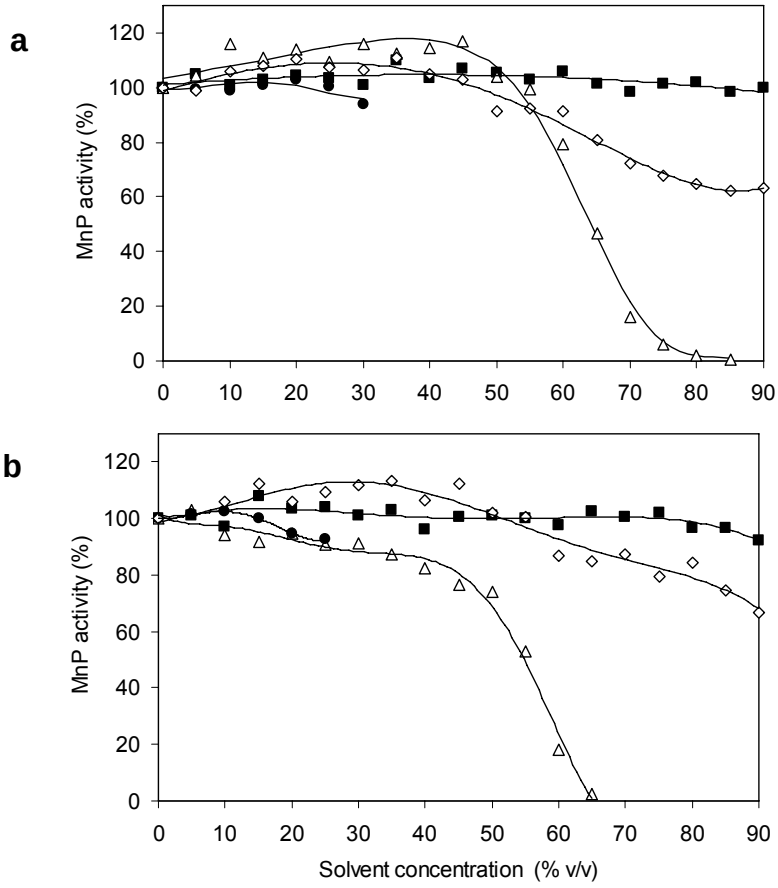

Figure 2-2. Inactivation of MnP from *Bjerkandera* sp. BOS55 (a) and *Phanerochaete chrysosporium* (b) in solvent: water mixtures at different concentrations. Symbols: methyl-ethyl-ketone (●), acetone (■), ethanol (◇), methanol (△).

Similar results were obtained for MnP from *P. chrysosporium* (Fig. 2-2b), where methanol even at lower volumes exerted a remarkable detrimental effect on MnP activity.

2.3.3. Long-term stability of MnP in water:solvent mixtures

The stability of MnP during long-term incubations was evaluated in a series of experiments with different proportions of solvent:water mixtures. Initially, the effect of the solvents on MnP activity at concentrations of 10% (v/v) was evaluated. Thereafter, the effect of the solvent concentration attaining anthracene solubilization up to 10 mg/L was studied. Finally, MnP stability in different proportions of acetone, from 0 to 90%, was evaluated.

MnP stability in the presence of 10% solvent (v:v)

10% (v:v) solvent:water mixtures were incubated for several days at 30ºC in order to determine the effect of the solvent on MnP from *P. chrysosporium* and *B*. sp. BOS55 (Fig. 2-3). The control experiment, performed without solvent, showed that MnP from *P. chrysosporium* was less stable than that from *B.* sp. BOS55. An activity loss of 10 and 48% of the initial activity of MnP from *P. chrysosporium* and *B.* sp. BOS55, respectively, was observed after 14 days of incubation. Moreover, the greatest inactivation of MnP from *P. chrysosporium* in all solvents occurred during the initial 24 h.

In experiments with MnP from *B.* sp. BOS55 all solvents had similar effect and only after 5 days, the acetone mixture maintained the enzyme stable (Fig.2-3a). In the case of MnP from *P. chrysosporium*, the presence of 10% of solvents such as ethanol or acetone produced an apparent stabilization of enzyme (Fig. 2-3b). The solvent which permitted better stability of MnP was ethanol and in fact, the enzyme in this medium maintained its activity 5.8-fold higher than the control after 15 days of incubation. On the other hand, the poorest stability occurred in presence of MEK mixtures.

Although it is not very usual that the presence of a solvent permitted a better stability of the enzyme, Khmelnitsky et al. (1988) reported numerous examples of enzyme activation by moderate concentration of solvents (10-30%), leading in some cases to strong activation effects of the enzyme (28-fold). This phenomenon was accounted for conformational changes in the enzyme molecule caused by introduction of the organic solvent into the system (Khmelnitsky et al. 1988). This effect was also described in recent works by Sana et al. (2006) and Liu et al. (2006).

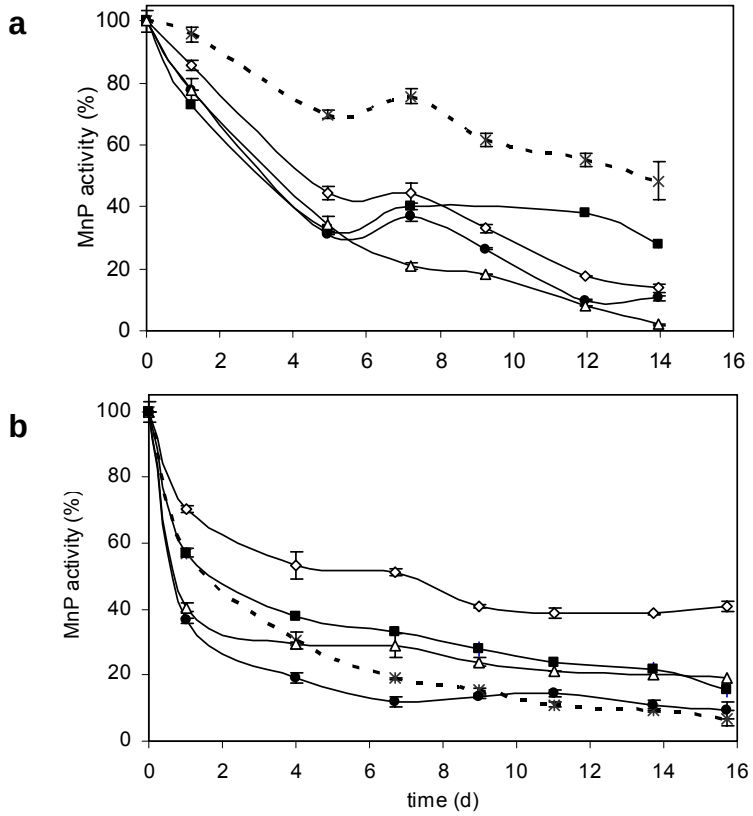

Figure 2-3. Stability of MnP incubations in solvent:water mixtures at 30ºC. MnP from *Bjerkandera* sp. BOS55 (a) and *Phanerochaete chrysosporium* (b). Symbols: control (×), methyl-ethyl-ketone (●), acetone (■), ethanol (◇), methanol (△)

MnP stability for a fixed concentration of anthracene (10 mg/L)

The stability of MnP was evaluated in prolonged incubations in the presence of solvents concentrations enabling to dissolve 10 mg/L of anthracene at 20ºC (bold values in Table 2-2). Figure 2-4 shows the MnP activity profile for the enzyme from *B.* sp. BOS55 (a) and *P. chrysosporium* (b).

The inactivation strength of the solvents on MnP from *B.* sp. BOS55 in decreasing order was: methanol, ethanol, MEK and acetone (Fig. 2-4a). Furthermore, a control assay performed in the absence of solvent maintained its initial MnP activity after 24 h. Control and acetone mixture followed similar trends and thus, at the end of the experiment, acetone mixture activity was 97% of the control activity. On the contrary, a remarkable deactivation of the enzyme was found in methanol: water mixtures. This instability is time-dependent leading to irreversible loss of enzymatic activity after only 20 min.

Similar assays were carried out to determine the stability of MnP from *P. chrysosporium* cultures (Fig. 2-4b), which was proved to be more affected than MnP incubations from *B.* sp. BOS55. Methanol also exerted significant inactivation, being already evident after the first minutes of incubation. Acetone mixture turned out to be the best solvent, in terms of enzyme inactivation, although a pronounced decrease in comparison with that of the control (48 %) was observed.

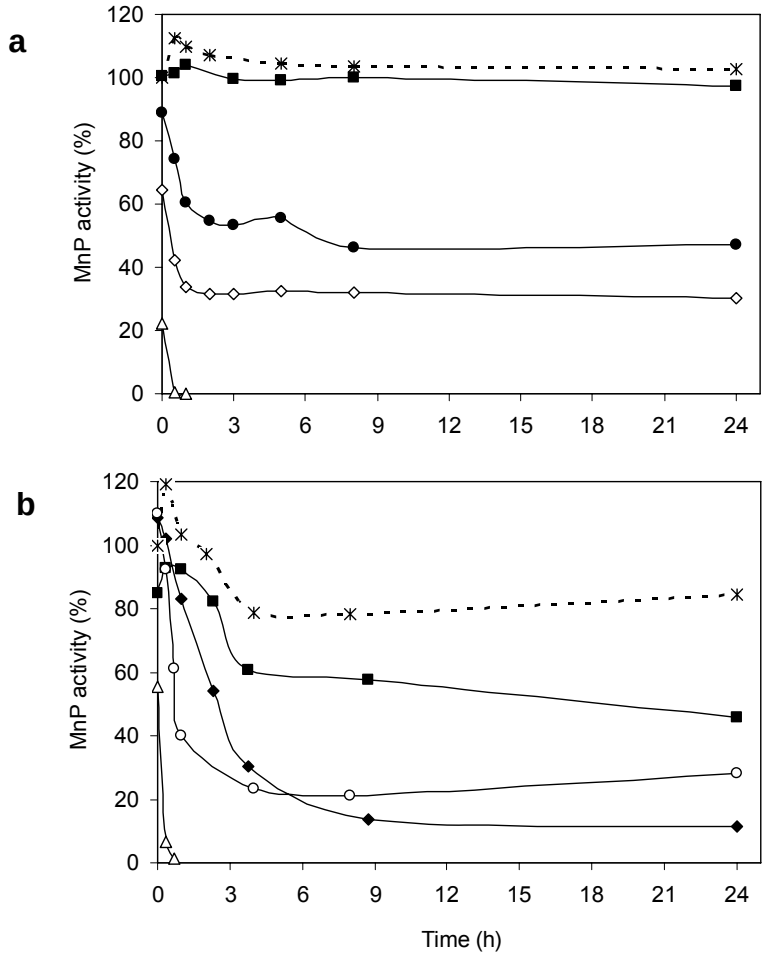

Figure 2-4. Stability of MnP incubations in solvent:water mixtures at room temperature. MnP from *Bjerkandera* sp. BOS55 (a) and *Phanerochaete chrysosporium* (b). Symbols: control (×), methyl-ethyl-ketone (●), acetone (■), ethanol (◇), methanol (△)

At 30ºC the rate of inactivation was higher in all cases and the deactivating action of solvents followed a similar trend (Fig. 2-5).

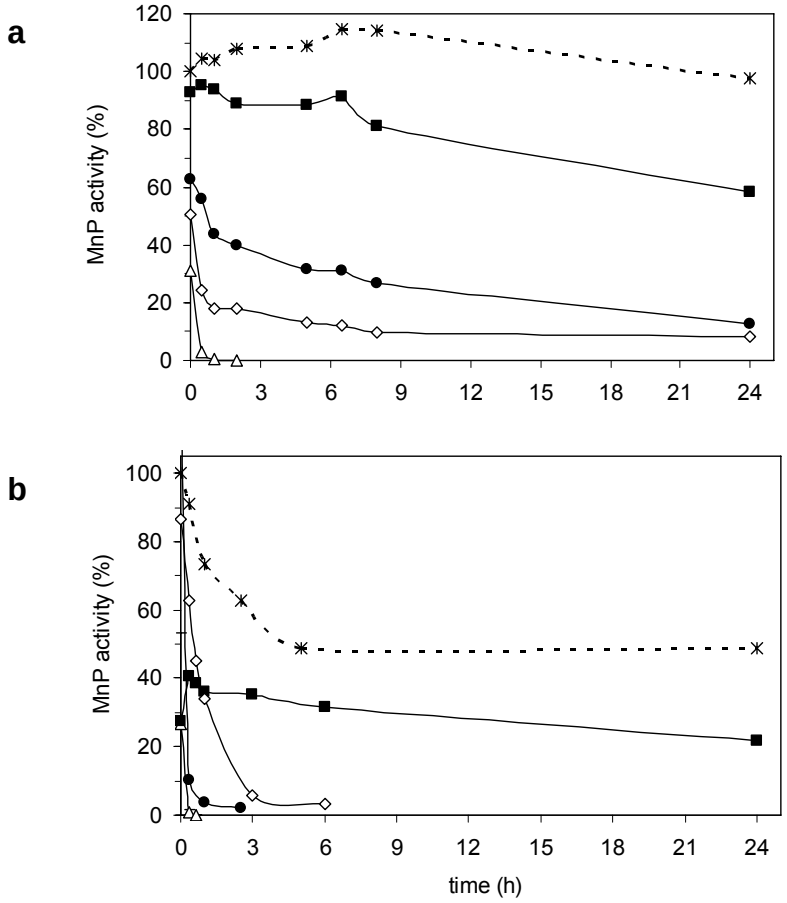

Figure 2-5. Stability of MnP incubations in solvent:water mixtures at 30ºC. MnP from *Bjerkandera* sp. BOS55 (a) and *Phanerochaete chrysosporium* (b). Symbols: control (×), methyl-ethyl-ketone (●), acetone (■), ethanol (◇), methanol (△)

The enzyme stability in the organic solvents seems not to be directly dependent on the water content, since the content of water for acetone was higher than that for MEK and lower for ethanol and methanol. In monophasic systems, loss of enzymatic activity has been mainly attributed to the fact that water molecules in the enzyme are stripped away or replaced by solvent molecules causing deformation and denaturation of the enzyme (Gorman and Dordick 1992; Schulze and Klibanov 1991). Laane et al. (1987a) also found a quantitative correlation between the hydrophobicity of the solvent and the activity retention of the

biocatalyst. Therefore, solvents with high values of water and n-octanol partition coefficient (K_{OW}) are more favorable for preserving enzymatic activity. Methanol, the most hydrophilic solvent (log K_{OW}: -0.72), caused stronger inactivation of MnP than ethanol (log K_{OW}: -0.19), acetone (log K_{OW}: -0.16) and MEK (log K_{OW}: 0.37). Whereas MEK, the solvent with the highest hydrophobicity, caused higher enzyme inactivation than acetone.

For those reasons it is important to take into account other characteristics of solvents that may influence on enzyme stability. Gorjup et al. (1999) studied the influence of 102 compounds (most of them, organic solvents) on lignin peroxidase (LiP) deactivation, evaluating 16 solvent property parameters such as log K_{OW}, dielectric constant, refractive index, dipole moment, surface tension, etc. The analysis showed that no single property of solvents explains their influence on peroxidase activity. The solvent influence is complex, but hydrogen bonding and anion stabilization seem particularly important. The physical properties of the solvents studied in this paper are too similar to come a conclusion.

Acetone was selected as the most appropriate solvent as it attained both higher solubilization of anthracene and minimal MnP deactivation. MnP from cultures of *B.* sp. BOS55 had been described to present superior resistance to hydrogen peroxide (Palma et al. 1997). The results presented in this chapter suggested that it is also more tolerant to solvent:water mixtures than the enzyme from *P. chrysosporium*. Taking this into account, the following experiments were carried out with MnP from *B*. sp.

Incubations of acetone:water

With the aim of a better knowledge into the inactivation caused by acetone to MnP from *B.* sp. BOS55, long-term incubations in mixtures with acetone were assayed. The concentration of solvent ranged from 0 to 90% (step 10%) and some activity profiles are shown in Fig. 2-6.

The inactivation produced by 90% of solvent was very low (at 22 h the enzyme in the mixture maintained 90% of the activity related to the control). Therefore, it can be concluded that MnP from *B.* sp. BOS55 is quite stable in acetone:water mixtures. In literature, total inactivation of dissolved enzymes, at concentrations of organic cosolvent exceeding 80-90 volume percent, was avoided only in few cases when a favorable combination of the specific properties of a particular enzyme and cosolvent was found (Khmelnitsky et al. 1988; Vázquez-Duhalt et al. 1993).

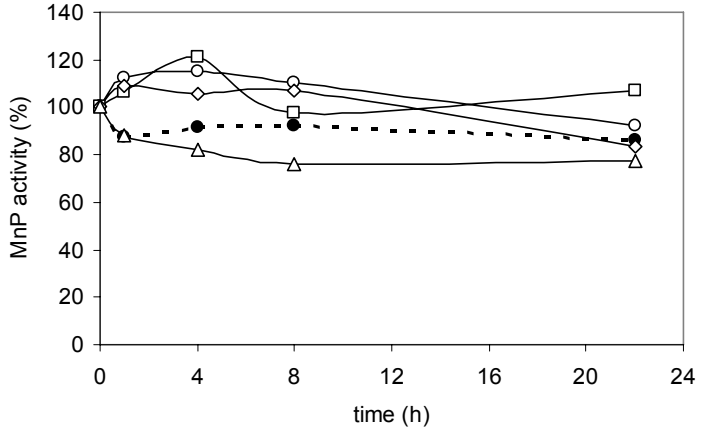

Figure 2-6. Stability of MnP incubations in acetone:water mixtures at room temperature. MnP from *Bjerkandera* sp. BOS55. Symbols: control (●), 20% (o), 50% (□), 70% (◇), 90% (△) acetone:water (v:v)

2.3.4. Toxicity of acetone in aerobic and anaerobic cultures

Acetone (36% v:v) was selected for the enzymatic treatment of anthracene due to its good characteristics in terms of solubility of anthracene and stability of enzyme. However, the use of solvents for environmental processes may be constrained by its biodegradability. Non-biodegradable solvents should be avoided since they could constitute a risk for the environment. For these reasons, acetone toxicity was evaluated in both anaerobic and aerobic cultures and its biodegradability is discussed.

Anaerobic toxicity

Experiments in order to check the toxicity of acetone on anaerobic populations were carried out. The production of CH_4 was measured during the time course of the experiment, and once stabilized, a second addition of volatile fatty acids (VFAs) was made to test the adaptation of the culture (Fig. 2-7).

10% of acetone inhibited completely methanogenic activity of the bacteria, even after the second addition of VFAs. 5% of acetone slowed down methane production, but the total production after 4 d was the same as the produced by the control. 0.5% and 1% of acetone run parallel to the control, which indicated no inhibition of the methanogenic cultures. The second addition did not show culture adaptation to acetone, because methane production in the media with 5% of acetone was also slower than control.

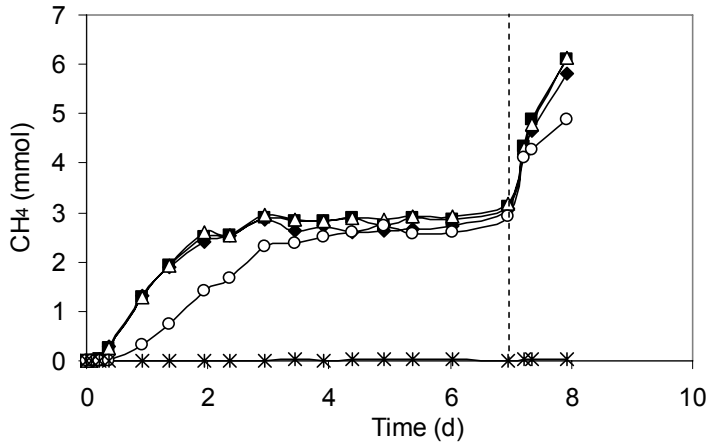

Figure 2-7. Toxicity of acetone at different concentrations in anaerobic cultures. Symbols: 0% (◆) 0.5% ■, 1% △, 5% ○, 10% ✳. Discontinuous line shows the time when the second addition of VFAs was added.

As there was a strong inhibition in the range 5% to 10% of acetone, intermediate concentrations of acetone were studied: 6, 7, 8 and 9% of acetone (Fig. 2-8). All acetone concentrations inhibited methane production, but concentrations higher than 8% completely inhibited the bacterial cultures.

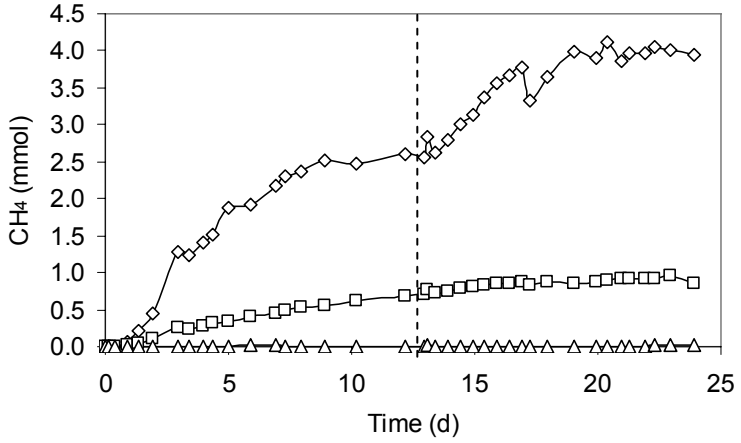

Figure 2-8. Toxicity of acetone at different concentrations in anaerobic cultures. Symbols: 6% ◇, 7% □, 8% △. Discontinuous line shows the time when the second addition of VFAs was added.

For concentrations of acetone around 5%, the volume of methane produced was

similar to the control. Therefore, 5% of acetone was considered as the maximum amount of solvent that can be released to an anaerobic treatment plant.

Regarding the anaerobic biodegradability of acetone, studies with several different strains of anaerobic bacteria from municipal waste water treatment plants have shown that acetone is degraded to CO_2 following aceto-acetate formation through an initial carboxylation reaction and incorporated into the carbon cycle (Platen and Schink 1989).

Aerobic toxicity

The effect of 5% of acetone was also tested in aerobic cultures. In this case, the oxygen consumption in two media (a control experiment, without acetone and a experiment with 5% of acetone) was measured (Fig. 2-9). This fraction of acetone led to a partial inhibition of the culture after the third day. In terms of activity, the sludge with 5% of acetone had an inhibition value of 44%.

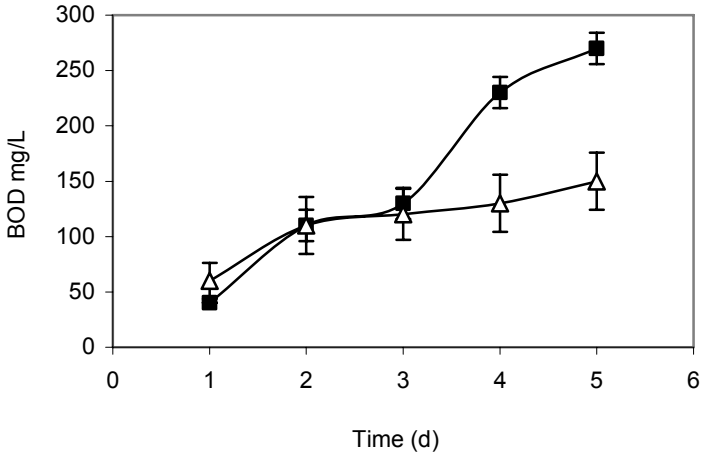

Figure 2-9. BOD5 assays for media with 5% of acetone (△) and in absence of acetone (■)

The reported values of EC_{50} (concentration of a substance that causes a 50% reduction in oxygen uptake by the micro-organisms) for acetone to activated sludge differ depending on the source of sludge. The higher EC_{50} values reported in the literature corresponded to municipal sludge and the average value was 7.7% (Kilroy and Gray 1992).

Biodegradability studies on acetone (10 mg/L) indicated a ready degradation after an initial lag period of 2 days (Young et al. 1968). Acetone meets the OECD definition of readily biodegradable which requires that the biological oxygen demand (BOD) is at least 70% of the theoretical oxygen demand within the 28-day test

period. Studies by the standard dilution method have shown greater than 75% of the acetone is biodegraded when using non-acclimated sewage sludge from either a freshwater or a sea water sanitary waste treatment plant (Price et al. 1974). These results compare favorably with the values from biodegradability tests performed according to OECD 301D guidelines. Using the OECD method, the BOD5, BOD15, and BOD28 for acetone were found to be 14%, 74%, and 74%, respectively (Waggy et al. 1994).

In conclusion, the effluent from the enzymatic reactor, which contains 36% of acetone, should be diluted with other effluents of the plant in order to reduce its concentration to values below 5% (v:v).

2.4. Conclusions

Enzymes in organic media can afford many advantages such as the oxidation of poorly soluble compounds which increase their bioavailability by using cosolvents. However, the nature of solvents influences the activity and stability of enzymes and consequently, the presence of organic solvents always constitutes a risk of enzyme inactivation. The use of water-miscible solvents was first considered since mass transfer limitations are avoided in monophasic systems.

The selection of an adequate miscible organic solvent was based according to three criteria: i) enhanced solubility of anthracene, ii) stability of MnP in their mixtures and iii) toxicity of the solvent. Four solvents, acetone, MEK, ethanol and methanol, were pre-selected taking into account that they are easily available, safe, relatively inexpensive and with low environmental toxicity.

Although MEK permitted the highest solubility of anthracene in the range 1 to 30% (v:v), proportions over this value led to formation of two phases. Acetone followed MEK in terms of anthracene solubilization capacity, whereas methanol was the solvent dissolving less anthracene. Increasing the temperature from 20 to 30ºC implied a reduction of the organic solvent between 7-12% for a certain concentration of anthracene dissolved.

Regarding MnP inactivation in solvent:water mixtures, short-term experiments showed that methanol (and ethanol, but to a lesser extent) produced an immediate inactivation of MnP at fractions higher than 50% (v:v). It was quite unexpected that long-term stability experiments at 10% ethanol or acetone led to activation of MnP from *P. chrysosporium*. However, MnP from *B.* sp. BOS55 suffered inactivation, similar for all mixtures at 10% solvent. A great difference was observed in long-term experiments at solvent fractions dissolving 10 mg/L of anthracene. In this case, MnP from *B.* sp BOS55 run parallel to the control for 24 h in mixtures with acetone. MnP from *P. chrysosporium* was also more stable in acetone mixtures. MnP

from cultures of *B.* sp. BOS55 was more stable than the enzyme from *P. chrysosporium*. The inactivation effect of acetone mixtures is very low since incubations of enzyme in medium containing 90% of acetone for 22 h confirmed that MnP was scarcely deactivated.

Acetone was selected as the most appropriate solvent as it attained both higher solubilization of anthracene and minimal MnP deactivation. The environmental risks of using acetone were checked by means of anaerobic and aerobic toxicity assays. From this study, we can conclude that the effluent from the enzymatic reactor containing 36% of acetone should be diluted with other streams not to have a detrimental effect on bacterial cultures. Below this threshold value, biodegradability studies have demonstrated that acetone is readily biodegradable by both aerobic and anaerobic cultures.

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Chapter 3

In vitro degradation of anthracene by MnP in batch reactors containing acetone:water mixtures[2]

Summary

The *in vitro* degradation of anthracene by MnP in batch reactors containing acetone:water mixtures was investigated for different concentrations of the main cofactors and substrates that affect the catalytic cycle of MnP (Mn^{2+}, H_2O_2 and organic acids) as well as for other environmental parameters (temperature, air/oxygen atmosphere and light/dark conditions). The optimization of these parameters was carried out in terms of efficiency, having into account not only the extent of degradation or products formation, but also the inactivation of the enzyme. The operation was performed till complete oxidation under optimal conditions, attaining a nearly complete degradation of 5 mg/L of anthracene after 6 h of operation. This oxidation rate was superior to those described in the literature for the degradation of anthracene by MnP.

[2] Part of this chapter has been published as:

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Outline

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3.1. Introduction

There are several studies of *in vitro* incubations of polycyclic aromatic hydrocarbons with crude and purified LiP or MnP (Bogan and Lamar 1996; Günther et al. 1998; Vázquez-Duhalt et al. 1994). The assays reported were performed on a very small scale (1 mL), and only a limited removal yield was achieved. Table 3-1 summarizes the results of anthracene degradation by MnP reported in literature. The low efficiency achieved, especially in the absence of mediating agents, may be due to either some compound added in scarce amounts, lower than required or to the no optimized physicochemical conditions.

Table 3-1. Degradation rate of anthracene in organic solvents: water mixtures

Solvent	Mediating agent	Degradation rate (μM/h)	Reference
40% acetone	-	0.96	Field et al. 1996
5% DMF[a]	-	0.33	Sack et al. 1997
5% DMF	-	0.70	Günther et al. 1998
5% DMF	5 mM GSH[b]	1.15	Sack et al. 1997
5% DMF	5 mM GSH	2.34	Günther et al. 1998

[a] dymethylformamide

[b] glutathione

The action of MnP depends on the combined action of several compounds, referred to as substrates, cofactors and mediators, which initiate, participate in, and allow the completion of the catalytic cycle. The optimization of the degradation process was conducted taking into account specific physico-chemical factors which may directly affect the activation of the MnP catalytic cycle and the degradation rate of anthracene: (a) the concentration of cofactors and substrates required for the action of MnP (Mn^{2+}, H_2O_2, organic acids) (Martínez 2002; Wariishi et al. 1992) and (b) operating parameters such as temperature, light source and maintenance of air or oxygen atmosphere (Mielgo et al. 2003).

Another important factor to be considered is the loss of enzymatic activity. In the works mentioned above, the enzyme, which was only added at the beginning of the reaction, was supposed to be sufficient to complete the reaction (from 2 to 7 days). The cost of the enzyme will determine the operability of a system in many cases (Buchanan et al. 1998). Therefore it is important to take into consideration the enzyme consumed during the reaction. The efficiency, as the substrate degraded per activity consumed, was considered in this work as a key factor to

balance the adequate conditions of operation in terms of degradability and economic feasibility.

3.2. Materials and methods

3.2.1. Enzyme and chemicals

MnP was obtained from *Bjerkandera* sp. BOS55 (ATCC 90940) as described in Chapter 2.

Anthracene and anthraquinone were obtained from Janssen Chimica (99% purity). Acetone was purchased from Panreac (chemical purity). H_2O_2 (30% v:v), sodium malonate and manganese sulphate were from Sigma-Aldrich.

3.2.2. Anthracene biodegradation assays

Effect of H_2O_2, Mn^{2+} and sodium malonate

Oxidation of anthracene was carried out in 100-mL Erlenmeyer flasks, sealed with Teflon plugs, with magnetic stirring at room temperature, i.e. 22ºC ± 1ºC. The reaction mixture (50 mL) consisted of acetone 36% (v:v), anthracene 5 mg/L (from a stock solution of 1 g/L prepared in acetone), crude MnP 200 U/L and different concentrations of the main cofactors and substrates reported for MnP: Mn^{2+}, H_2O_2 and organic acid: malonic, oxalic, citric and tartaric acid. No volatilization of acetone took place as observed in experiments at the same conditions. Samples were withdrawn periodically to determine anthracene and anthraquinone concentrations as well as the evolution of MnP activity. To verify that degradation took place only due to an enzymatic oxidation, controls were run in parallel using thermal inactivated MnP. No change in anthracene concentration after 6-8 h of incubation was observed in any controls (data not shown).

The experimental design considered three factors: i) Mn^{2+} concentration was assayed at 20 μM and 100 μM, ii) H_2O_2 was added continuously at 5 and 25 μmol/L·min, and iii) sodium malonate was assayed at 1 and 10 mM. Experiments were run in triplicate. Two experiments in the central point were also carried out (60 μM Mn^{2+}, 15 μmol/L·min H_2O_2 and 5 mM sodium malonate). A peristaltic pump was used to feed H_2O_2 at a flow rate around 15-25 μL/min. The dilution effect was taken into account to calculate the concentration of the compounds in the medium. The analysis of the experimental design was carried out with a statistical software package.

Effect of the organic acids

The effect of oxalic, citric and tartaric acid on the extent of degradation and the enzymatic activity was also assayed, at concentrations ranging from 1 mM to 30 mM. The conditions were the following. 36% of acetone, 20 μM Mn^{2+} and an

addition rate of 5 µmol/L·min of H_2O_2.

Stability of MnP in the reaction media

Inactivation of MnP was determined in 100-mL Erlenmeyer flasks, sealed with Teflon plugs, with magnetic stirring at room temperature, i.e. 22ºC ± 1ºC. The reaction mixture (50 mL) consisted of crude MnP, 20 mM sodium malonate, 20 µM Mn^{2+} and, when indicated, acetone 36% (v:v) and the addition of 5 µmol/L·min H_2O_2. Samples were withdrawn periodically during 24 h to determine spectrophotometrically the evolution of MnP activity.

Degradation of anthracene (20 mg/L)

100-mL Erlenmeyer flasks sealed with Teflon plugs were used to degrade, at room temperature, 20 mg/L of anthracene in medium with 50% of acetone, 200 U/L of crude MnP, 20 mM sodium malonate, 20 µM Mn^{2+} and the addition of 5 µmol/L·min H_2O_2. The duration of the experiment was 2 h of treatment.

Optimization of environmental parameters

The possible effects of other environmental parameters, such as temperature, light and oxygen atmosphere, on the degradation of anthracene were also investigated. The influence of temperature was evaluated in assays performed at 23ºC, 30ºC and 40ºC. An oxygen atmosphere was also investigated by flushing industrial oxygen at periodic intervals (3 min every 30 min). Dark conditions were obtained by covering the reactors with aluminium foil. The duration of the experiments was 2 h.

Complete degradation of anthracene

Two long-term experiments were assayed in 100-mL Erlenmeyer flasks, sealed with Teflon plugs, at room temperature and the conditions following described: 36% acetone, 20 µM Mn^{2+}, 20 mM sodium malonate, the addition of 5 µmol/L·min H_2O_2 and 200 U/L of crude MnP. One of them was carried out under oxygen atmosphere (flushing industrial oxygen at periodic intervals).

3.2.3. Analytical determinations

MnP activity was measured spectrophotometrically, anthracene and anthraquinone were determined by liquid chromatography as described in Chapter 2.

3.3. Results and discussion

3.3.1. Effect of substrates and co-substrates of MnP

Experimental design

In order to analyze the effect of the three main factors affecting the action of MnP (malonate, Mn^{2+} and H_2O_2), a 2^3 factorial experimental design was planned with analysis of the two factors at two levels (-1 and +1). Additionally, two central points were assayed to give an estimate of the experimental error (0). The conditions evaluated are summarized in Table 3-2. Three experiments for each condition were carried out, summing up a total of 30 experiments. The factorial design allows obtaining the effect of each factor and their interactions as crossed effects.

Table 3-2. Experimental plan of the factorial design 2^3 with repetition on centre point

Exp	A1	A2	A3	Malonate (mM)	Mn^{2+} (μM)	H_2O_2 (μM/min)
1	-1	-1	-1	1	20	5
2	-1	1	-1		100	5
3	-1	-1	1		20	25
4	-1	1	1		100	25
5	1	-1	-1	10	20	5
6	1	1	-1		100	5
7	1	-1	1		20	25
8	1	1	1		100	25
9	0	0	0	5	60	15
10	0	0	0		60	15

The degradation is a widely used parameter to determine the suitability of an oxidative reaction. However, the enzyme deactivation is also a very important issue which may likely determine if a technology is economically viable. Therefore we introduced additionally the term efficiency as the amount of anthracene degraded per unit of activity consumed. Consequently, three objective functions were considered: anthracene degradation rate, enzyme deactivation and efficiency. The mean of the triplicates obtained for each condition are shown in Table 3-3.

Table 3-3. Results obtained from the set of 2-h experiments described in Table 3-2

Exp.	Degradation rate (μM/h)	Enzyme deactivation rate (U/L·h)	Efficiency (μmol/U)
1	1.41	30	0.047
2	1.37	23	0.059
3	1.54	56	0.028
4	1.23	57	0.022
5	**4.12**	**61**	**0.068**
6	3.56	44	0.081
7	3.99	69	0.058
8	4.05	51	0.079
9	2.52	35	0.072
10	3.32	42	0.080

In terms of degradation rate it is clear that the concentration of sodium malonate was determinant. When comparing the set of the experiments performed at concentrations of the organic acid of 1 mM and 10 mM, the degradation rate was observed to increase 3-fold at the higher concentration. However the loss of MnP activity was also increased, therefore, the use of the efficiency as parameter had great significance. Figure 3-1 shows these effects for the specific conditions of 5 μmol/L·min H_2O_2 and 20 μM Mn^{2+}. In this case the degradation rate with 10 mM was 2.9-fold superior (Exp 5) than with 1 mM (Exp 1) but the activity loss rate was also higher (2.0-fold), which finally resulted into an improved efficiency: 1.45-fold higher.

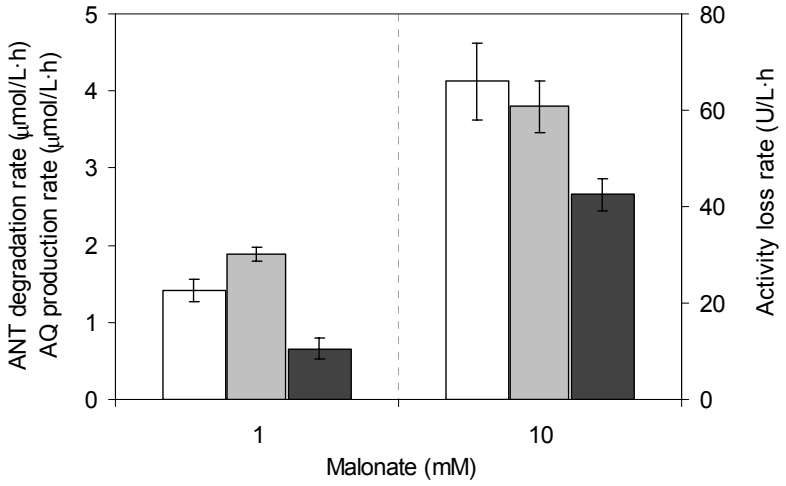

Figure 3-1. Effect of the concentration of malonate in experiments at 5 µmol/L·min H_2O_2 and 20 µM Mn^{2+}. White bars: anthracene degradation rate; grey bars: activity loss rate; dark bars: anthraquinone production

The three objective functions (OF) were modeled to a mathematical function given by:

$$OF = A_0 + A_1 \cdot X + A_2 \cdot Y + A_3 Z + A_{12} XY + A_{13} XZ + A_{23} YZ \qquad (3\text{-}1)$$

where X is the concentration of sodium malonate, Y the concentration of Mn^{2+} and Z the H_2O_2 addition rate. All of them are dimensionless parameters. A_i represents the coefficients for the individual effects and A_{ii} the double effects. The coefficients obtained from this model are shown in Table 3-4.

Table 3-4. Coefficients of the objective functions (OF)*

OF	A_0	A_1	A_2	A_3	A_{12}	A_{13}	A_{23}	r^2
Degradation	2.71	**1.27**	-0.11	0.04	-0.02	0.05	0.04	0.93
Activity loss	46.7	**7.3**	**-5.1**	**9.3**	-3.6	**-5.5**	0.9	0.84
Efficiency	0.059	**0.017**	0.006	**-0.008**	0.004	0.006	-0.001	0.74

* Subscripts: 0 refers to the independent term, 1 refers to malonate, 2 refers to Mn^{2+} and 3 refers to H_2O_2

Bold figures: significant coefficients (α=0.01)

Figure 3-2 shows the plot of the response surfaces for the three objective functions: degradation, enzyme deactivation and efficiency.

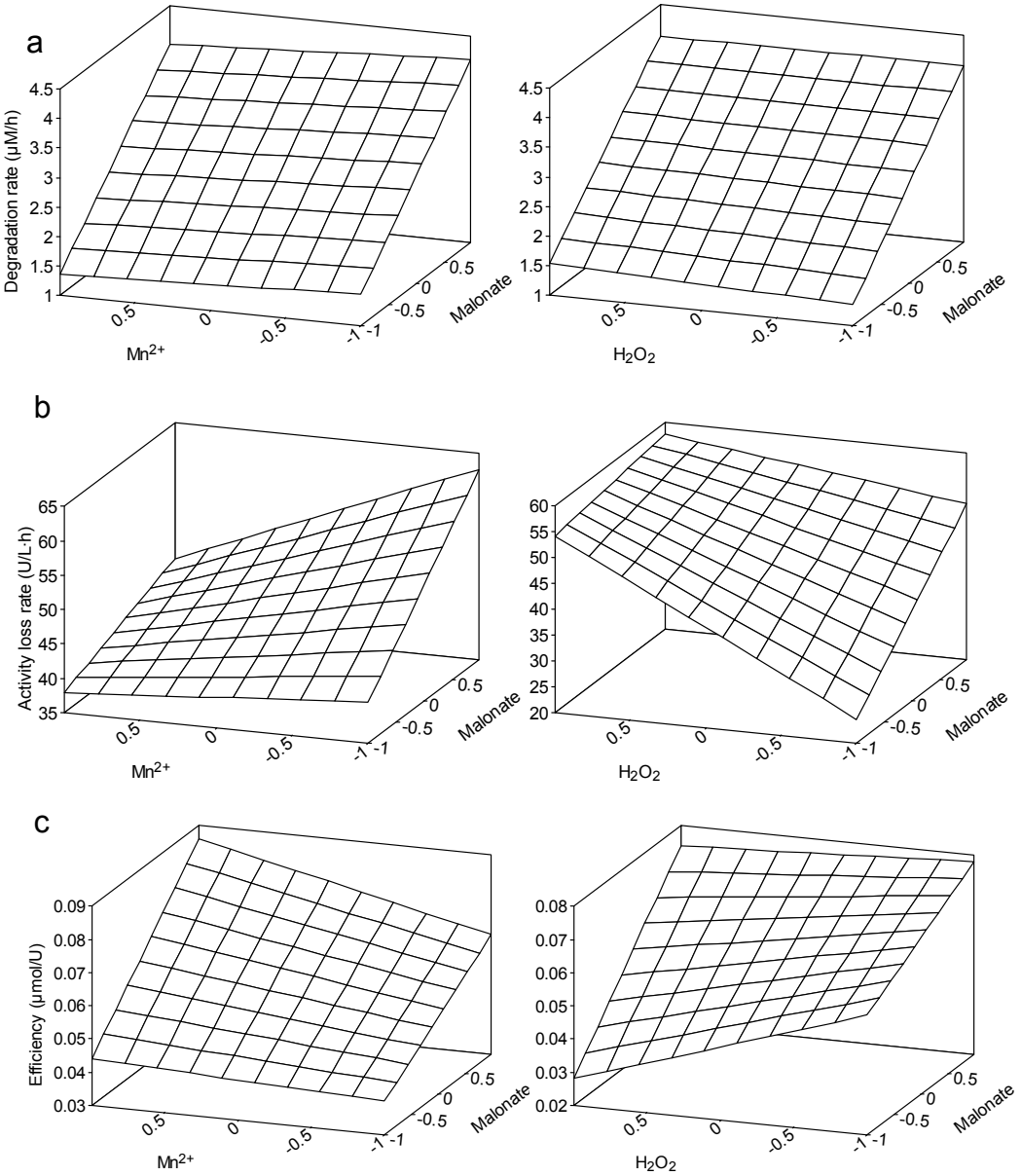

Figure 3-2. Response surfaces of the objective functions: (a) degradation rate (µM/h), (b) enzyme deactivation rate (U/L·h) and (c) efficiency (µmol/U)

The analysis of variance is a way of presenting the calculations for the significance of the effect related to a particular factor, especially for data in which

the influence of several factors is being considered simultaneously. Analysis of variance decomposes the sum of squared residuals from the mean into non-negative components attributable to each factor, or combination of factor interactions. The F-test was applied for the 1% of significance level (α=0.01) (Fig. 3-3).

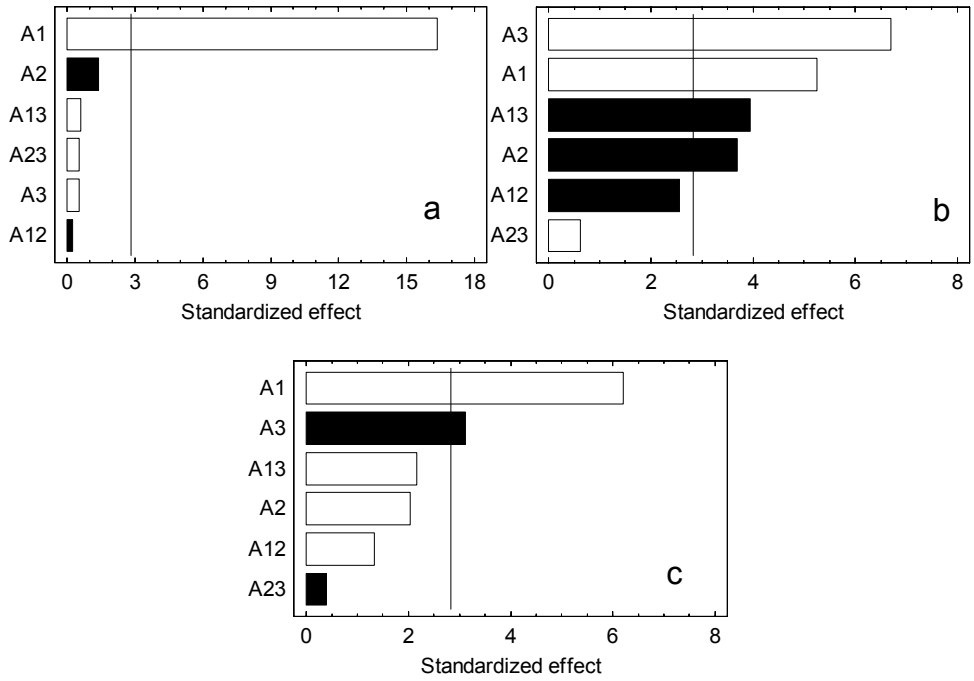

Figure 3-3. Standardized pareto chart for (a) degradation, (b), enzyme deactivation and (c) efficiency. White bars: + effect, black bars: - effect

From the analysis, the subsequent conclusions can be derived:

- In the case of degradation, only the coefficient related to malonate (A_1) was significant. The effect of Mn^{2+} was very low and that of H_2O_2 even lower.
- Production of anthraquinone was parallel to degradation of anthracene.
- Regarding enzyme deactivation, H_2O_2 exerted the major influence but the other parameters including double effects were also important. As it was expected, the higher were the concentration of malonate and the addition of H_2O_2, the higher the activity loss was. But Mn^{2+} had the opposite effect, stabilizing the enzyme at high concentrations.
- The efficiency was extensively dependent on both the concentration of the organic acid and the addition rate of hydrogen peroxide.

Summarizing, the best results in terms of efficiency (0.081 μmol/U) were obtained in exp. 6 with 10 mM of sodium malonate, 100 μM of Mn^{2+} and the addition of H_2O_2 at 5 μmol/L·min. However, the best results in terms of degradation (4.12 μM/h) were obtained at the same conditions except for Mn^{2+}: 20 μM (exp. 5). As the compulsory limit of manganese concentration in the effluents is 36 μM, the concentration of Mn^{2+} considered for the following experiments will be 20 μM for a practical application of the process. In this case the efficiency was not the decisive parameter because the highest efficiency did not imply the lowest costs, since an additional process to remove manganese from the effluent should be considered.

In order to improve the efficiency obtained in exp. 5 (0.068 μmol/U), the concentration of the organic acid should be increased and the addition rate of hydrogen peroxide should be decreased, since they were the major parameters obtained from the analysis of variance. Although in the experiment with a slower H_2O_2 addition rate (1 μM/min) the activity loss was decreased, the efficiency was not improved, as a consequence of a decrease of the anthracene degradation (3-fold lower).

Effect of organic acids

Sodium malonate concentration was the main factor affecting the degradation of anthracene and the efficiency of the process, as demonstrated in the experimental design. Organic acids are essential in the catalytic cycle of MnP because they facilitate the release of Mn^{3+} from the active site and also stabilize this species in aqueous solution (Banci et al. 1998; Martínez 2002). In addition, Kuan et al. (1993) reported that complexed Mn^{2+} is the preferred substrate for the oxidized form of MnP compound II. Experiments with concentrations of organic acids of 20 and 30 mM of malonate were carried out. Other organic acids such as oxalic, tartaric and citric were also assayed at 10 and 20 mM (Fig. 3-4).

Regarding the anthracene degradation, the best result corresponded to 20 mM malonic acid (43.3%), followed by oxalic (32.6%). Tartaric acid seemed not to be involved in the MnP catalytic cycle, attaining similar degradations as observed in absence of organic acid, and surprisingly, the addition of citric acid (both 10 and 20 mM) caused a reduction on the degradation extent (2.9 and 3.8%, respectively). Taking the loss of MnP activity into consideration, the addition of any organic acid increased MnP inactivation. Oxalic acid 20 mM caused the greatest activity loss, leading to a total inactivation of MnP after 90 min. Tartaric and citric acid, in both concentrations, affected MnP activity in a similar way (activity loss around 50 U/L·h).

Oxalic and malonic acids have been shown to be oxidatively decarboxylated by Mn^{3+} (Van Aken and Agathos 2002), generating a carbon dioxide anion radical

which permits the endogenous formation of H_2O_2 via Mn^{2+} and a superoxide radical. The resulting accumulation of H_2O_2 may explain the greatest activity loss for both acids at high concentrations, specially oxalate which produces higher H_2O_2 concentrations (Schlosser and Hofer 2002). Moreover, the carboxyl radical formed during the mechanism, could modify the heme group, resulting in a loss of catalytic activity, as reported for horseradish peroxidase (Huang, 2004).

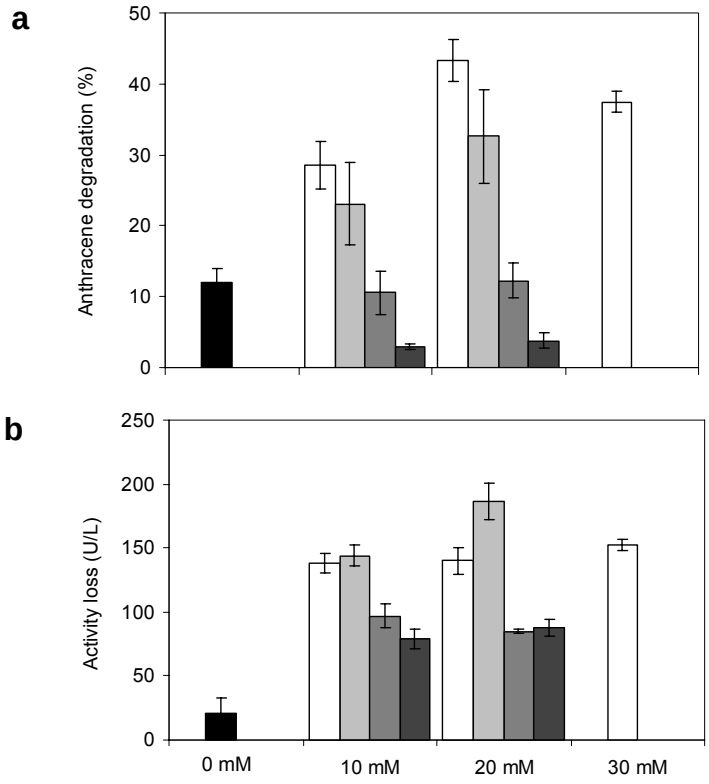

Figure 3-4. Effect of different organic acids on anthracene degradation (a) and MnP activity consumption (b) in 2-h reactions. Symbols: control (■), malonic (□), oxalic (▫), tartaric (▪) and citric acid (▪)

The efficiencies of this set of experiments are summarized in Table 3-5. The result of the efficiency obtained in the control experiment (with no organic acid) was very high (0.168 μmol/U) with a minimum degradation of anthracene (12%). The crude MnP contains lactic acid in a concentration of 1 mM from the fermentation medium, which would be enough to permit a low degradation extent.

Table 3-5. Comparison of the efficiency for experiments with different organic acids

Concentration (mM)	Efficiency (μmol/U)			
	Malonic	Oxalic	Tartaric	Citric
10	0.068	0.045	0.034	0.010
20	0.083	0.049	0.041	0.012
30	0.077	-	-	-

The values obtained with malonic acid were higher than those from the other compounds. The increase of the concentration of all organic acid led to higher efficiencies, except for malonate 30 mM, which caused higher activity loss and did not improve the degradation. The highest value of efficiency, 0.083 μmol anthracene/U MnP, was obtained when 20 mM malonic acid was applied, due to the superior degradation achieved (43.3%).

The higher extent of degradation obtained with the higher concentration of organic acids, could be due to the high reactivity of peroxyl radicals derived from the organic acids, used by MnP in a partly autocatalytic process (Hofrichter, 1998). However, in the case of tartaric and citric acid, the degradation extent was lower than that obtained without exogenous organic acid. Wariishi et al. (1989) reported that chelation of Mn^{3+} by organic acids facilitates its release from the enzyme-Mn complex. It is possible that the binding of tartaric and citric acid (C4 and C6, respectively) to the enzyme is sterically hindered, being therefore, the extent of degradation even lower than the corresponding to the control.

3.3.2. Evaluation of MnP stability in the reaction media

In order to elucidate the role of each component of the medium in the inactivation of MnP, stability assays were carried out without anthracene and varying the conditions of the reaction media. Table 3-6 summarizes the conditions of the experiments and the results of the activity loss rate. Run 0 presents data obtained in Chapter 2 (Fig. 2-6).

The differences of MnP inactivation in run 1 and 2, compared to run 0, could be due to the different conditions of the experiments and, specifically, due to the higher concentration of malonate, which was shown to inactivate the enzyme at a higher extent. Figure 3-5 shows the MnP activity profile in each set.

Table 3-6. Composition of the media in the experiments of MnP stability

Run	Acetone (%)	Malonate (mM)	Mn^{2+} (μM)	H_2O_2 (μM/min)	MnP (U/L)	Act loss rate (U/L·h)
0	10-50	10	-	-	100	≈ 0
1	36	20	20	-	439	6
2	45	20	20	-	383	10
3	-	20	20	5	392	12
4	36	20	20	5	389	59
5	45	20	20	5	225	65

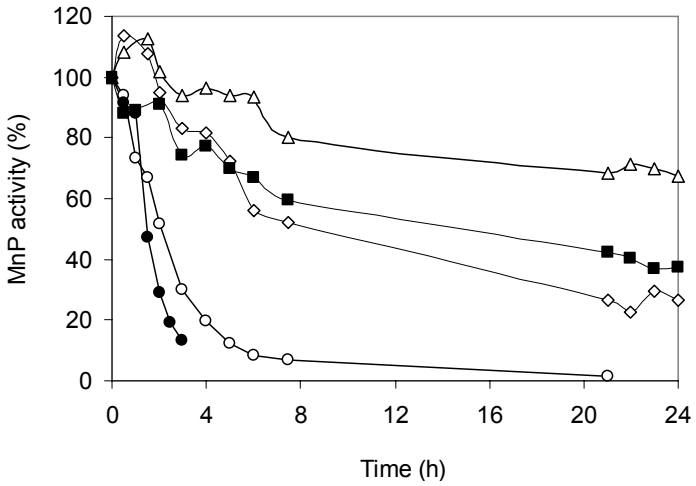

Figure 3-5. Profile of MnP activity in media described in Table 5-6.
Runs: △ 1, ■ 2, ◇ 3, ○ 4, ● 5

The highest inactivation occurred in reaction media with 45% of acetone and hydrogen peroxide addition (65 U/L·h). When 36% of acetone was present and H_2O_2 was added, the inactivation was slightly lower (59 U/L·h). Comparing these experiments with run 3, where no acetone was present, the stability of the enzyme was much higher (around 5-fold). From these results we could deduce that acetone produced high inactivation of the enzyme; thus, the higher acetone concentration present in the medium, the higher enzymatic inactivation. However, if we compare run 1 with run 4 the difference was the addition of H_2O_2, which led to an inactivation 10-fold higher. The same behavior was observed when 45% of acetone was used (exp 2 and 5). A possible explanation could be based on the reaction of H_2O_2 with

acetone, leading to the formation of compounds which could inactivate the enzyme. We could, therefore, conclude that the degradation products are the main responsible of enzymatic inactivation better than the acetone itself.

There have been only a few reports of acetone degradation by means of H_2O_2 in aqueous solution (Stefan and Bolton 1999; Stefan et al. 1996). These studies considered the removal and mineralization of acetone by the UV-H_2O_2 process. They found that the decay of acetone led to the formation of carboxylic acids such as acetic, formic and oxalic. These reactions proceeded with the formation of carboxyl radicals, the same as those described by Huang et al. (2004) which have been shown to inactivate peroxidases by modification of the heme group.

3.3.3. Degradation of anthracene (20 mg/L)

A higher anthracene concentration was assayed in order to evaluate the efficacy of the system. Acetone was added at a 50% concentration to ensure a concentration of 20 mg/L of anthracene in the medium (Fig. 2-1 in Chapter 2). The average results obtained from the three experiments compared to the degradation of 5 mg/L of anthracene are shown in Table 3-7.

Table 3-7. Results of the degradation of anthracene at two concentrations

Anthracene (mg/L)	Anthracene degraded (µM/h)	Anthraquinone produced (µM/h)	Activity loss (U/L)	Efficiency (µmol/U)
20	4.70	2.34	153	0.063
5	5.81	1.58	140	0.083

The activity loss slightly increased with 50% acetone whereas the degradation of anthracene was lower. Therefore, the efficiency of the degradation of 20 mg/L of anthracene was 70% of the efficiency obtained at 5 mg/L of anthracene.

The decrease of the efficiency in the system with 20 mg/L of anthracene could be related to the presence of 50% of acetone, which could affect the completion of the degradation.

3.3.4. Effect of environmental parameters

Other parameters such as oxygen concentration, temperature and light were evaluated using the optimized conditions.

Oxygen and air atmosphere

As it can be seen in Fig. 3-6, dissolved oxygen (up to 25 mg/L in the reaction media) improved the anthracene degradation (50.5%) and anthraquinone production (19.0%) whereas the enzymatic activity loss was not affected.

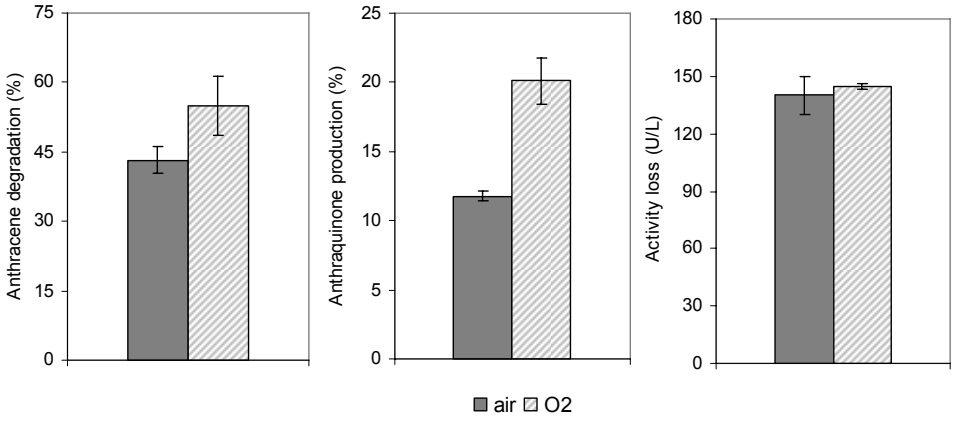

Figure 3-6. Effect of the oxygen atmosphere on the anthracene degradation by MnP

Temperature

The increase of temperature to 30ºC led to a reduction of the anthracene degradation (34.9%), as well as to a greater activity loss (83 U/L·h) (Fig. 3-7). Operation at 40ºC exerted a very severe activity loss (MnP was totally inactivated after 1 h reaction), being therefore the oxidation of anthracene very low (5.5%).

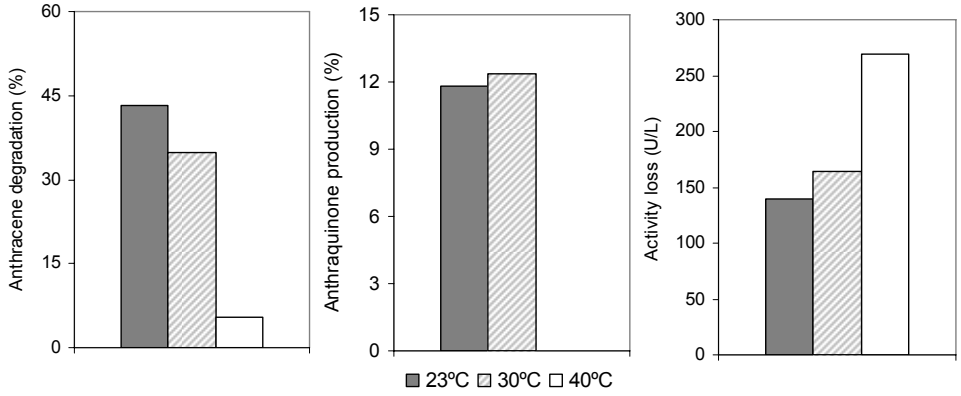

Figure 3-7. Effect of the temperature on the anthracene degradation by MnP

Light and dark

Experiments in complete darkness were also performed to check the effect of light on the anthracene oxidation (Fig. 3-8). It was observed that the extent of anthracene degradation was slightly lower in darkness (83% of that in presence of light), whereas no changes in activity loss were observed.

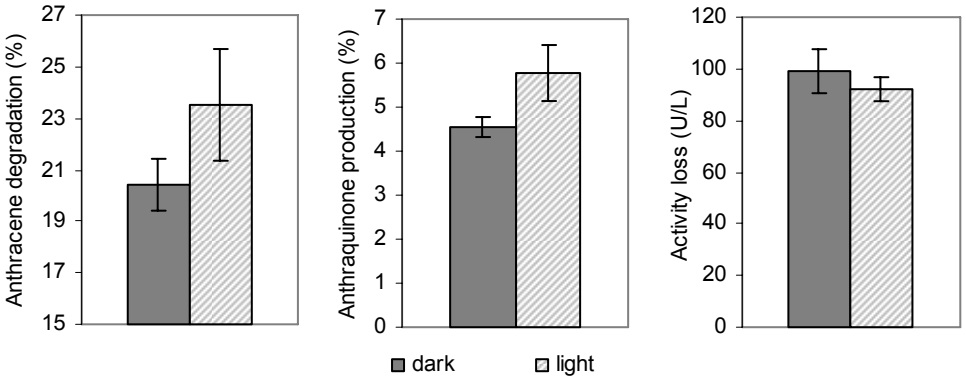

Figure 3-8. Effect of the light on the anthracene degradation by MnP

The efficiencies of the experiments at different environmental conditions are summarised in Fig. 3-9. The highest value (0.090 µmol/U) was obtained at 22ºC, under oxygen atmosphere and light.

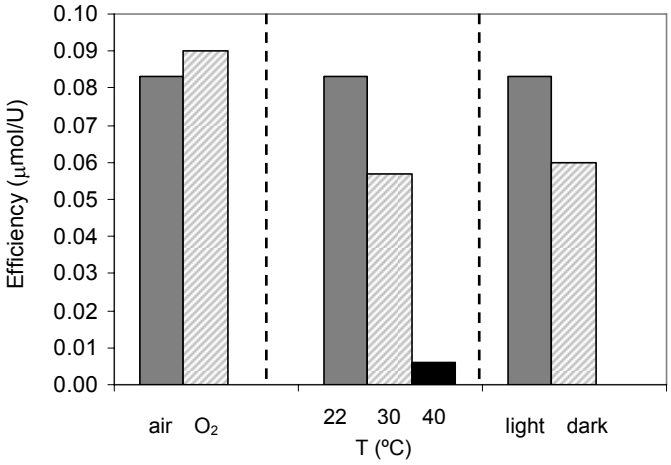

Figure 3-9. Evaluation of the efficiency in experiments at different environmental conditions

Oxygen atmosphere increases the anthracene oxidation. This fact which has been observed in degradation of azo dyes in water may be attributed to the catalase-type activity of MnP (López et al. 2004). MnP releases atomic oxygen which could be directly used for the degradation of anthracene. In this case, it is interesting to see that the maximum degradation rate was coincident with the highest dissolved oxygen concentration in the medium (27.9 mg/L).

Enzymes effectively work at mild conditions, and under temperatures around 20 to 30ºC the behavior of MnP is very similar (Mielgo et al. 2003). Temperatures above 40ºC were shown to inactivate rapidly the enzyme (Sutherland and Aust 1996).

Multiple studies have demonstrated that PAHs, and particularly anthracene, undergo fairly rapid transformations when exposed to light in an aqueous medium and also in organic solvents and solvent–water mixtures (Bertilsson and Widenfalk 2002; Lehto et al. 2000). In the present work, the difference of the degradation extent was not as notable as expected, since the reaction mixtures were not subjected to direct light from UV-lamps as happened in the mentioned studies.

3.3.5. Complete degradation of anthracene

So far, experiments to determine the optimal conditions for the *in vitro* oxidation of anthracene have been conducted for 2 h. In order to quantify the maximum extent of anthracene degradation, the operation was performed till complete oxidation. The degradation profile of 5 mg anthracene/L (28 μM) in a medium containing 36% acetone (v:v), malonic acid 20 mM, Mn^{2+} 20 μM, continuous addition of H_2O_2 at 5 μmol/L·min working under oxygen atmosphere is shown in Fig. 3-10 (a). The anthracene degradation was nearly complete after 6 h. During the first 2 h of the experiment, a marked activity loss and anthracene degradation were observed. A parallel experiment was carried out under an air atmosphere instead of oxygen, attaining in this case, a nearly complete oxidation of anthracene (98%) after 8 h (Fig. 3-10 (b)).

The degradation of anthracene resulted in its total oxidation to anthraquinone (Field et al. 1992; Hammel et al. 1991). The degradation mechanism, probably arising via one-electron oxidative pathway, is quite complex, implying the generation of intermediate compounds such as anthrol and anthrone (Haemmerli 1988). The apparent discrepancy between the expected ratio 1:1 of anthraquinone and anthracene and that obtained in this experimental work, around 1:2, indicates the presence of relative amounts or these or other intermediate compounds. In fact, the final step to anthraquinone is likely to be limiting the overall reaction rate of the process, as we determined an increase of the anthraquinone concentration around 10% in samples measured after 24 h. In this sense, ongoing research has as an

objective the deeper knowledge of the degradation mechanism and kinetics and the way to enhance the rate of the whole process.

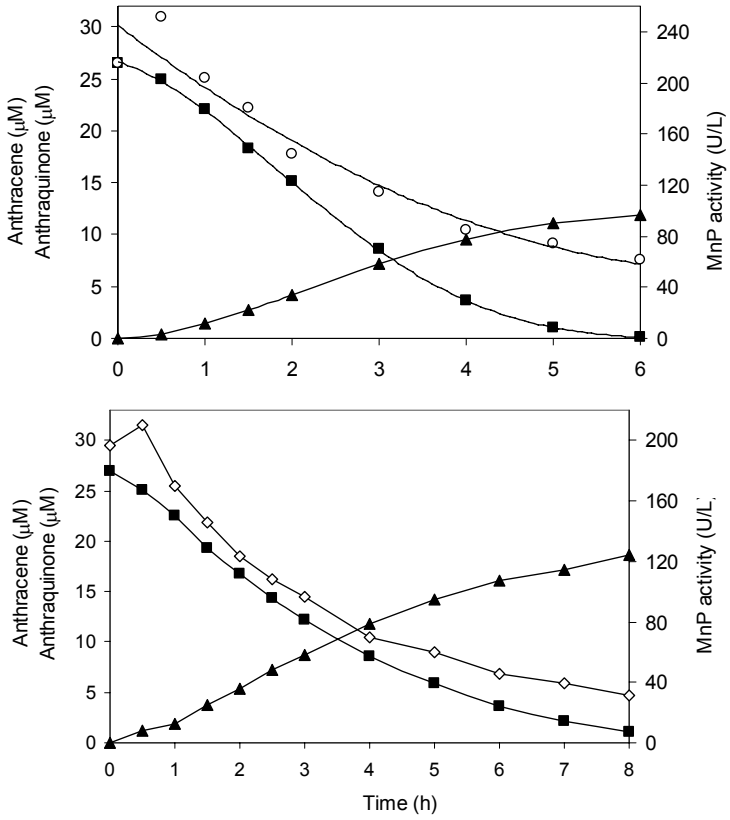

Figure 3-10. Time course of anthracene degradation in oxygen (a) and air atmosphere (b). Symbols: MnP activity (○), Anthracene (■), Anthraquinone (▲)

3.4. Conclusions

By improving the understanding of the main factors affecting anthracene degradation, an efficient treatment based on the use of free MnP may be defined. The completion of the catalytic cycle of MnP depends on the combined action of its cofactors, cosubstrates and mediators. Therefore, for optimizing the catalytic action of the enzyme, special attention was paid to study the influence of the following main factors: H_2O_2 and Mn^{2+} concentrations, organic acids and other operating parameters such as temperature and oxygen atmosphere.

The continuous addition of H_2O_2 at a controlled flow (5 μmol/L·min) permits the progressive participation of H_2O_2 in the catalytic cycle through a suitable

regeneration of the oxidized form of the enzyme, minimizing the peroxide-dependent inactivation of the peroxidase (Moreira et al. 1997).

Our results confirmed that the concentration of the organic acid (e.g. malonic) is decisive on the action of the enzyme: on the one hand, degradation extent is improved, but on the other hand, activity loss also increases. The optimization of the concentration of malonic acid permits a high extent of degradation with no compromise to the stability of the enzyme.

Unlike the results discussed in Chapter 2 where acetone concentrations as higher as 90% scarcely inactivated MnP, increasing the concentration of acetone in media containing all the compounds involved in the catalytic cycle, led to higher inactivation of the enzyme. This negative effect was related to the presence of degradation products from the reaction of acetone with H_2O_2.

Environmental factors such as oxygen atmosphere, temperature and irradiation, were analyzed and the results obtained compare favorably with those obtained in the literature: irradiation favors the degradation of anthracene; mild temperatures are preferred for the action of the enzyme and working under oxygen atmosphere increases the extent of oxidation.

The optimization of the parameters involved in the enzymatic degradation of anthracene in mixtures acetone:water led to the complete degradation of 5 mg/L after 6 h of operation. Comparing these results with previous works (Table 3-1), the average degradation rate achieved here, 4.40 μM/h, was the highest, being 4.6-fold higher than that obtained by Field et al. (1996) at similar conditions.

3.5. References

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Chapter 4

Degradation of anthracene, pyrene and dibenzothiophene in batch reactors containing acetone:water mixtures. Mechanisms of degradation[3]

Summary

The optimization of the degradation of anthracene by manganese peroxidase in batch reactors containing acetone:water mixtures has been described in the previous chapter. In the present chapter this technology was applied for the elimination of other PAHs, obtaining evidences of degradation for dibenzothiophene and pyrene. These compounds were degraded to a large extent, even completely after a short period of time (around 24 h), at conditions that allowed the MnP-oxidative system to be optimized. The initial amount of enzyme present in the reaction medium was essential for the kinetics of the process. With respect to the kinetics, anthracene is the compound which degrades faster, however dibenzothiophene is 12-fold slower and pyrene 34-fold.

The degradation products were determined using gas chromatography-mass spectrometry and the degradation mechanisms were proposed. Anthracene was degraded to phthalic acid. A product derived from the ring cleavage of dibenzothiophene, 4-methoxybenzoic acid, was also observed. In the degradation of anthracene, it was also detected a structure with ortho hydroxyl radicals that was assigned as dihydroxyanthrone. This compound, together with production of 1-hydroxypyrene from pyrene, indicated a direct hydroxylation by $^{\bullet}OH$ radicals during oxidative process.

[3]Part of this chapter has been published as:

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4.1. Introduction

PAHs are environmental contaminants from natural or anthropogenic sources, resulting from the combustion of organic matter. Their concentration in crude oil and fuel is commonly relevant, and subsequently they are present in oil spills. The fuel from the Prestige oil spill, (Galicia, 2002) contained 53% of aromatic compounds and the composition of this fraction is presented in Fig. 4-1 (Data from Ministry of Health and Consumer Affairs):

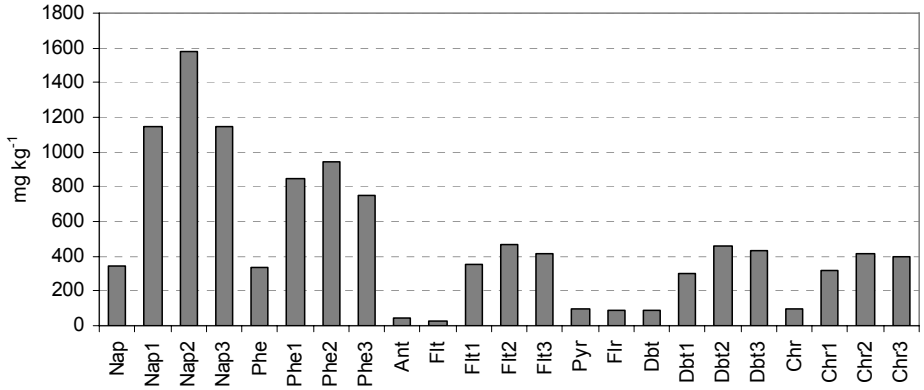


Figure 4-1. Relevant PAHs found in fuel from Prestige. Nap: naphthalene; Phe: phenanthrene; Ant: anthracene; Flt: fluoranthene; Pyr: pyrene; Dbt: dibenzothiophene; Chr: chrysene; PAH-number from 1 to 3 means: methyl, dimethyl and trimethyl-PAH, respectively.

Naphthalene is a highly volatile compound, and this characteristic greatly hampers its study. Phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), fluorene (FLR), dibenzothiophene (DBT), chrysene (CHR) and their derivatives, represented 60% of the PAHs present in the fuel. These compounds, except their methylated forms, were selected to study their degradation by the enzyme MnP.

One characteristic indicative of the persistence of a molecule is its ionization potential (IP) which is the energy required to remove an electron. It is a significant parameter of the reluctance of the molecule to transfer an electron. Therefore, molecules with lower values of IP are likely to be more reactive. The oxidative activity of manganese peroxidase (MnP) is mediated through the production of manganese ions, acting as freely diffusible oxidants. In a way to reproduce the degradative action of MnP, manganic acetate was found to be incapable of oxidizing PAHs with IPs equal or greater than 7.8 eV (IP of chrysene), which gives an idea

about the threshold value for the PAH degradation by the catalytic action of MnP (Cavalieri and Rogan 1985). However, when lipid peroxidation was involved, the degradation was evident for those PAHs not oxidized directly by MnP. This process occurs when unsaturated lipids are present, generating powerful oxidative radicals which help to decompose the recalcitrant compound.

In the last years, the *in vitro* degradation of PAHs has been focused on the determination of the threshold IP under different conditions and the effect of mediating agents (Bogan and Lamar 1995; Bogan and Lamar 1996; Bogan et al. 1996; Sack et al. 1997b; Wang et al. 2003) whereas little attention has been paid to the optimization of the system. Günther et al. (1998) have reported the degradation of 30% ANT and 12% PYR by MnP from *Nematoloma frowardii* after 24 h of reaction (initial concentration: 10 mg/L). In another work, the degradation of fluoranthene was evaluated to follow a much slower rate (only 10% of degradation after 96 h), and in the case of PHE and CHR no degradation was observed in comparison with the control (Sack et al. 1997b). The poor degradation attained in experiments with crude MnP suggests that the operational conditions were not optimized. The first objective of this chapter is to apply the technology and the appropriate conditions used in the degradation of ANT for the oxidation of DBT, FLR, FLT, PYR, PHE and CHR, as examples of PAHs.

The precise role of individual ligninolytic enzymes in the degradation of PAHs by white-rot fungi has set controversial opinions. On the one hand several authors support the function of these enzymes as the initiators of the degradation, converting the PAH into its quinone (Hammel 1995; Hammel et al. 1991). Further steps which lead to the ring cleavage and mineralization could be carried out by a non-ligninolytic system (Hammel et al. 1992). On the other hand, Schützendübel and coworkers have not found a direct correlation of the metabolization of PAHs with the production of the ligninolytic enzymes (Schutzendubel et al. 1999). Several authors suggested that cytochrome P-450 monooxygenase could be the responsible of the initial step of PAH degradation (Bezalel et al. 1996; Gramss et al. 1999; Verdin et al. 2004). The second objective of this chapter is to elucidate the pathways in which MnP is involved. Moreover, the mechanisms of degradation of each PAH will be discussed.

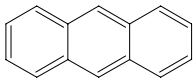
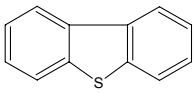
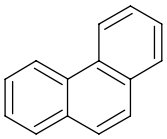
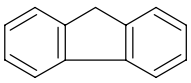
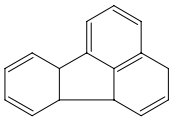
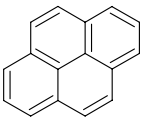
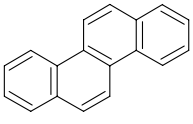
Finally, the application of the enzymatic system for the degradation of PAHs will be compared with the chemical process, utilizing directly manganese(III) acetate as the oxidizing agent in absence of H_2O_2.

4.2. Materials and methods

4.2.1. Enzyme and chemicals

The main characteristics of the PAHs under study are presented in Table 4-1. All PAHs present a complex structure, low water solubility and high ionization potentials in a range between 7.4 and 8.1 eV.

Table 4-1. Structure, aqueous solubility and ionization potential of PAHs.

PAH	Structure	Solubility[1] (mg/L)	IP[2] (eV) [range]	Genot	Carcin
Anthracene (ANT)		0.07	7.41 ± 0.08 [7.15-7.55]	-	-
Dibenzothiophene (DBT)		1.47	8.14 ± 0.21 [7.90-8.44]	-	-
Phenanthrene (PHE)		1.29	7.94 ± 0.14 [7.60-8.25]	(?)	(?)
Fluorene (FLR)		1.98	8.03 ± 0.28 [7.78-8.52]	-	-
Fluoranthene (FLT)		0.26	7.84 ± 0.10 [7.72-7.95]	+	(+)
Pyrene (PYR)		0.14	7.50 ± 0.12 [7.31-7.72]	(?)	(?)
Chrysene (CHR)		0.002	7.73 ± 0.14 [7.59-8.00]	+	+

[1] Mackay and Shiu (1977) and Hassett et al. (1980)

[2] Average values calculated with different methods (http:/webbook.nist.gov)

Crude MnP was obtained from cultures of *Bjerkandera* sp. BOS55 (ATCC 90940) as described in Chapter 3. All PAHs were obtained from Janssen Chimica (95-99% purity). Acetone was obtained from Panreac (chemical purity). Manganese(III) acetate dihydrate was obtained from Aldrich.

4.2.2. Operation in batch reactors

Acetone concentration

To attain a PAH concentration in liquid phase of 5 mg/L, acetone was added in different proportions to ensure total solubilization of the added PAH. A proportion of 36% of acetone, which was used in the previous chapter for solubilizing ANT, was selected to dissolve DBT, PHE, FLR and FLT, all of them having a solubility in water higher than 2-times the solubility of ANT.

Chrysene is the less water soluble compound. Experiments of solubilization of CHR at different mixtures of acetone:water were carried out at room temperature following the same procedure as described in chapter 2. The results of CHR solubility in a logarithmic scale are presented in Fig. 4-2. 45% of acetone dissolved 13.9 mg L^{-1} of CHR and this amount was selected for the experiments of degradation. PYR is a four-ringed PAH with a water solubility slightly higher than that of ANT. In order to avoid experiments of solubility with PYR, 45% of acetone was used for the *in vitro* degradation.

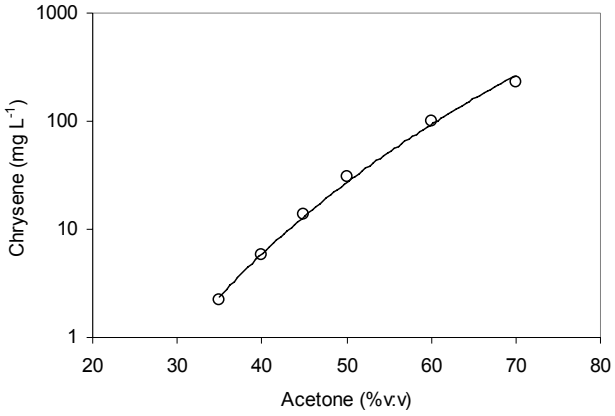


Figure 4-2. Solubility of chrysene in mixtures acetone:water at room temperature

Degradation experiments

Oxidation of PAHs was carried out in 100-mL Erlenmeyer flasks, sealed with Teflon plugs, with magnetic stirring at room temperature (22ºC ± 1ºC). The reaction

mixture (50 mL) at pH 4.5 consisted of acetone 36% (ANT, PHE, FLR, FLT and DBT) or 45% (PYR and CHR), 5 mg/L PAH, 20 μM Mn^{2+}, 20 mM malonic acid, continuous addition of 5 μmol/L·min H_2O_2 and MnP activities specified for each case. Samples were withdrawn periodically and disappearance of each PAH was determined by HPLC. The evolution of MnP activity was spectrophotometrically determined. To verify that degradation took place only due to an enzymatic oxidation, controls were run in parallel in absence of MnP. The dilution effect caused by the continuous addition of H_2O_2 was corrected in the final value of PAH concentrations.

4.2.3. Chemical oxidation of PAHs by Mn^{3+}

The degradation of ANT, DBT and PYR was chemically carried out by means of the oxidizing agent Mn^{3+}. Immediately prior to be used, manganese(III) acetate was dissolved in ethanol at a concentration of 20 mM. Reaction mixtures (50 mL) contained 5 mg/L PAH, 36% acetone (ANT and DBT) or 45% (PYR), and 20 mM sodium malonate (pH 4.5). Two concentrations of Mn^{3+} were considered: 20 and 1000 μmol/L. Samples were withdrawn periodically and disappearance of each PAH was determined by HPLC.

4.2.4. Sample preparations

The concentrations of PAHs were directly measured by HPLC. However, the samples used for the determination of the degradation products of ANT, DIB and PYR, were prepared as follows: The whole content of each reaction was initially acidified with 0.5 mL of HCl 1 M and then extracted with 20 mL of ethyl acetate for 20 min in a horizontal shaker. In order to favor the separation of the two phases, samples were introduced in an ultrasound bath for 5 min. After removing the organic phase, the aqueous layer was extracted 3 subsequent times with the solvent. Then, all the ethyl acetate fractions were concentrated in a rotary evaporator and the final volume, 2-3 mL, was dried by passing the sample through a cartridge filled with $NaSO_4$.

4.2.5. Analytical determinations

MnP activity was measured spectrophotometrically as described in Chapter 2. A HP 1090 HPLC, equipped with a diode array detector, a 4.6×200 mm Spherisorb ODS2 reverse phase column (5 μm; Waters) and a HP ChemStation data processor were used for determining PAH concentrations. The injection volume was set at 10 μL and the isocratic eluent was pumped at a rate of 1 mL/min. The conditions of the mobile phase and the wavelengths used to measure the PAH concentrations are described in Table 4-2.

Table 4-2. HPLC conditions for the determination of each PAH.

PAH	Mobile phase ACN:H_2O (v:v)	λ (nm)	Retention time (min)
ANT	80:20	254	7.9-8.1
PHE	80:20	254	6.3-6.6
PYR	80:20	240	10.6-10.7
FLT	80:20	240	9.3-9.4
FLR	80:20	260	6.0-6.2
DBT	80:20	260	6.8-6.9
CHR	95:5	268	5.3-5.5

Degradation products of ANT, PYR and DBT were analyzed by gas chromatography coupled with mass spectrometry (GC-MS, GCQ, Finnigan, USA) in the Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague. For structure elucidation, electron impact and chemical ionization mass spectrometry as well as MS-MS technique were used. The GC instrument was equipped with split/splitless injector and a DB-5MS column was used for separation (30 m, 0.25 mm id, 0.25 μm film thickness). The temperature program started at 60°C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 150°C at a rate of 25°C/min. The second temperature ramp was up to 260°C at a rate of 10°C/min, this temperature being maintained for 20 min. The solvent delay time was set to 4 min. The transfer line temperature was set to 280°C. Mass spectra were recorded at 1 scan/sec under electron impact at 70 eV, mass range 50–450 amu. The excitation potential for the MS/MS product ion mode applied was 0.5 V, and 0.9 V in the case of more stable ions. Methane was used as a medium for chemical ionization (CI). The extracts were directly injected with no derivatization. Moreover, the samples were trimethylsilicated with aliquot volume of N,O-bis(trimethylsilyl)trifluoroacetamide (60 min, 60°C) (Cajthaml et al. 2002).

4.3. Results and discussion

4.3.1. Biodegradation of PAHs

Experiments of degradation of phenanthrene (PHE), fluorene (FLR), fluoranthene (FLT), pyrene (PYR), dibenzothiophene (DBT) and chrysene (CHR) by MnP in media containing acetone were carried out, as well as the control experiments in absence of MnP to evaluate the possible oxidation by H_2O_2.

No clear evidences of degradation were obtained for PHE, FLR, FLT and CHR, in 24 h-experiments. For these compounds, the differences between the final concentrations of each PAH in the *in vitro* experiment and the control were lower than 5%. Moreover, the GC/MS analysis of the samples at the end of the reaction did not show any possible intermediate of their degradation. These results were the expected since the IPs of those PAHs are higher than the threshold value established by Cavalieri and Rogan: 7.8, which was chrysene IP (Cavalieri and Rogan 1985). Günther et al. (1998) evaluated the *in vitro* degradation by MnP of PHE and FLT among other PAHs, and their disappearance was lower than 5%.

PYR and DBT were degraded by MnP but to a lower extent than ANT at the same conditions. In order to enhance their conversion, the initial concentration of MnP was increased and its effect was evaluated in terms of degradation rate and the value of the kinetic constant (Table 4-3).

Anthracene. Four initial enzymatic activities were assayed to determine ANT degradation rates and the kinetic constants. The higher enzymatic activity (550 U/L) led to a higher ANT degradation rate (3.22 μmol/L·h) and consequently, to a higher kinetic constant (first order kinetics, 0.488 h^{-1}). In these conditions, 23 μM of ANT were degraded after 7 h (Fig. 4-3). Anthraquinone, the main reaction product, was measured during the experiment, and the final concentration was 12 μM, which represented 52% of the degraded ANT (data not shown). A control experiment was performed in absence of MnP where only a slight decrease of ANT concentration (9%) was observed with no traces of anthraquinone. The continuous addition of hydrogen peroxide reduced the acetone concentration from 36% to 28% which caused a slight diminution of soluble ANT in the control composition.

The experiments at lower MnP activities (60, 140 and 210 U/L) were stopped when MnP activity decreased below 10 U/L (4, 5 and 6 h, respectively), which corresponded with a distinct change in the slope of ANT degradation. From these results we can conclude that the minimum enzyme requirements for ANT degradation were beyond 10 U/L.

Table 4-3. Biodegradation of ANT, PYR and DBT with MnP at different initial enzymatic concentration

PAH	Initial enzyme E_0 (U/L)	Reaction duration (h)	Average PAH degradation rate (μmol/L h)	First order kinetics	
				k (h^{-1})	r^2
ANT	60	4	1.78	0.081	0.98
	140	5	2.04	0.114	0.99
	210	6	2.15	0.140	0.98
	550	7	3.22	0.488	0.98
PYR	210	6	0.28	0.012	0.94
	540	9	0.55	0.023	0.98
	1180	24	0.65	0.034	0.99
	1310	24	0.54	0.040	0.98
DBT	170	6	1.06	0.023	0.99
	570	24	0.93	0.055	1.00
	1340	24	1.24	0.121	0.96

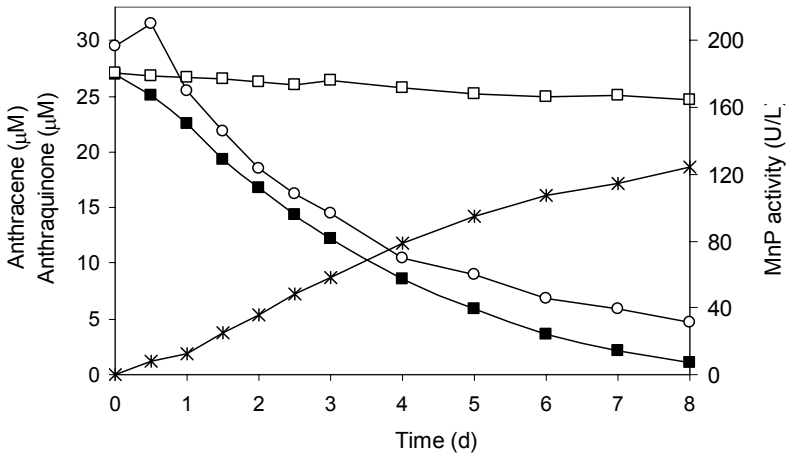


Figure 4-3. Time course of anthracene disappearance (■), anthraquinone formation (∗) and MnP enzymatic activity (○) during *in vitro* treatment. A control assay without MnP was run in parallel (□)

Pyrene. The experiments with PYR were carried out under the same optimized environmental conditions considered in the assays with ANT at various initial enzymatic concentrations: 210, 540, 1180 and 1310 U/L (Table 4-2). In comparison with ANT, the amounts of MnP assayed were higher, not only because of lower degradation percentages (11, 19, 53 and 61%, respectively) but also higher inactivation rates of MnP (data not shown). Figure 4-4 shows the degradation of 13 μM PYR after 24 h, at an initial MnP concentration of 1180 U/L. The control in absence of enzyme showed no change in PYR concentration in the course of the experiment, this verifying the degradative action of MnP for the *in vitro* system. In this case the dilution effect did not affect the PYR concentration of the control experiment since the initial concentration of acetone was higher than for the other compounds.

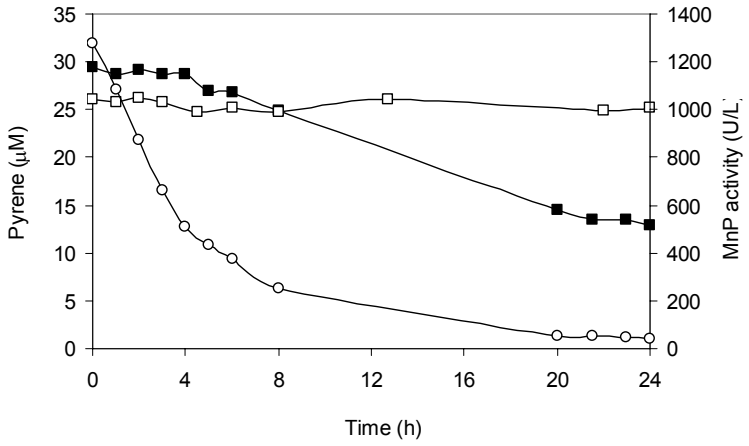


Figure 4-4. Time course of pyrene disappearance (■), MnP enzymatic activity (○) during *in vitro* treatment. A control assay without MnP was run in parallel (□).

Dibenzothiophene. Three initial MnP concentrations were assayed for experiments of DBT degradation: 170, 570 and 1340 U/L (Table 4-2). The last experiment led to a nearly complete degradation after 24 h (95%) (Fig. 4-5). The slight diminution of the DBT concentration in the control experiment (15%) was probably due to the reduction in solvent concentration during the experiment (from 36 to 20%) as a result of the hydrogen peroxide addition.

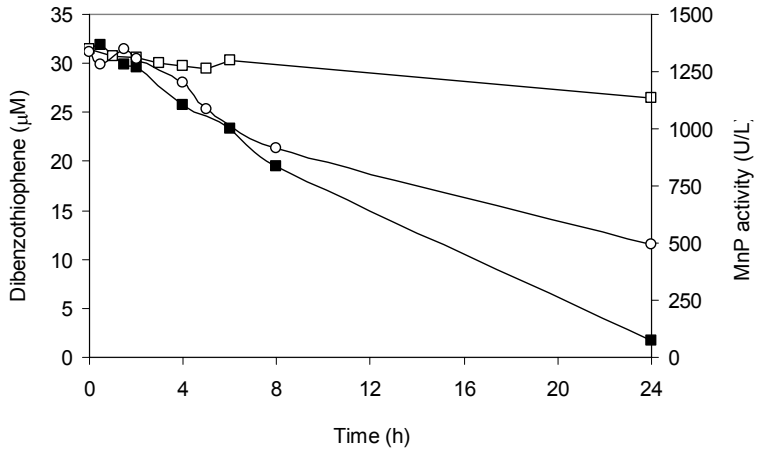


Figure 4-5. Time course of dibenzothiophene disappearance (■), MnP enzymatic activity (○) during *in vitro* treatment. A control assay without MnP was run in parallel (□).

Theoretically, the enzymatic system should be efficient provided that the oxidation potential of a particular compound is lower than the oxidative potential of the enzymatic cycle. The *in vitro* system was proven to oxidize ANT and PYR with crude MnP with an IP lower than that of chrysene (7.73 ± 0.14 eV). However, this limitation was overcome when the system was capable to oxidize DBT efficiently (IP: 8.14 eV), yielding an almost total degradation after 24 h. In the case of LiP, Vázquez-Duhalt et al. (1994) demonstrated that its IP threshold value, 7.6 eV (Hammel et al. 1992), was slightly higher for alkylaromatic and heteroaromatic polycyclic compounds (8.0 eV). However, the IP threshold should be considered as a range, not a value, due to the disparity of IP values (Table 4-1). Even if we compare IP values obtained using the same method, the disparity is high. As an example, pyrene IP determined using charge transfer method varies from 7.31 (Finch 1964) to 7.72 eV (Briegleb 1964). When the average of the different methods is calculated, the standard deviations can be as high as 0.28 or 0.21 eV in the case of FLR and DBT respectively. Therefore, it is difficult to establish a threshold IP value for the oxidation of PAHs by MnP.

4.3.2. Effect of initial MnP activity on the kinetics

The initial enzymatic activity greatly affected the degradation kinetics for the studied PAHs. Therefore, in order to determine the relationship between these two variables (E_0 and k), linear regressions were considered for the experiments with ANT, PYR and DBT (Fig. 4-6). The regression coefficients ranged from 0.97 to 1.0, indicating that the data fitted well to the linear equation.

The IP values of each PAH (Table 4-1) give insight of the recalcitrant character of each compound: ANT is the less recalcitrant one, followed by PYR and finally DBT is the most recalcitrant one. With respect to the kinetics, the slope of the equations in Fig. 4-6, gives an idea about the degradation rates of each compound: ANT is the compound which degrades faster, however DBT is 12-fold slower and PYR 34-fold.

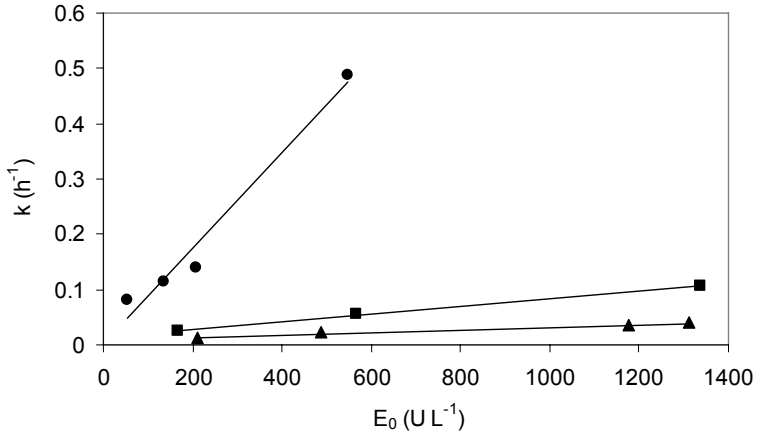


Figure 4-6. Kinetics constants of anthracene (●), pyrene (▲) and dibenzothiophene (■) as a lineal function of initial enzymatic activity. $k_{ANT} = 8.7 \cdot 10^{-4} \cdot E_0 + 0.0014$ ($r^2 = 0.97$); $k_{PYR} = 2.4 \cdot 10^{-5} \cdot E_0 + 0.0084$ ($r^2 = 0.97$); $k_{DBT} = 7.0 \cdot 10^{-5} \cdot E_0 + 0.0139$ ($r^2 = 0.99$);

4.3.3. Mechanisms of biodegradation

Table 4-4 lists the retention data and mass spectral characteristics of the detected degradation products. Possible degradation sequences are given in Fig. 4-7. In all cases, except for anthraquinone, only traces of intermediate compounds were detected (0.5-1% of the stoichiometric concentration expected for total degradation), indicating that no significant accumulation of these compounds took place and immediate degradation occurred after formation.

The intermediates were identified by comparing the mass spectra with data in the NIST 98 library, and independently by interpreting the fragmentation pattern. Additionally, unknown structures of metabolites were explored using MS/MS (product ion scan) to clarify the fragmentation sequence. Most of the intermediates were confirmed by comparison with chemical standards (Table 4-4). Phthalic acid was identified as dehydrated form and trimethylsilyl derivative. A structure of dihydroxyanthrone was suggested using electron impact fragmentation. The fragmentation pathways of MS-MS generated product ions showed a loss of water

molecules from the molecular ion indicating possible ortho position of two hydroxyl groups (M-H_2O= m/z 210$^{+\bullet}$). Other fragmentations suggested a loss of one hydroxyl (m/z 209) and further a loss of carbonyl group (m/z 181). Ion m/z 152 (m/z 181-COH) appeared to be stable under our MS-MS conditions. Another fragmentation could be explained by a loss of oxygen from m/z 209 producing ion m/z 193 and further formation of m/z 165 after a loss of carbonyl.

Table 4-4. Retention data and electron impact mass spectral characteristics degradation products

t_R (min)	MW (CI)	Parent compound	m/z of fragment ions (relative intensity)	Compound suggestion
6.72	148	ANT	148 (2.3), 104 (100), 76 (41.2), 50 (20.4)	phthalic anhydride*§
10.65	310	ANT	310 (3.7), 295 (57.6), 265 (6.4), 221 (27.5), 193 (3.8), 147 (100), 73 (53.1)	phthalic acid di-TMS*
13.00	194	ANT	194 (100), 165 (98.4), 139 (49.6), 81 (37.1)	Anthrone*
13.51	208	ANT	208 (100), 180 (64.2), 152 (58.8), 126 (4.4), 76 (5.9)	9,10-anthracenedione*
14.83	226	ANT	226 (100), 210 (41.5), 209 (44.7), 208 (36.8), 194 (21.1), 193 (23.7), 165 (34.2), 152 (52.6)	(ortho) ?,? -dihydroxyanthrone
19.23	218	PYR	218 (100),189 (40.3), 95 (13.9)	1-hydroxypyrene*
7.7	152	DBT	152 (74.6), 135 (100), 107 (14.5), 92 (10), 77 (20.5)	4-methoxybenzoic acid*
15.03	216	DBT	216 (100), 187(27.6), 168 (17), 160 (21.3), 139 (18.4), 136 (20.2)	dibenzothiophene sulfone*

* structures were later identified by comparison with standards

§ dehydrated form of the metabolite

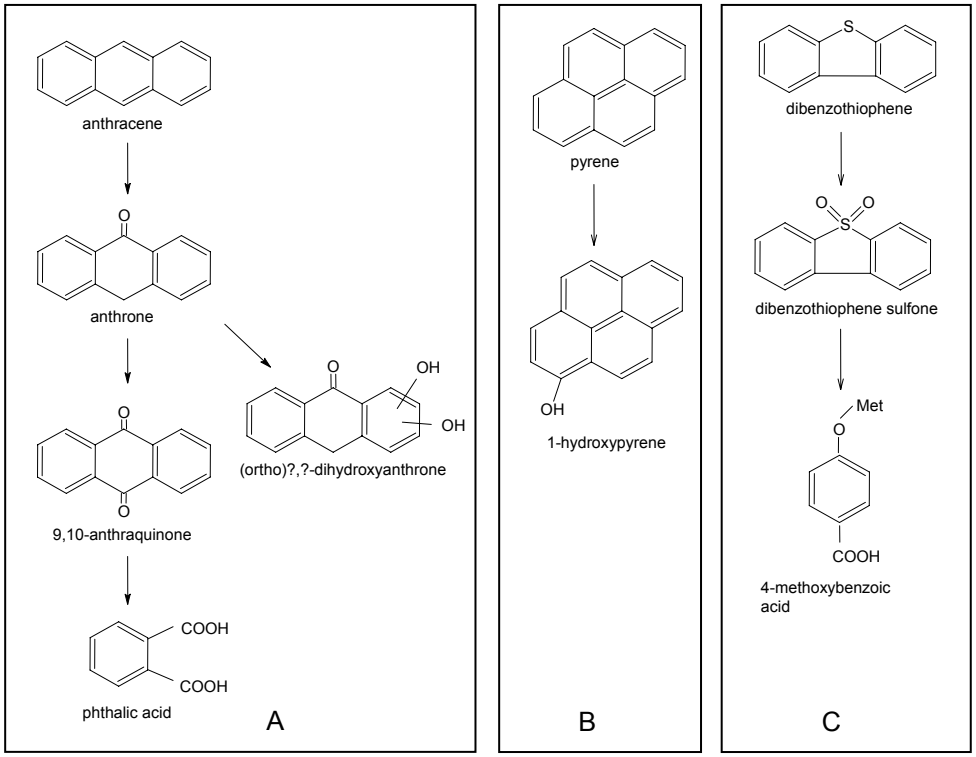


Figure 4-7. Intermediate compounds from the degradation of anthracene (A), pyrene (B) and dibenzothiophene (C) in experiments with MnP from *Bjerkandera* sp. BOS55

During the degradation of ANT by MnP, the formation of anthrone was detected, which was an expected intermediate, and it was followed by the appearance of 9,10-anthraquinone (Cerniglia 1992). This compound was produced at high molar yields, around 50%. Anthraquinone has been earlier described as the common oxidation product in *in vitro* reactions of peroxidases (Hammel, 1995). Further oxidation resulted in the ring cleavage, forming phthalic acid. The biological ring cleavage of PAHs was first considered as a purely bacterial phenomenon. Their metabolism involves a dioxygenase-catalyzed oxidation which leads to the ring fission (Gibson and Subramanian 1984). However, ligninolytic fungi are the only eukaryotic cells that have been shown to form quinones and a subsequent ring cleavage from the degradation of PAHs (Hammel 1995). This process had been considered independent from the ligninolytic system (Hammel et al. 1992) or at least, the presence of a redox mediator like glutathione or unsaturated lipids was necessary to carry out the cleavage (Moen and Hammel 1994; Sack et al. 1997a).

The present work and the recent one presented by Baborova et al. (2006), concluded that MnP can lead to the ring fission of the PAHs in the absence of any mediator.

It was also detected a structure that was assigned as dihydroxyanthrone with ortho hydroxyl radicals. This compound, together with production of 1-hydroxypyrene from PYR, indicates a direct hydroxylation by •OH radicals during oxidative process. On the other hand, it was not detected any formation of pyrenediones (Kästner 2000). DBT was transformed to dibenzothiophene sulfone (Bezalel et al. 1996; Ichinose et al. 2002) and, a ring cleavage product 4-methoxybenzoic acid, was detected.

4.3.4. PAH oxidation by Mn^{3+}

It was investigated the chemical oxidation of compounds of this nature by Mn^{+3} in an experiment with manganese(III) acetate. The conditions were the same as the described for the enzymatic assays (5 mg/L PAH, 36% or 45% acetone, 20 mM malonic acid) but in absence of enzyme and hydrogen peroxide. Two concentrations of Mn^{3+} were assayed: 20 μM, which was the amount used for the *in vitro* experiments, and a much higher concentration, 1000 μM.

When the concentration of Mn^{3+} was 20 μM there was not appreciable reduction on DBT and PYR concentration after 24 h of reaction (Table 4-5). In the case of ANT, 8% of degradation was observed after 2 h of the experiment and no higher oxidation was produced in 24 h. Experiments with 1000 μM Mn^{3+} showed an oxidation of 29% for ANT and 21% for DBT after 2 h, but in the case of PYR no degradation was achieved. After 24 h, there was an extra oxidation for ANT and DBT.

Table 4-5. Residual PAH (in percentage) after 2 and 24 h in experiments with manganic acetate

PAH	20 μM Mn^{3+}		1000 μM Mn^{3+}	
	2 h	24 h	2 h	24 h
ANT	92	91	71	68
DBT	97	97	79	75
PYR	100	100	100	100

As stated by Paice et al. (1995), it can be argued that the Mn^{3+} complex can be more easily generated by chemical or electrochemical means, avoiding the difficulties involved in working with the enzymes. However, the obtained results

showed that the concentration of Mn^{3+} ions required for the degradation of the three PAHs was higher than the concentration used for *in vitro* assays. A concentration of 1000 μM Mn^{3+} (50 fold the concentration used in the *in vitro* experiments) only degraded 32% and 25% of ANT and DBT, respectively and, therefore, its catalytic formation by MnP seems a better option. In the case of PYR higher concentrations of Mn^{3+} should be used, because no oxidation was detected for the concentrations studied. Moreover, it has been shown that the chemical reaction with manganic acetate was considerably rapid since no significant differences were found after 2 and 24 h of reaction. Finally, the order of degradability was in agreement with the results obtained in the experiments with the enzyme, but not with the expected order related to their IP.

4.4. Conclusions

The first objective of this chapter was to evaluate the oxidative action of MnP from *Bjerkandera* sp. BOS55 for the degradation of PAHs. Several aromatic compounds with different physical characteristics, such as number of rings (3 or 4), water solubility (from 0.02 to 1.98 mg/L) or IP (from 7.4 to 8.2), were assayed. PAHs with IPs higher than 7.7 (FLT, FLR, PHE and CHR) were not degraded in experiments of 24 h.

In the case of ANT and PYR (IPs: 7.4 and 7.5 eV, respectively), crude MnP was sufficient to initiate and promote their degradation. Even more, the heterocyclic compound DBT with an IP much higher (8.1 eV) could be degraded by MnP, which suggests that the limit established by Cavalieri and Rogan (1985) in 7.8 eV is not definite. Moreover it is not recommended to set a threshold value due to the high variability of the IPs. The degradation attained in the present work was optimized and we presented results of degradation.

Chemical oxidation experiments showed that higher concentrations of Mn^{3+} are required to imitate the enzymatic reaction of MnP. Even more, the higher concentration assayed, 1000 μM was not enough to initiate the oxidation of PYR, whereas for *in vitro* experiments 20 μM of Mn^{3+} led to a 55% of degradation after 24 h.

Anthraquinone was the main product detected in the degradation of ANT. The other intermediates from the degradation of DBT and PYR were detected in small traces. The *in vitro* degradation of ANT and DBT led to the ring cleavage of both molecules, process which had been conventionally considered independent of the ligninolytic system, or at least, related to the presence of a redox mediator. From the intermediate compounds detected in the degradation of ANT and PYR, we concluded that $^{\bullet}OH$ radicals were involved during oxidative process.

4.5. Acknowledgments

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Chapter 5

Enzymatic degradation of anthracene in fed-batch and continuous reactors containing acetone:water mixtures. Modeling

Summary

The optimization of the degradation of anthracene by MnP in batch reactors containing acetone:water mixtures was described in previous chapters. In order to scale-up the process a comprehensive knowledge of kinetics is essential for the design and optimization of the operation. In the present chapter the kinetics of the degradation of anthracene by MnP was studied in fed-batch reactors and the obtained equation was then applied to semi-continuous and continuous reactors. Although H_2O_2 and Mn^{2+} are the primary substrates of MnP, anthracene was considered as the substrate of the enzymatic reaction. Fed-batch experiments, where MnP was added in order to maintain the activity in a specific range, showed that degradation rates increased with time, which could be explained by an autocatalytic process due to the formation of the degradation products, such as anthraquinone. The proposed model, together with the MnP decay kinetics, was applied to predict the time course of anthracene in a semi-continuous (with continuous addition of all compounds with the exception of MnP) and continuous reactor. Results in both cases showed that MnP activity in the reactor is a factor to consider in the model of the process. The operation of the continuous reactor for 108 h demonstrated the feasibility of the system.

Outline

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5.1. Introduction

The enzymatic bioconversion processes are of increasing use in the production, transformation and valorization of raw materials. The reactions are usually carried out in a batch reactor where the enzymes are dissolved in an aqueous reaction medium. The use of such reactors is relatively simple at any scale. Nevertheless, this type of bioreactor presents a certain number of disadvantages, especially for the processing of large quantities of raw materials in industrial practice. Their relatively high labor, operational costs, low productivity, great variability of the product quality and the time required for shutdowns are the main disadvantages in batch processes (Ríos et al. 2004).

These drawbacks can be partially solved by means of continuous reactors which provide products with homogeneous quality at higher yields, lower operational costs and an improved control of the process (López et al. 2002). In an industrial application the economic feasibility of the enzymatic process will be likely influenced by the lifetime of the enzyme (Buchanan et al. 1998). In order to achieve an economically viable process, the inactivation or loss of enzyme in the effluent should be minimized. Several approaches were carried out in batch experiments with the aim of minimizing the enzymatic inactivation (Chapter 3).

In continuous processes the optimization of the enzymatic reactor is of major importance. Rigorous design and operation under controlled optimized conditions must be undertaken (Illanes and Wilson 2003), and for this purpose, a comprehensive knowledge of kinetics is essential. A Michaelis-Menten model is the most widely used one to predict the kinetics of enzymatic reactions. The rate of the reaction is defined by:

$$r = \frac{r_m \cdot S}{K_M + S} \tag{5-1}$$

The model was developed based on the assumed hypothesis that the free enzyme is combined with the substrate to form an enzyme-substrate complex, which is further dissociated into free enzyme and product. The validity of this approach requires a high substrate-enzyme ratio, considering that the enzyme is not prone to significant inactivation or inhibition, and that the formation and decay of the enzyme-substrate complex occurs at steady-state conditions (Bailey and Ollis 1986). Nonetheless, the enzyme deactivation is generally significant and especially evident when the enzymatic process is performed for an extended period of reaction. Moreover, in some processes the enzymatic reactions do not follow a simple sequence of events as those described by Michaelis-Menten (Segel 1993). That is the case of reactions carried out by MnP, where two different substrates are used during the catalytic cycle (Fig. 1-3). These steps include the reduction of H_2O_2,

the oxidation of Mn^{2+} and the formation of the complex Mn^{3+}-organic acid. In this case, as was mentioned in previous chapters, the enzyme inactivation is significant and thus, this consideration should be present in the model.

The objective of this Chapter is to develop a kinetic model of the degradation of anthracene by MnP. By means of the analysis of the substrate conversion, products generation and enzyme consumption, a model describing those parameters was proposed. Different configurations of the reactor were also taken into account to determine the influence of other factors affecting the process kinetics.

5.2. Materials and methods

5.2.1. Enzyme and chemicals

Crude MnP was obtained from cultures of *Bjerkandera* sp. BOS55 as described in previous chapters.

Anthracene was obtained from Janssen Chimica (95-99% purity). Acetone was obtained from Panreac (chemical purity). H_2O_2 (30% v:v), sodium malonate and manganese sulphate were from Sigma-Aldrich.

5.2.2. Fed-batch reactors

Oxidation of anthracene was carried out in 100-mL Erlenmeyer flasks sealed with Teflon plugs, under magnetic stirring and at room temperature, i.e. 22ºC±1ºC. The reaction mixture (50 mL) consisted of acetone 36% (v:v), anthracene (5 mg/L), MnP (200 U/L), Mn^{2+} (20 μM) and malonic acid (20 mM) at pH 4.5. The reaction started with the continuous addition of 5 μmol/L·min of H_2O_2 with a peristaltic pump at low flow (around 20 μL/min). The dilution effect caused by H_2O_2 addition was considered to calculate the concentrations in the reactor.

Anthracene (250 μL from a stock solution of 1 g/L in acetone) or MnP (2.5 mL of enzymatic crude) were periodically added in the reactor when concentrations of these compounds were negligible. Samples were withdrawn periodically to determine anthracene and anthraquinone concentrations by high pressure liquid chromatography (HPLC), and evolution of MnP activity was spectrophotometrically determined. To verify that degradation took place only due to an enzymatic oxidation, controls were run in parallel using boiled MnP. No change in anthracene concentration after 6-8 h of incubation was observed in any controls (data not shown).

5.2.3. Semi-continuous reactor

Oxidation of anthracene was carried out in 250-mL Erlenmeyer flasks sealed with Teflon plugs, with magnetic stirring at room temperature, i.e. 22ºC±1ºC. The

volume of the reactor was 150 mL and the hydraulic retention time (HRT) was 11.5 h. The concentrations of the different compounds in the reactor at the beginning of the reaction were as follows: anthracene 5 mg/L, acetone 36% (v:v), sodium malonate 20 mM, Mn^{2+} 20 μM and MnP 200 U/L. The process was initiated by the addition of two solutions:

i) a solution containing anthracene 6.03 mg/L, acetone 42%, Mn^{2+} 23.4 μM and sodium malonate 23.4 mM (at pH 4.5) was pumped at 10 mL/h using a high precision pump P-500 (Pharmacia) with Teflon tubes to avoid anthracene adsorption.

ii) H_2O_2 5 μmol/L·min was pumped at 2 mL/h using a Masterflex peristaltic pump (Cole Palmer). The solution was stored in a cool box and periodically changed.

During the time course of the experiments, MnP pulses were regularly added once the activity into the reactor reached zero to restore the enzyme concentrations to levels around 200 U/L.

5.2.4. Continuous reactor

Oxidation of anthracene was carried out in a continuous reactor (Fig. 5-1) under identical conditions to those of semi-continuous reactor. In this case, the process was initiated by the addition of three solutions:

i) a solution containing anthracene 7.3 mg/L, acetone 52%, Mn^{2+} 28.9 μM and sodium malonate 28.9 mM (at pH 4.5) was pumped at 9 mL/h using a high precision pump P-500 (Pharmacia) with Teflon tubes to avoid anthracene adsorption.

ii) H_2O_2 5 μmol/L·min was pumped at 2.5 mL/h through a Masterflex peristaltic pump (Cole Palmer).

iii) MnP addition rate was varied throughout the experiment: 36, 0, 50 and 75 U/L·h. Different stock solutions of crude (3350, 4630 and 7500 U/L for 36, 50 and 75 U/L·h, respectively) were added at 1.6 mL/h using a Masterflex peristaltic pump (Cole Palmer). MnP crude was stored in a cool box to avoid thermal inactivation.

5.2.5. Analytical techniques

The concentrations of anthracene and anthraquinone were measured by HPLC. MnP activity was determined spectrophotometrically following the oxidation of 2,6-dimethoxyphenol as described in Chapter 3.

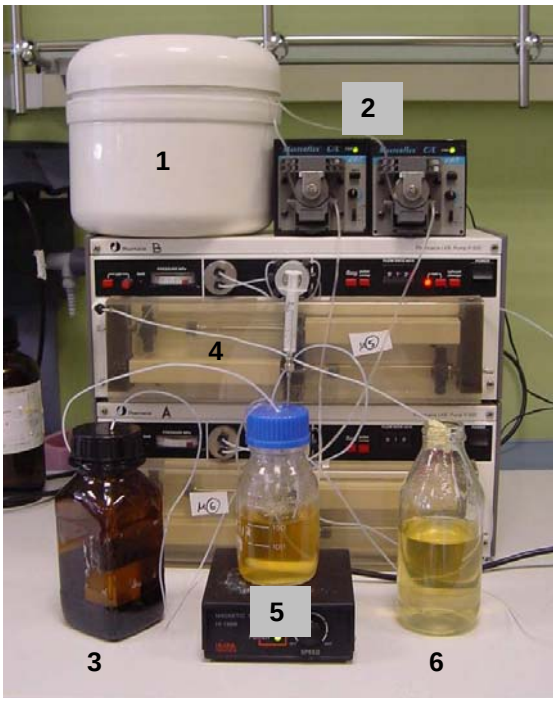

Figure 5-1. Picture of the continuous reactor scheme. 1: Cooler box containing H_2O_2 and crude MnP, 2: Peristaltic pumps for H_2O_2 and enzyme, 3: Solution of anthracene, acetone, malonate and Mn^{2+}, 4: Precision pumps with Teflon tubes for the input and output flow, 5: Magnetically stirred reactor, 6: Effluent.

5.2.6. Method of numerical integration

A software package using an algorithm based on a Runge-Kutta formula (the Dormand-Prince pair) was used to solve the set of nonlinear ordinary differential equations. It solves the equations in one step: computing $y(t_n)$, the solution at the immediately preceding time point, $y(t_{n-1})$, is only required.

5.3. Results and discussion

5.3.1. Development of the kinetic model and enzyme decay equation

Batch experiments were performed in order to evaluate the kinetic parameters of the enzymatic reaction and the inactivation kinetics of MnP (Exp 1.1 and 1.2). MnP was added in pulses in order to maintain an enzymatic activity in the range 100-200 U/L, as was previously described for the treatment of dyes when MnP activities below 100 U/L were found to limit the extent of the reaction (Mielgo et al. 2003).

Figure 5-2 shows the time course profiles of two identical experiments performed during 7 h. A pulse of enzyme was added after 3 h when the MnP activity was below 100 U/L.

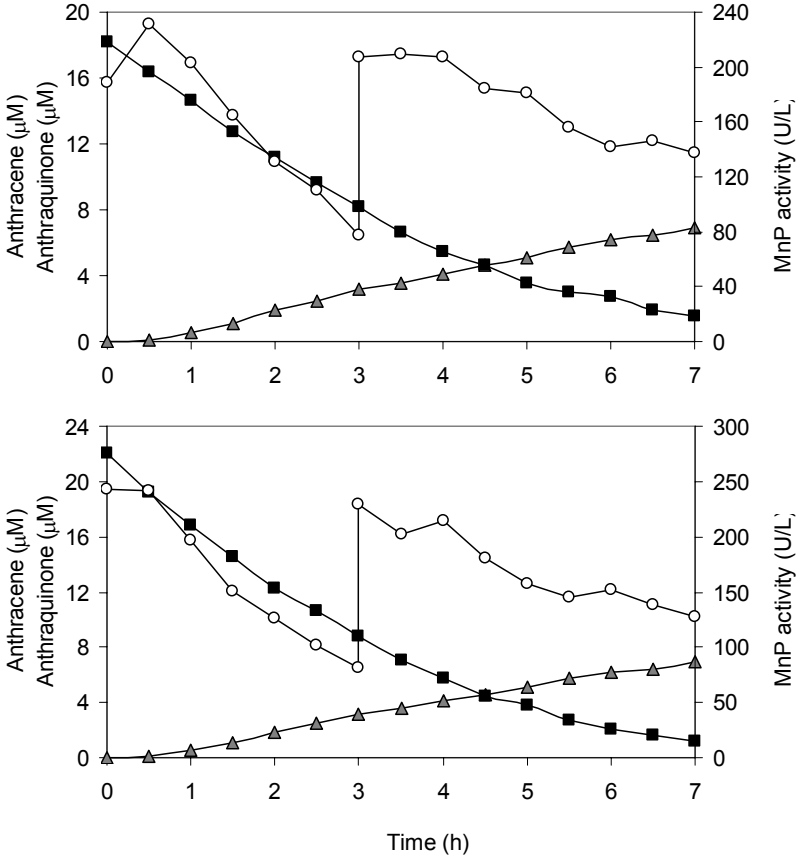

Figure 5-2. Time course of anthracene disappearance (■), anthraquinone production (▲) and MnP enzymatic activity (○) during fed-batch experiments of MnP (Exp. 1.1 and 1.2)

Kinetic model

Kinetic model reported for horseradish peroxidase (HRP) in the degradation of phenol considers all the steps in the catalytic cycle of the enzyme (Nicell 1994). Both substrates, H_2O_2 and the aromatic compound, are included in the kinetic equation, as well as the different forms of the enzyme. However, in the present work, the concentration of H_2O_2 in the medium was nearly zero. The addition rate was previously optimized to a flow rate of 5 μmol/L·min (Chapter 3), resulting in non-detectable concentrations. The other substrate, Mn^{2+} which is converted to

Mn^{3+}, is regenerated in each cycle of the enzyme. The real oxidizing agent of anthracene is the complex Mn^{3+}-malonate. When the enzymatic reaction is faster than the oxidation of the final compounds, the latter reaction turns out to be the limiting step in the degradation process, and therefore, the global kinetics matches up with the degradation kinetics of the final substrates. For these reasons we consider anthracene as the substrate of the enzymatic reaction.

As a preliminary approach, first-order kinetics was considered. The integrated form of the kinetic equation would permit to obtain the catalytic constant, k_{cat} (Eq. 5.2):

$$\ln S = \ln S_0 - k_{cat} \cdot t \qquad (5\text{-}2)$$

Figure 5-3 shows the adjustment to first-order kinetics. Although the experimental data fit well to the model (r^2=0.99), it is important to highlight that there is an increase of the degradation rate with time. This would mean that the first-order model related to the substrate concentration is not accurate, indicating that the degradation rate is not only dependent on the substrate concentration. However, the enzyme does not seem to be responsible of this increase of velocity, as can be deduced by the fact that the highest values of MnP activities were present at the beginning and in the middle of the reaction and they were not coincident with the highest rates.

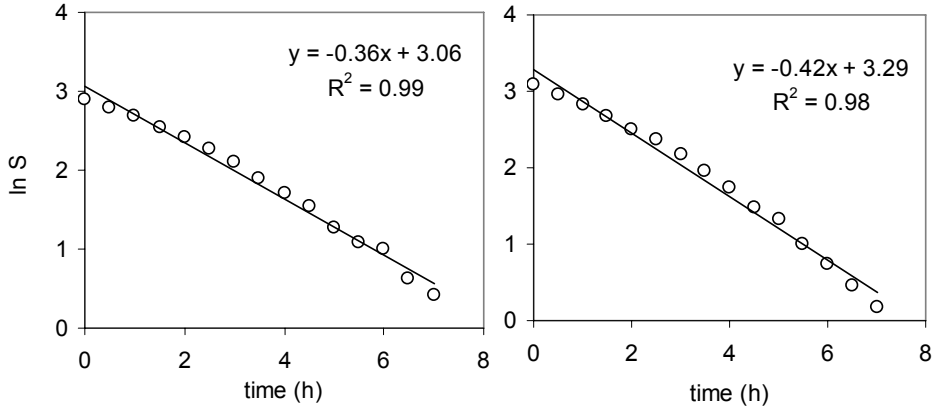

Figure 5-3. Linearization of the first-order kinetic model for the anthracene degradation (Exp. 1.1 and 1.2)

A possible explanation could arise from the autocatalytic effect of the products formed in the reaction. Anthraquinone is the main metabolite produced in the degradation of anthracene by MnP (Eibes et al. 2006) and as it can be seen in Figure 5-2, 40% of anthracene was converted to anthraquinone. In the literature, quinones were described to play a role as electrons carriers, thus increasing overall

degradation rates (Méndez-Paz et al. 2005). In order to include this autocatalytic effect, the products of the reaction were considered in the model; therefore not only anthraquinone but also other intermediates present in the mechanism of degradation were taken into account. The equation having into account first-order kinetics related to the substrate and the autocatalytic process is given by Eq. 5-3:

$$r_S = -(a + b \cdot P) \cdot S \tag{5-3}$$

In a batch experiment and considering $P=\Sigma S_0-S$:

$$\frac{dS}{dt} = -\left[a + b \cdot \left(\sum S_0 - S\right)\right] \cdot S \tag{5-4}$$

After the integration:

$$S = \frac{\frac{a}{b} + \sum S_0}{1 - \left(1 - \frac{a}{b \cdot S_0} - \frac{\sum S_0}{S_0}\right) \cdot \exp\left[\left(\frac{a}{b} + \sum S_0\right) \cdot b \cdot (t - t_0)\right]} \tag{5-5}$$

Equation 5-6 indicates the profile of anthracene in a batch reactor, depending on two parameters: *a* and *b*. In the present case there was not an extra-addition of anthracene, therefore $\Sigma S_0=S_0$ and $t_0=0$. By fitting the data from the two identical experiments to the equation 5-6, the values obtained for each parameter can be obtained (Table 5-1).

Table 5-1. Parameter estimation for the experiments with one pulse of MnP (Exp. 1.1 and 1.2)

Parameter	Exp	Estimation	Std deviation	Confidence interval 95%	
				Lower limit	Upper limit
a	1.1	0.192	0.004	0.183	0.200
	1.2	0.225	0.006	0.212	0.238
b	1.1	0.015	0.001	0.014	0.017
	1.2	0.013	0.001	0.011	0.014

Regression coefficients: $r^2_{1.1} = 0.999$; $r^2_{1.2} = 0.999$

The mean value of the parameters was calculated: a = 0.209, b = 0.014 and they were used to fit data in both experiments (Fig. 5-4).

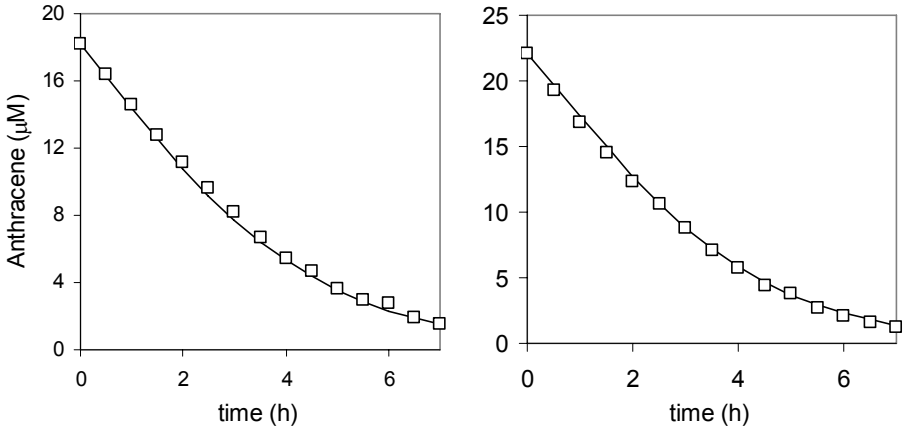

Figure 5-4. Fitting of anthracene concentration to the model given by Eq. 5-5 (Exp. 1.1 and 1.2)

Enzyme decay model

Although the enzymatic activity was not considered in the model, the inactivation of MnP was evaluated as first-order decay as commonly described in literature (Baldascini and Janssen 2005; Buchanan and Nicell 1997; Wu et al. 1999) (Eq. 5-6):

$$\ln E = \ln E_0 - k_d \cdot (t - t_0) \tag{5-6}$$

Figure 5-2 shows the data of enzymatic activity in experiments 1.1 and 1.2. There were two additions of MnP (at the beginning and after 3 h) and the analysis of each period led to different decay constants (Fig. 5-5). In the first phase, MnP activity suffered from higher inactivation than in the second phase. A similar behavior was observed in both experiments and the values of k_d for each period were comparable.

We considered the inactivation of MnP as a first order decay kinetics. The inactivation caused by temperature, pressure, H_2O_2 or the presence of solvents of different peroxidases such as HRP or prostaglandin H synthase has also been described as single exponential kinetics (Buchanan and Nicell 1997; Wu et al. 1999). It is noteworthy that enzyme inactivation had two different periods in batch experiments: during the first period (the three first hours) the decay constant was higher than the second one. Inactivation kinetics of MnP were described as biphasic, suggesting a sequential two-step process, which is related with the loss of Ca^{2+} ions (Reading and Aust 2000; Sutherland and Aust 1996).

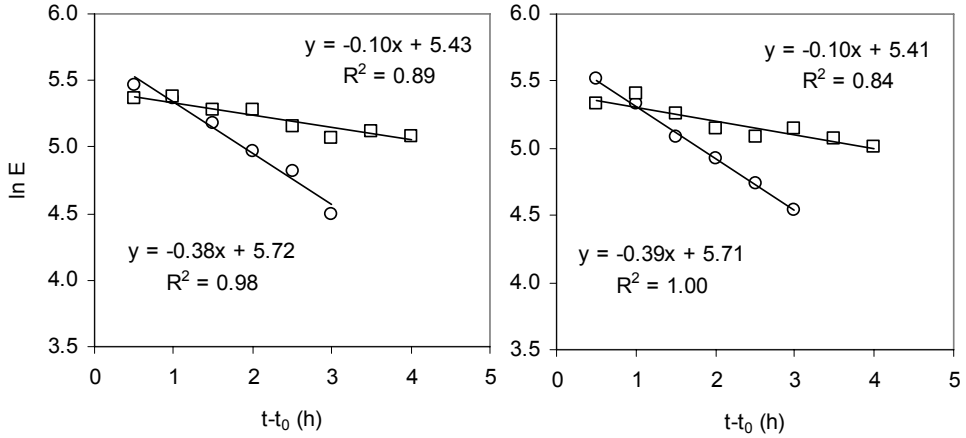

Figure 5-5. First order decay kinetics in Exp. 1.1 and 1.2. Symbols: ○ first-stage experimental data; □ second-stage experimental data

5.3.2. Verification of the model in fed-batch reactors

MnP and anthracene fed-batch reactor

The next experiment was similar to the previous one but with the addition of anthracene in two pulses at 3 and 6 h (Exp. 2). The enzymatic activity was maintained above 100 U/L by means of the addition of a pulse of MnP. The time-course of the reaction is plotted in Fig. 5-6.

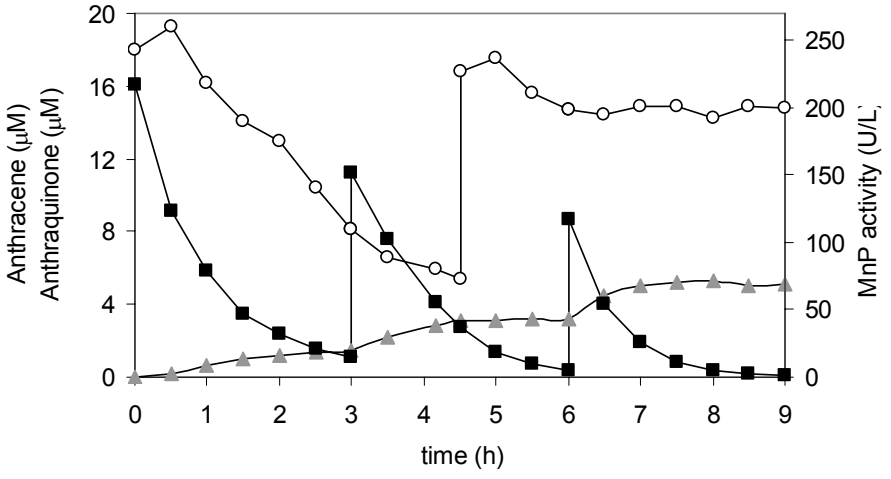

Figure 5-6. Time course of Anthracene (■), Anthraquinone (▲) and MnP enzymatic activity (○) during the fed-batch experiment of MnP and anthracene (Exp. 2)

The model proposed by equation 5-6 was applied to the data obtained in this experiment. The regression was evaluated in three sections, since different initial concentrations of substrate were present. The products at a given time were a function of the sum of the substrates added till this moment ($P=\Sigma S_0-S$). The kinetic parameters obtained from the model proposed by equation 5-6 are summarized in Table 5-2 and the fitting of anthracene is shown in Fig. 5-7.

Table 5-2. Parameter estimation for Exp. 2

			Confidence interval 95%	
Parameter	Estimation	Std error	Lower limit	Upper limit
a	0.604	0.015	0.573	0.635
b	0.019	0.001	0.017	0.021

Regression coefficient: r^2= 0.998

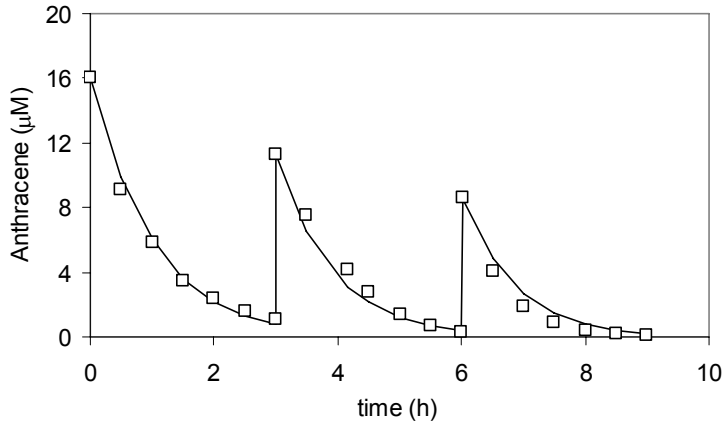

Figure 5-7. Fitting of anthracene concentration to the autocatalytic model proposed by Eq. 5-6 (Exp. 2)

Although the parameter *b* is fairly similar to that obtained in the first experiments (0.014 and 0.019), the parameter *a*, which refers to first-order kinetics related to substrate, was almost 3-fold higher than the previous one.

A first-order decay model was applied to the enzymatic activities from Figure 5-6. As was observed in the previous experiments, there was a marked difference between the two decay constants during the reaction (Fig 5-6). During the second stage, the enzymatic activity practically was maintained at 200 U/L, which resulted in bad fitting (r^2=0.34) and a decay constant practically zero (0.03).

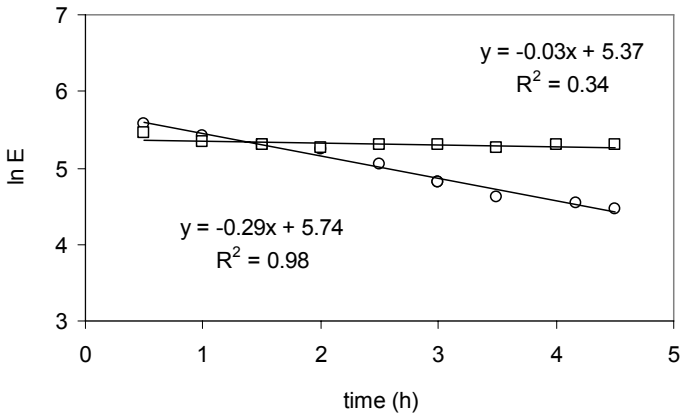

Figure 5-8. First order decay kinetics in Exp. 2. Symbols: ○ first-stage experimental data the first stage; □ second-stage experimental data

Anthracene fed-batch reactor

The effect of two pulses of anthracene with no addition of MnP was studied (Exp. 3). In this case, the effect of low values of enzymatic activity at the end of the reaction was evaluated. The time-course of the reaction is plotted in Figure 5-9.

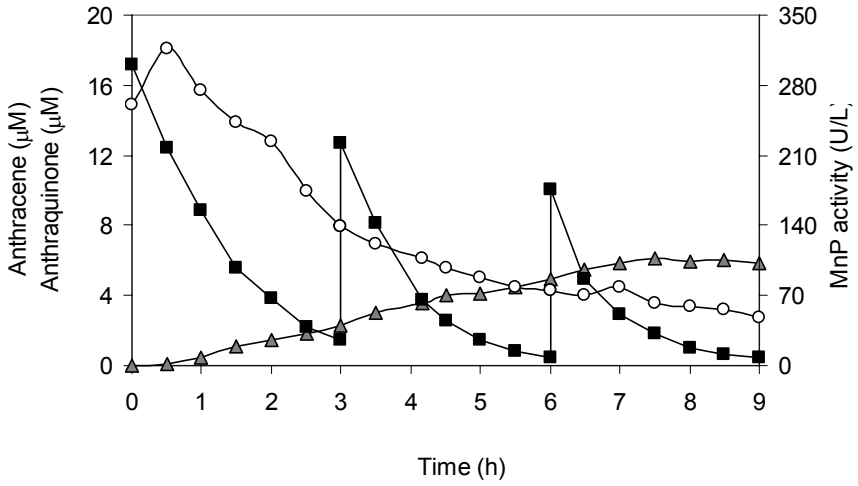

Figure 5-9. Time course of Anthracene (■), Anthraquinone (▲) and MnP enzymatic activity (○) during Exp. 3

The kinetic parameters obtained from the fitting to the model proposed by Eq. 5-6 are shown in Table 5-3. Figure 5-10 illustrates the data and the prediction.

Table 5-3. Parameter estimation for Exp. 3

			Confidence interval 95%	
Parameter	Estimation	Std error	Lower limit	Upper limit
a	0.605	0.015	0.573	0.637
b	0.019	0.001	0.016	0.021

Regression coefficient: r^2= 0.998

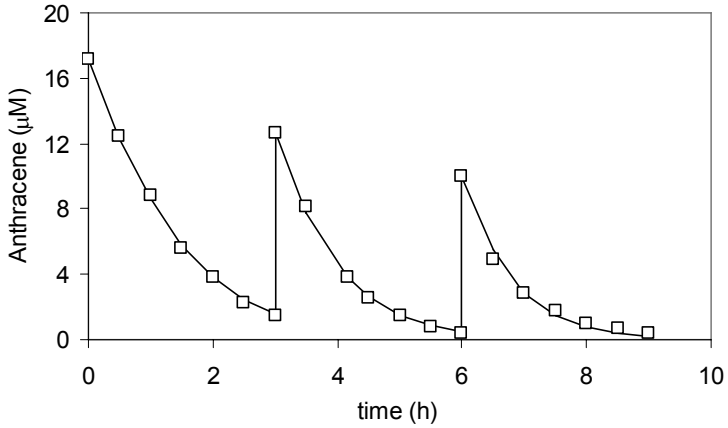

Figure 5-10. Experimental (□) and fitted (-) data of anthracene disappearance in Exp. 3 (r^2 = 0.998)

Although the activity into the reactor decreased below 100 U/L after 5 h, the profile of anthracene was very similar to that predicted by the model. From this experiment it seems that enzymatic activity does not play an important role in the kinetics of degradation. The effect of low enzymatic activities will be studied later in semi-continuous and continuous reactors.

In the present experiment, in spite that the enzyme was not added during the reaction, two stages for enzymatic decay can be identified (Fig. 5-11). The value of the first-decay constant was quite similar to Exp. 2, (0.29 and 0.28, respectively) while in this case the inactivation in the second stage was higher (0.03 and 0.12, respectively).

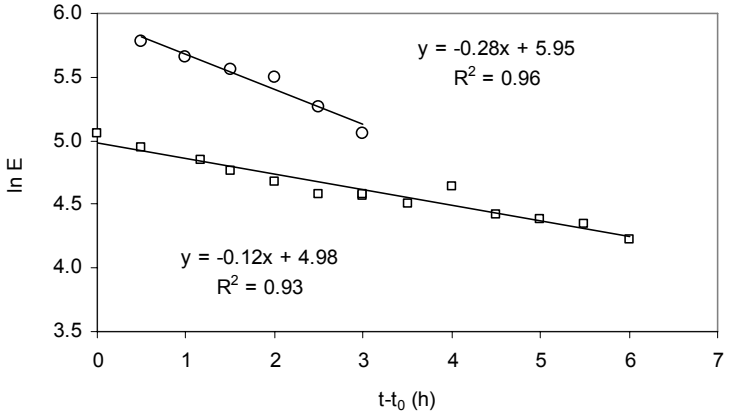

Figure 5-11. First order decay kinetics in Exp. 3. Symbols: ○ first-stage experimental data; □ second-stage experimental data

The values of the catalytic parameters obtained from Exp. 2 and 3 are higher than those obtained from MnP fed-batch experiments (Exp. 1.1 and 1.2), because the reaction rates observed in these experiments were very high. When comparing the first 3 h of reaction, the net anthracene degradation was greater than that obtained in all previous results (see Chapter 3). Enzymatic inactivation in the first stage was also lower than in previous experiments (1.4-fold). Although the initial enzymatic activity in all the experiments was nearly the same, crude MnP from different batches of enzyme could have different properties, since the enzyme was not purified. A non-standard batch of enzyme could cause unexpected degradation rates of anthracene. The proposed model fitted well the data for these experiments, but the values of the kinetic parameters and decay constants obtained in the fitting were not taken into account for the following experiments.

5.3.3. Semi-continuous reactor

In the semi-continuous reactor all the components were added continuously except MnP, which was added (at different concentrations) when the activity in the reactor was nearly zero. Figure 5-12 illustrates the anthracene, anthraquinone and activity profiles. As it can be observed, the enzymatic activity had an influence on the degradation achieved: when the activity decreased below 10 U/L the enzymatic reaction stopped (for example at 5 h) and if no addition of MnP was performed, the anthracene concentration in the reactor increased and anthraquinone decreased (for example at 15 h). The highest values of enzymatic activity in the reactor enabled to obtain the highest values of degradation (82 h).

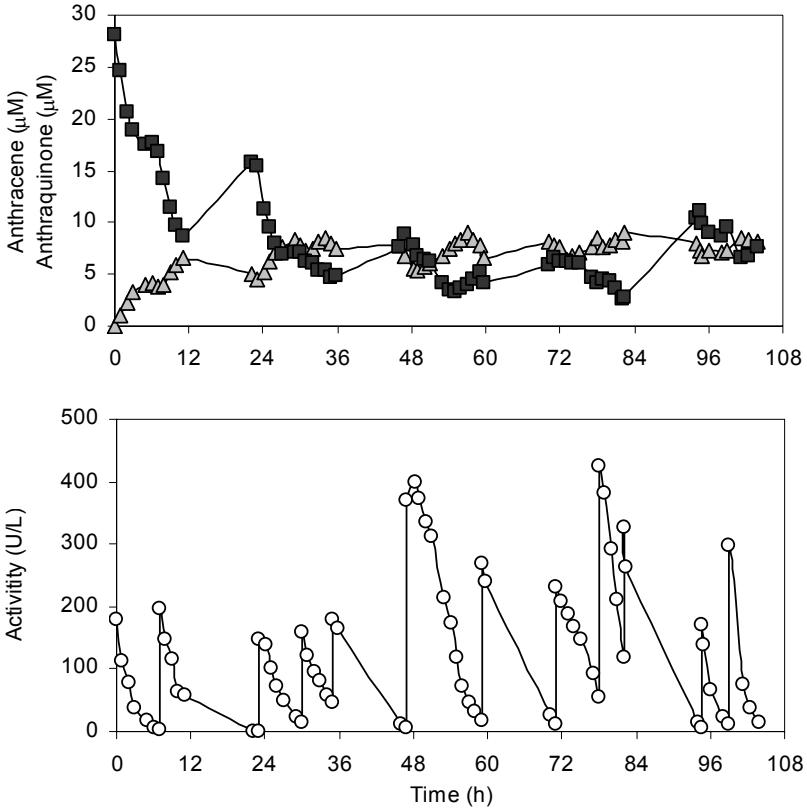

Figure 5-12. Time course of anthracene disappearance (■), anthraquinone production (▲) and MnP enzymatic activity (○) in the operation of the semi-continuous reactor

Modeling

The three differential equations which describe anthracene, products and enzyme in the semi-continuous reactor are given by:

$$\frac{dS}{dt} = \frac{Q_i}{V_R}\cdot S_i - \frac{1}{\tau}\cdot S - (a + b\cdot P)\cdot S \qquad (5\text{-}7)$$

$$\frac{dP}{dt} = -\frac{1}{\tau}\cdot P + (a + b\cdot P)\cdot S \qquad (5\text{-}8)$$

$$\frac{dE}{dt} = -\frac{1}{\tau}\cdot E - k_d\cdot E \qquad (5\text{-}9)$$

where Q_i: 0.01098 L/h, is the input flow of the substrate; V_R: 0.150 L, is the volume of the reactor; S_i: 34.9 μM, is the concentration of the substrate in the input flow; τ: 11.5 h^{-1}, is the hydraulic retention time (HRT) and k_d = 0.25 h^{-1} (calculated

as the average value of k_{d1} and k_{d2}), except for the for the initial period (0-7 h) and the last period (94.5-108 h) where k_d was increased to 0.55 h^{-1} since the inactivation was much higher than in the course of the reaction. The kinetic constants were defined according to the enzymatic activity into the reactor:

i) When the enzymatic activity into the reactor was higher than 10 U/L, the values of the kinetic constants were those obtained from fed-batch experiments: For E>10 U/L, a = 0.209 μM^{-1} h^{-1}; b = 0.014 h^{-1};

ii) When the enzymatic activity was below 10 U/L, the enzymatic reaction stopped: E<10 U/L, a=b=0.

The equations 5-7 to 5-9 were integrated for each stage of the semi-continuous process using a numerical integration method (Fig. 5-13). For each period, after the addition of enzyme, the initial MnP activity was included.

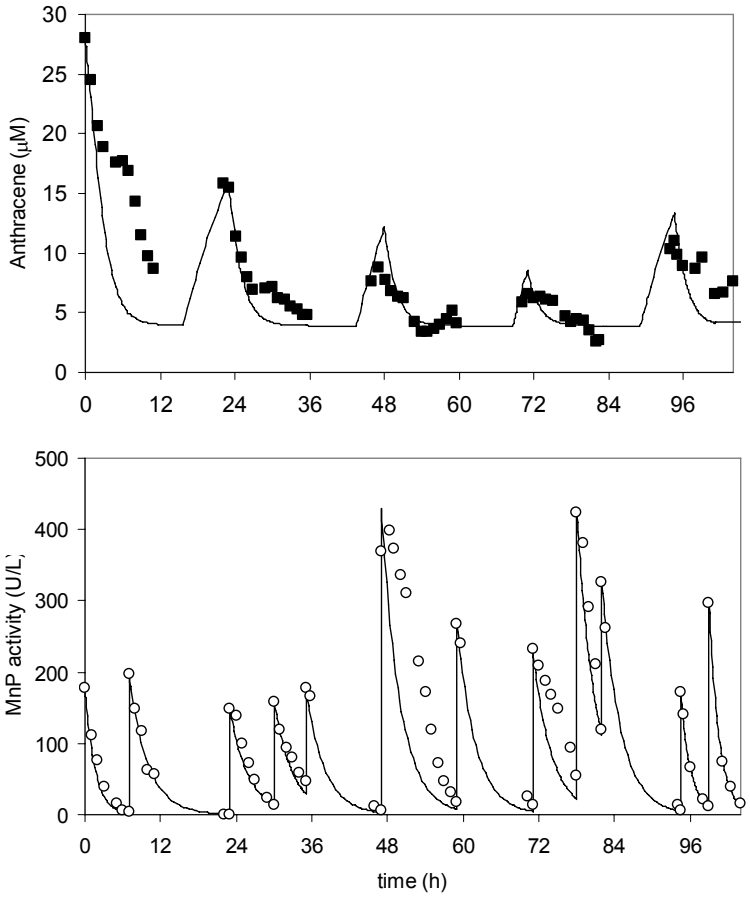

Figure 5-13. Fitted (-) and experimental data of anthracene disappearance (■) and MnP enzymatic activity (○) during semi-continuous experiment

5.3.4. Continuous reactor

Experiment

A continuous reactor operating at a HRT of 11.5 h was performed. Different MnP addition rates were studied: I) 36, III) 50 and IV) 75 U/L·h, in order to evaluate the effect of different stationary activities of MnP inside the reactor (Fig. 5-14). During stage II there was no addition of MnP, which led to an accumulation of anthracene in the reactor.

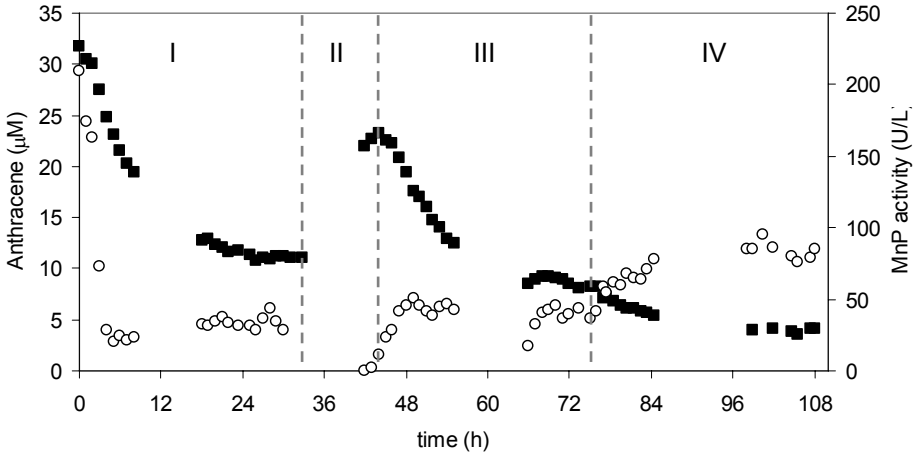

Figure 5-14. Time course of anthracene (■) and MnP enzymatic activity (○) at different addition rates of MnP: I) 36 U/L·h, II) no addition of MnP, III) 50 U/L·h and IV) 75 U/L·h

We observe that steady state was achieved after 24 h in the first stage and less than 20 h for III and IV. In stage III, a failure of enzyme pump was detected (around 66 h), reaching again the stationary conditions after 5 h. Data showed that this slight difference of enzymatic activity in the reactor implied different degradation extents. The steady state values for the concentration of anthracene and enzyme are shown for each stage in Fig. 5-15. The higher the enzymatic activity was into the reactor, the lower anthracene concentration.

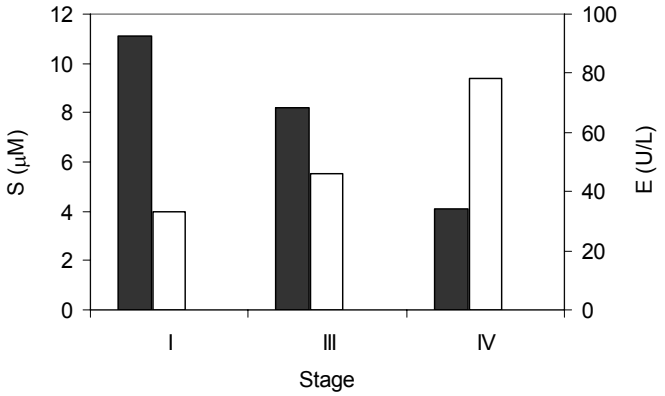

Figure 5-15. Steady state values of the Substrate (■) and Enzyme (□) for the three different stages of the continuous process

Modeling

Since degradation of anthracene is dependent on enzymatic activity into the reactor, a new unknown function, Y(E), was included in the kinetics. Mass balances of anthracene and products in the continuous reactor are given by equations 5-10 and 5-11:

$$\frac{dS}{dt} = \frac{Q_i}{V_R}\cdot S_i - \frac{1}{\tau}\cdot S - y(E)\cdot(a + b\cdot P)\cdot S \qquad (5\text{-}10)$$

$$\frac{dP}{dt} = -\frac{1}{\tau}\cdot P + y(E)\cdot(a + b\cdot P)\cdot S \qquad (5\text{-}11)$$

By applying the conditions of steady state for substrate and products (dS/dt=dP/dt=0), and using the stationary values of S and E from Fig. 5-15, the stationary values of Y(E) and P were obtained for each steady state condition (Table 5-4).

Table 5-4. Values of products concentration and Y(E) for the three steady states corresponding to the three stages

Stage	I	III	IV
P (μM)	17.2	20.1	24.2
Y(E)	0.299	0.434	0.936

The correlation of Y(E) was formulated considering two additional conditions: a) when no enzyme is present in the reactor, no enzymatic reaction took place, i.e. y(0)=0; and b) considering that this function was taken out in the fed-batch

experiments where the activity was maintained in the range 100-200 U/L, its value for the average enzymatic activity of those experiments, 167 U/L, would be Y(167)=1.

The equation which fulfils all the requirements is given by equation 5-12 (r^2=0.98), and is represented in Fig. 5-15. The function has a sigmoid shape, which agrees with results found in literature, which reported that MnP increase did not improve the degradation at levels higher than the optimal (Mielgo et al. 2003). In Chapter 3, where different initial MnP activities were assayed, the maximum degradation was obtained for an average activity of 185 U/L (the initial activity was 550 U/L) and the increase of MnP activity did not improve degradation. However, low enzymatic activities (below 100 U/L) greatly influenced the extent of degradation.

$$y(E) = -0.027 + \frac{1.043}{1 + 26.822 \cdot \exp(-0.070 \cdot E)} \qquad (5\text{-}12)$$

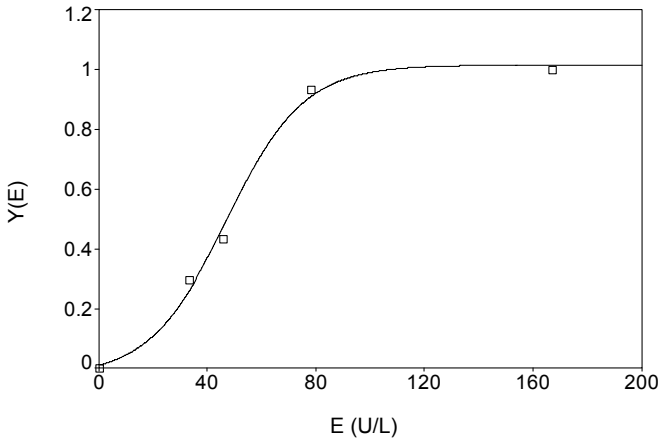

Figure 5-15. Function dependent on the enzymatic activity: Y(E)

The complete model of the process is represented by the set of the three following differential equations (Table 5-5):

Table 5-5. Continuous reactor modeling

$$\frac{dS}{dt} = \frac{Q_i}{V_R}\cdot S_i - \frac{1}{\tau}\cdot S + \left(-0.027 + \frac{1.043}{1 + 26.822 \cdot \exp(-0.070 \cdot E)}\right)\cdot(0.209 + 0.014\cdot P)\cdot S \quad (5\text{-}13)$$

$$\frac{dP}{dt} = -\frac{1}{\tau}\cdot P + \left(-0.027 + \frac{1.043}{1 + 26.822 \cdot \exp(-0.070 \cdot E)}\right)\cdot(0.209 + 0.014\cdot P)\cdot S \quad (5\text{-}14)$$

$$\frac{dE}{dt} = \frac{Q_e}{V_R} \cdot E_i - \frac{1}{\tau}\cdot E - k_d\cdot E \quad (5\text{-}15)$$

where Q_i: 0.009 L/h, is the input flow of the substrate; V_R: 0.150 L, is the volume of the reactor; S_i: 41.0 μM, is the concentration of the substrate in the input flow; τ: 11.5 h^{-1}, is the hydraulic retention time (HRT) and k_d=1 h^{-1}. Enzymatic flow varied according to the stages, as shown in Table 5-6:

Table 5-6. Characteristics of the MnP input flow during the different stages

Stage	I	II	III	IV
Q_e (L/h)	0.0016	0	0.0016	0.0017
E_i (U/L)	3350	3350	4630	7500

The numerical integration in the four periods of the operation gave the fitting shown in Fig. 5-16. The inactivation of the enzyme in the continuous reactor (k_d = 1 h^{-1}) was greater than in the previous experiments, being 4-fold higher than that obtained before.

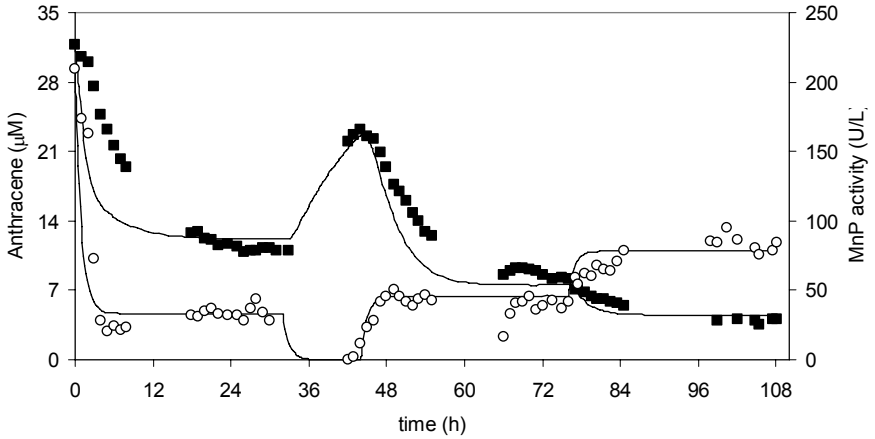

Figure 5-16. Fitting of anthracene (■) and MnP enzymatic activity (○) at different addition rates of MnP in the continuous reactor

Validation

Finally, in order to check the kinetic equation with all the terms (substrate, enzyme and products), the semi-continuous reactor was modeled using equations 5-7, 5-8 and 5-9 and including the sigmoid function given by equation 5-12 (Fig 5-17). After each MnP pulse, the substrates and products concentrations were corrected according to the dilution caused by the enzyme addition.

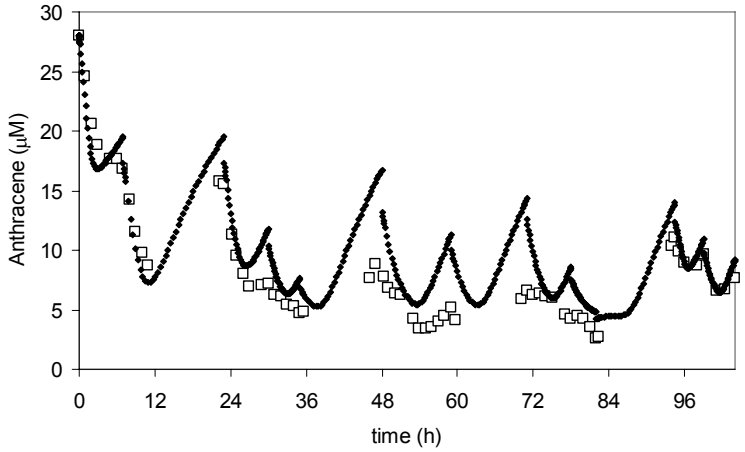

Figure 5-17. Validation of the model in the semi-continuous experiment

The higher discrepancies between the model and experimental data occurred during the period where the adjustment of enzymatic activity to a first-order decay model was less satisfactory, i. e., from 42 to 78 h (Fig. 5-13), and thus indicating that the knowledge of enzymatic inactivation kinetics is essential for the global model of the process.

5.4. Conclusions

A deep knowledge of kinetics is essential for a rigorous design of bioreactors and for their operation under controlled optimized conditions. In enzymatic processes the complexity of the models is defined by the number of parameters involved in the reaction as well as the possibility of enzymatic inhibition. Although complex models are, in many cases, highly precise, they may turn out to be less practical for the design of the process than the simpler ones.

A simple model for the degradation of anthracene by MnP may consider anthracene as the only substrate. Operation in fed-batch reactors showed that first-order kinetics related to anthracene fits experimental data inaccurately, since anthracene degradation rate increases throughout the reaction. The increase of degradation rate is likely related to the formation of reaction products, mainly

anthraquinone, producing an autocatalytic effect on the oxidation rate. The kinetic model considering this autocatalytic process predicts anthracene concentration in experiments where MnP is maintained in a range (100-200 U/L) in order to avoid the effect of low enzymatic activities values.

An enzyme decay model was proposed from the results of MnP inactivation in fed-batch experiments. Two stages are observed during the enzymatic decay, being the inactivation higher during the initial period of the reaction. The model is first-order decay and the decay constant is around 3.5-fold higher during the first period of reaction.

A complete model for anthracene and products was proposed for the semi-continuous reactor with the addition of MnP, showing that enzymatic activity is determinant on degradation rates. The highest degradations of anthracene are attained at the highest MnP activities in the reactor.

This observation was confirmed when the continuous reactor was operated at different MnP addition rates, leading to lower stationary values of anthracene concentration when higher stationary values of MnP activity are reached in the reactor. The analysis of the different steady states in the continuous reactor enabled to define a function dependent on enzymatic activity. The function, with a sigmoid shape, reaches its maximum at high enzymatic activities (around 200 U/L), which means that the enzyme is not the limiting factor for the conditions in the reactor. However, low enzymatic activities (0-100 U/L) greatly influence the extent of degradation. Once defined the kinetics of the reaction, the model of the continuous reactor was described for substrate, products and enzyme. The decay constant was 4-fold higher than in batch and semi-continuous reactors, suggesting that the mechanism of MnP inactivation is not still clear for this system and further research should follow to understand the whole process. The model was validated with data from the semi-continuous reactor.

In conclusion, the kinetic model proposed in this chapter is based on the three main parameters of the reaction: the substrate, the products and the enzyme. The degradation rate has a direct relation with the substrate and the products; however, it follows a sigmoid function related to the enzyme. These assumptions permitted to fit the data obtained in a continuous reactor operated for 108 h. The implementation of the continuous reactor for the enzymatic degradation of anthracene in an industrial scale must include the recovery of the enzyme. Next work should be focused on this subject. The most common systems to recover the enzyme consist on the use of an ultrafiltration membrane coupled to the reactor (López et al. 2002) or the immobilization of the enzyme in the reactor (Tischer and Kasche 1999).

5.5. Nomenclature

a	1^{est} catalytic constant related to the first-order kinetics (h^{-1})
b	2^{nd} catalytic constant related to the autocatalytic process ($h^{-1}\ M^{-1}$)
E	Enzymatic activity in the reactor (U/L)
E_0	Initial enzymatic activity in the reactor (U/L)
E_i	Enzymatic activity in the input flow (U/L)
k_{cat}	Catalytic constant of first-order kinetics (h^{-1})
k_d	Decay constant (h^{-1})
K_M	Michaelis-Menten constant (μM)
P	Concentration of products in the reactor (μM)
Q_i	Input flow (L/h)
r	Reaction rate (μM/h)
r_m	Maximum velocity (μM/h)
S	Concentration of anthracene in the reactor (μM)
S_0	Initial concentration of anthracene in the reactor (μM)
S_i	Concentration of anthracene in the input flow (μM)
t	Time (h)
t_0	Initial time (h)
τ	Hydraulic retention time (h)
V_R	Volume of the reactor (L)

5.6. References

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Chapter 6

Operation of a two phase partitioning bioreactor for the oxidation of anthracene by MnP[4]

Summary

A study was conducted to determine the potential of a two-phase partitioning bioreactor (TPPB) for the treatment of a poorly-soluble compound, anthracene, by the enzyme manganese peroxidase (MnP) from the fungus *Bjerkandera* sp BOS55. Silicone oil was used as the immiscible solvent, which allowed the solubilization of anthracene at high concentrations. The optimization of the oxidation process was conducted taking into account the factors which may directly affect the MnP catalytic cycle (H_2O_2 and malonic acid concentrations) and those that affect mass transfer of anthracene between the organic and the aqueous phase (solvent selection and agitation rate). The main objective was to maximize the anthracene oxidized per unit of enzyme used defined as efficiency. A nearly complete oxidation of anthracene at a conversion rate of 1.8 mg/L·h during 56 h was attained. The obtained results suggest the good option of enzymatic TPPBs for the removal of poorly soluble compounds.

Modeling of the reactor is also proposed, comprising mass transfer process and kinetics of the enzymatic reaction as an autocatalytic reaction. The simulation was validated with batch experiments at different agitation speeds and fractions of solvent.

[4] Parts of this chapter have been published as:

Eibes G., Moreira M.T., Feijoo G., Daugulis A.J. and Lema J.M. (2007) Operation of a two phase partitioning bioreactor for the degradation of anthracene by the enzyme manganese peroxidase. Chemosphere 66:1744-1751.

Eibes G., López C., Moreira M.T., Feijoo G. and Lema J.M. (2007) Strategies for the design and operation of enzymatic reactors for the degradation of highly and poorly soluble recalcitrant compounds. Biocatalysis and biotransformation (in press).

Outline

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6.2.2. Determination of partition coefficients
6.2.3. Stability assays
6.2.4. Anthracene oxidation assays
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6.2.6. Analytical determinations

6.3. Results and discussion

6.3.1. Solvent selection
6.3.2. Effect of substrates and co-substrates of MnP
6.3.3. Optimization of mass transfer
6.3.4. Model of the process

6.4. Conclusions

6.5. Nomenclature

6.6. Acknowledgements

6.7. References

6.1. Introduction

Monophasic reactors have been successfully utilized for the oxidation of anthracene and other PAHs by the ligninolytic enzyme MnP. However, this system presented a number of drawbacks:

i) The concentration of anthracene in the medium is limited by the concentration of the cosolvent. Higher fractions of solvent could lead to a higher inactivation of the enzyme, as discussed in Chapter 3.

ii) The enzyme could not be recycled unless a system to separate it from the reaction medium is introduced. As examples immobilization or ultrafiltration membranes could be used (López et al. 2002; Mielgo et al. 2003).

iii) The solvent should be recovered to avoid its discharge for effluent post-treatment. The separation process increases the cost of the process.

In order to oxidize higher concentrations of anthracene by MnP, the addition of a second immiscible phase in a two-phase partitioning bioreactor (TPPB) was considered. The use of this type of reactors tries to overcome the restrictions described for monophasic reactors: i) the concentration of anthracene in biphasic reactors could be increased even to g/L (instead of mg/L), depending on the solubility of anthracene in the immiscible solvent utilized (much higher than in mixtures miscible solvent: water); ii) the enzyme remains in the aqueous phase and can be easily recycled; iii) the solvent, after the enzymatic treatment and depleted in anthracene, could be separated from the aqueous phase, re-contaminated with the PAH and returned to the aqueous phase for a further batch treatment.

The use of microbial TPPBs has allowed the biological treatment of many toxic and recalcitrant pollutants, such PAHs, at unprecedented loads and rates. Janikowski et al. (Janikowski et al. 2002) have successfully used this technology to degrade anthracene and other PAHs by *Sphingomonas aromaticivorans* cultures in biphasic reactors with dodecane as organic phase. The volumetric degradation rate of anthracene was 5.5 mg/L h after approximately 30 h.

Although the efficacy of microbial TPPBs for PAHs degradation is very high, the application of enzymatic reactors may be an interesting alternative, as evidenced by the advantages of this type of reactors. The use of biphasic enzymatic reactors is relatively recent and, as mentioned in Chapter 1, they have been mostly applied for synthesis of organic compounds. However, application of TPPBs for *in vitro* degradation of environmental pollutants is still lacking.

This chapter deals with the oxidation of anthracene by the enzyme MnP in a TPPB (Fig. 6-1). In TPPBs the concentration of the toxic compound in each phase is determined by the partition coefficient of the solvent. The substrate diffuses from the organic phase, as the enzyme degrades the pollutant in the aqueous phase, in order to re-establish the equilibrium (Vrionis et al. 2002). The operation of this enzymatic reactor allows working at high concentration of anthracene and the reuse of the enzyme in several batches.

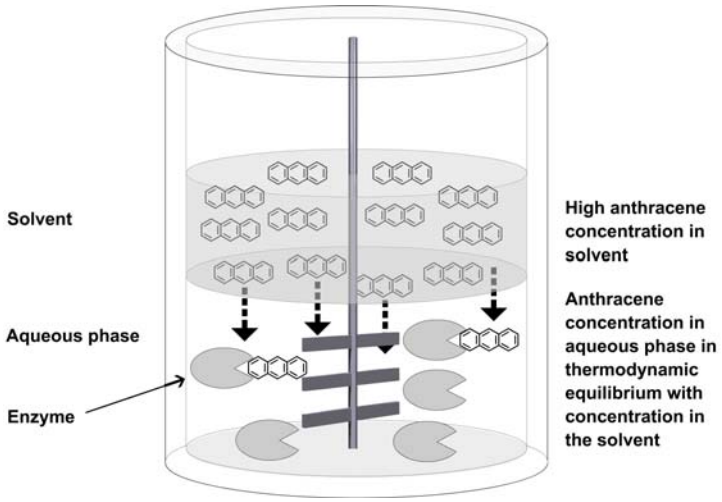

Figure 6-1. Scheme of enzymatic biphasic reactor for degradation of anthracene.

The use of TPPBs has some limitations that have to be overcome. The selection of the appropriate solvent is critical because it greatly influences mass transfer and consequently degradation rates. The selected solvent should be inexpensive, readily available, and exhibit suitable physical and chemical properties (be immiscible, non-volatile, etc.) (Déziel et al. 1999; MacLeod and Daugulis 2003; Marcoux et al. 2000; Villemur et al. 2000). Furthermore, the possible interaction between the solvent and enzyme is critical. It is important that the solvent is not a substrate of the enzyme (MacLeod and Daugulis 2003) and its effect on enzymatic activity is as low as possible (Ross et al. 2000). Besides, the partition coefficient of an appropriate solvent should enable the system to achieve the highest possible concentration of substrate in the aqueous phase. It has been stated that solvents with high partition coefficients, such as hydrocarbons, can sequester the target compound, thus decreasing their solubilization in the aqueous phase and limiting its biodegradation rate (Efroymson and Alexander 1995; Muñoz et al. 2003).

The substrate transfer rate from the organic to the aqueous phase is another essential factor that has to be enhanced so as not to limit the overall degradation

rate. Mass transfer is favoured by increased surface area for partitioning, and thus, the rate of biodegradation in a TPPB is governed by the size of the interface between the two liquid phases (Ascón-Cabrera and Lebeault 1995; Köhler et al. 1994), being the interfacial area defined by equation 6-1:

$$a = \frac{6 \cdot \varphi}{d_{sm}} \qquad (6\text{-}1)$$

Increasing the volume of organic solvent (φ) or decreasing the diameter of the drops (d_{sm}) by increasing the agitation rate would augment the interfacial area.

The aim of this chapter is the optimization of the operation of TPPBs, focused in four critical aspects:

i) Selection of the appropriate solvent: the partition coefficient of anthracene was evaluated as well as the influence of different solvents on the enzyme activity and stability;

ii) Study of the parameters involved in the catalytic cycle of the enzyme: hydrogen peroxide, malonate and pH control;

iii) Enhancement of mass transfer of the substrate from the organic phase, varying agitation and solvent fraction;

iv) Modeling of the reactor, comprising mass transfer and kinetics of the enzymatic reaction.

This system is, to our knowledge, the first attempt to degrade PAHs enzymatically in a biphasic reactor.

6.2. Materials and methods

6.2.1. Enzyme and chemicals

MnP was obtained from *Bjerkandera* sp. BOS55 (ATCC 90940) as described in Chapter 2. Anthracene (99% purity) as well as silicone oil 200 FLUID 20 cSt and all other chemicals used were obtained from Sigma-Aldrich.

6.2.2. Determination of partition coefficients

The partition coefficient of a substrate is the ratio between the concentration of the compound in the solvent and the concentration of the compound in water

$$K_{SW} = \frac{S_S}{S_W} \qquad (6\text{-}2)$$

Considering that this coefficient remains constant for all concentrations of the

substrate, it can be easily estimated by determining the saturation concentration in the solvent. Anthracene solubility at 25ºC is 0.07 mg/L (Mackay and Shiu 1977). In order to determine its saturation concentration in other solvents, the procedure of solubility of anthracene described in Chapter 2 was followed. Samples were diluted with acetone whenever required and then analyzed by HPLC.

6.2.3. Stability assays

Experiments with 10% silicone oil in absence of anthracene were performed to evaluate the effect of the solvent at three agitation rates. The aqueous phase consisted on 33 mM sodium malonate, 33 μM Mn^{2+} and 400 U/L of MnP in a total volume of 100 mL. No hydrogen peroxide was added. The agitator speed of Level 1 formed few medium droplets (visually > 5 mm diameter), while level 2 created droplets of variable diameter (1-5 mm) and at level 3 produced a complete dispersion (< 1 mm). Samples were withdrawn periodically in order to measure MnP activity.

MnP inactivation experiments with 10% solvent (silicone oil or dodecane) were performed at controlled agitation rates in a BIOSTAT®Q reactor (B. Braun-Biotech International, Melsungen, Germany). The agitations assayed were 400, 600 and 800 rpm. The aqueous phase, 25 mL, contained 33 mM sodium malonate, 33 μM Mn^{2+} and 100 U/L of MnP in a total volume of 250 mL.

6.2.4. Anthracene oxidation assays

Anthracene oxidation assays in serum bottles

Oxidation of anthracene was carried out in 500-mL glass bottles, with magnetic stirring at room temperature, i.e. 23ºC. The reaction mixture (100 mL) consisted of silicone oil (10 mL) saturated with anthracene (≈ 360 mg/L). The aqueous phase, 90 mL, consisted of 33 μM Mn^{2+}, 33 mM malonic acid and the continuous addition of 5 μM/min H_2O_2 (except when indicated). Samples were withdrawn periodically, centrifuged for 15 min at 3400 rpm to separate the two phases. Anthracene concentration in the organic phase was quantified using fluorescence spectroscopy while the concentration in the aqueous phase was assumed to be negligible (water solubility of anthracene: 0.07 mg/L (Mackay and Shiu 1977)). Fluorescence spectra were collected using a QuantaMaster QM1 fluorescence spectrometer (Photon Technology International, London, Ontario, Canada), equipped with a 75 W Xenon arc lamp, Czerney-Turner excitation and emission monochromators. Excitation and emission slits were set to 2 nm bandpass for all measurements. A solution sample holder was used to hold the quartz cuvettes in the path of the excitation radiation. The quartz cuvettes used were type 3H, with a path length of 10 mm, obtained from NSG Precision Cells, (Farmingdale, New York, USA). All samples taken from

the organic phase were diluted by a factor of 10,000 in anhydrous ethanol. The detection conditions were: Δλ = 125 nm, peak maximum = 377 nm and integration area = 360-390 nm.

Changes in MnP activity in the aqueous phase were spectrophotometrically determined, and pulses of MnP were added to maintain MnP activity in the reactor higher than 100 U/L. To verify that removal took place due only to an enzymatic oxidation, controls were run in parallel in absence of MnP.

Anthracene oxidation assays in batch reactors

The experiments with pH control and those at different agitation rates and solvent volumes were carried out in a BIOSTAT®Q reactor (B. Braun-Biotech International, Melsungen, Germany) (Fig. 6-2). It was equipped with pH, temperature and pO_2 sensors and a magnetic agitator. The temperature was set to 25ºC and pH was controlled at 4.5 by pumping HCl (1 M) or malonic acid (250 mM). Agitation rates varied from 200 to 300 rpm. The total reaction volume was 250 mL with different proportions of water:organic solvent. The aqueous phase contained 33 μM Mn^{2+}, sodium malonate, MnP and the continuous addition of H_2O_2. Before sampling, agitation was stopped for 2 min to equilibrate the system. Sampling of organic and aqueous phases was carried out by the bottom and by the top of the reactor.

Figure 6-2. Experimental set-up for parallel assays in batch reactors. Two peristaltic pumps added hydrogen peroxide continuously (left).

Anthracene concentration was only followed in the organic phase. Its concentration in the aqueous phase was considered to be negligible. 2 mL of organic sample were centrifuged for 5 min at 3000 rpm in order to separate tiny aqueous drops and 100 µL of the supernatant were added to a final volume of 10 mL of acetonitrile. After 5 min of extraction in a vortex, 1 mL of the sample in acetonitrile was then analyzed by HPLC as described in Chapter 2. The remaining volume of organic solvent was replaced in the reactor.

Aqueous samples were used to determine MnP activity and malonate concentration (by HPLC). Samples of 1 mL were centrifuged to separate and eliminate solvent drops. Pulses of MnP were added in order to maintain the activity in the reactor higher than 100 U/L.

6.2.5. Estimation of mass transfer coefficients

As k_La is dependent on the hydrodynamic of the system, experiments at different conditions of agitation and volume of solvent were carried out. 250 mL of different proportions of water:solvent saturated with anthracene were placed in a BIOSTAT Q reactor, and agitation was started. At a given time, agitation was stopped, the system was allowed to equilibrate and a sample (40 mL) was taken from the aqueous phase. The sample was then centrifuged at 5000 rpm for 10 min in order to remove tiny solvent drops. An aqueous sample of 10 mL was taken from the bottom of the vessel and, after adding 2 mL of hexane, was mixed in a vortex for 5 min. The concentration of anthracene in hexane was then analysed by GC-MS. The values of k_La obtained for each condition of agitation rate and volume of solvent were adjusted to a surface by means of the software Table Curve 3D.

6.2.6. Analytical determinations

MnP activity was measured spectrophotometrically as described in Chapter 2. Anthracene was determined either by liquid chromatography (HPLC) as described in Chapter 2 or gas chromatography coupled to mass spectrometry (GC/MS) when the concentration of anthracene in the media was below 1 mg/L.

GC (Varian Saturn 2100T) was equipped with a split/splitless injector and a CP-SIL 8 CB column was used for separation (30 m, 0.25 mm id, 0.25 µm film thickness). Temperature started at 60°C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 180ºC at a rate of 20°C/min. The second temperature ramp was up to 200°C at a rate of 5°C/min, and temperature was increased to 310ºC at a rate of 10ºC/min, being maintained for 5 min. The solvent delay time was set to 5 min. Transfer line temperature was set to 310°C. Mass spectra were recorded at 1 scan/s under electron impact at 70 eV, mass range 90–300 amu.

Malonic acid concentration was determined by a HP 1090 HPLC with a refractive index detector, using sulphuric acid as mobile phase (0.6 mL/min) and a Aminex-87H BioRad column (BioRad Laboratories, Madrid). The injection volume was set at 20 µL.

6.3. Results and discussion

6.3.1. Solvent selection

Determination of the partition coefficient of anthracene

Several solvents including mineral and vegetable oils, alcohols, alkanes, ketones and esters were considered due to its high boiling point, low water solubility, low cost, minimal toxicity and commercial availability. The partition coefficients were evaluated for each solvent (Table 6-1).

Table 6-1. Values of log K_{SW} obtained for 15 different solvents.

Solvent	log K_{SW}	Solvent	log K_{SW}
Silicone oil	3.7	Triacetin	4.8
Paraffin oil	4.3	Olive oil	4.9
Sunflower oil	4.3	Corn oil	4.9
Oleic alcohol	4.4	Ethyl acetate	5.0
Decanol	4.4	Biodiesel	5.0
n-hexadecane	4.5	Marc olive oil	5.0
Dodecane	4.5	Undecanone	5.2
Engine oil	4.6		

The values of log K_{SW} obtained ranged from 3.7 (silicone oil) to 5.2 (undecanone). Lower values of the partition coefficient are preferred, since it has been shown that solvents with high partition coefficient can sequester the substrate, thus limiting its concentration in the aqueous phase and consequently its oxidation rate (Efroymson and Alexander 1995; Muñoz et al. 2003). Taking this into account, two solvents were selected for further study: silicone oil, with the minimum log K_{SW} 3.7, and dodecane, with an intermediate value of log K_{SW} 4.5.

Interaction of the solvents with MnP

The second factor considered in the selection of the solvent was the interaction with the enzyme. Organic solvents can produce a deleterious effect on the biocatalyst,

which may be due to the interaction with dissolved solvent molecules or with the interface between the aqueous and organic phases (Ross et al. 2000). Silicone oil and dodecane are nearly insoluble in water with a high hydrophobicity: log K_{OW} of dodecane is 6.6 and log K_{OW} of silicone oil is higher than 11 (Bruggeman et al. 1984). Since the presence of dissolved solvent molecules in water is scarce, the main mechanism of inactivation seems to be the interfacial interaction.

The enzyme was subjected to different interfacial areas by modifying the agitation in the presence of 10% silicone oil. A non stirred control and 3 different levels were assayed (Fig. 6-3).

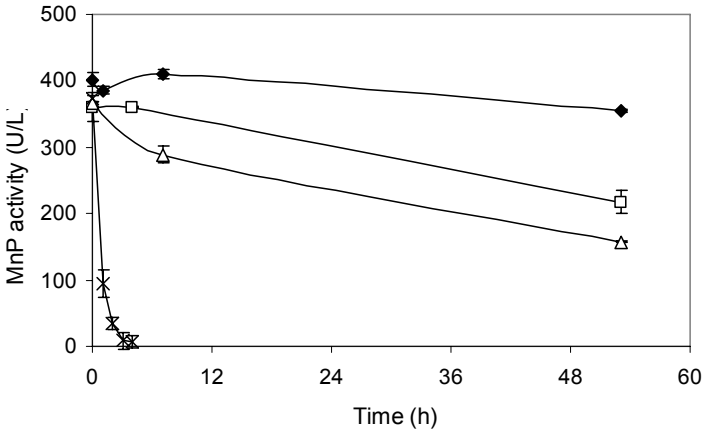

Figure 6-3. Effect of the agitation rate on MnP activity: ♦ no agitation, □ level 1 (mean droplet diameter around 0.5-1 cm), △ level 2 (mean diameter < 0.5 cm) and × complete agitation (homogeneous phase)

Level 1 of agitation formed solvent droplets dispersed on the water phase with a diameter between 5 and 10 mm. At level 2 the number of droplets increased and the diameter diminished (< 5 mm). Finally, higher agitation produced a visually homogeneous phase (droplet diameter < 1 mm) which could be related to an agitation speed higher than 500 rpm. This strong agitation resulted in complete inactivation of the enzyme after only 3 h, while MnP activity after 53 h at level 1 and level 2 was maintained at 61% and 44%, respectively, when compared with the control experiment.

In order to compare the detrimental effect of silicone oil and dodecane the agitation rate was controlled in the following short-term experiments (Fig. 6-4). Inactivation rates for silicone oil and dodecane were: 6.7 and 11.8 U/L·h at 400 rpm; 61 and 81 U/L·h at 600 rpm; and 138 and 143 U/L·h at 800 rpm, respectively, causing dodecane higher enzymatic inactivation at all agitation rates. As it is quite

difficult the measurement of the interfacial area, agitation rate was selected as the control parameter. Under the same agitation rate, silicone oil formed higher interfacial areas than dodecane due to its lower interfacial tension (20 and 53 mN/m for silicone oil and dodecane, respectively). In consequence, even at higher interfacial areas, enzyme inactivation in silicone oil was lower.

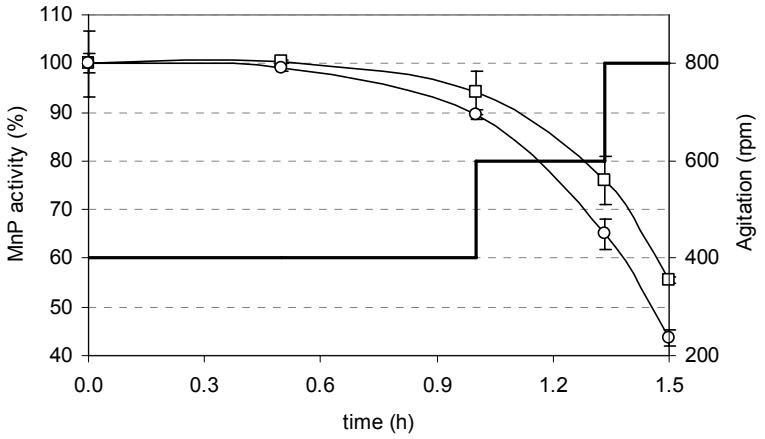

Figure 6-4. Effect of the agitation on MnP activity in media with dodecane (○) or silicone oil (□).

Solvent selection

Both factors, partition coefficient and enzyme inactivation, were more favorable in the case of silicone oil, which was selected for the following experiments. Silicone oil has been successfully used in TPPBs with various microorganisms for PAHs degradation (Bouchez et al. 1997; Marcoux et al. 2000; Muñoz et al. 2003) due to its hydrophobicity, biocompatibility, chemical stability, and resistance to hydrolytic and oxidative breakdown as discussed by Ascón-Cabrera and Lebeault (1993).

6.3.2. Effect of substrates and co-substrates of MnP

The reactions and processes involved in the enzymatic degradation of anthracene are shown in Fig. 6-5. The enzyme MnP is present in the aqueous phase with the cofactors and substrates required for the catalytic cycle. The anthracene molecules transferred from the organic to the aqueous phase are oxidized by Mn^{3+} ions generated during the catalytic cycle. The products formed, mainly anthraquinone, can be transferred to the organic phase (Eibes et al. 2006). The parameters affecting the catalytic cycle of MnP, those present in the aqueous phase, were investigated to optimize anthracene oxidation in TPPBs operated with silicone oil.

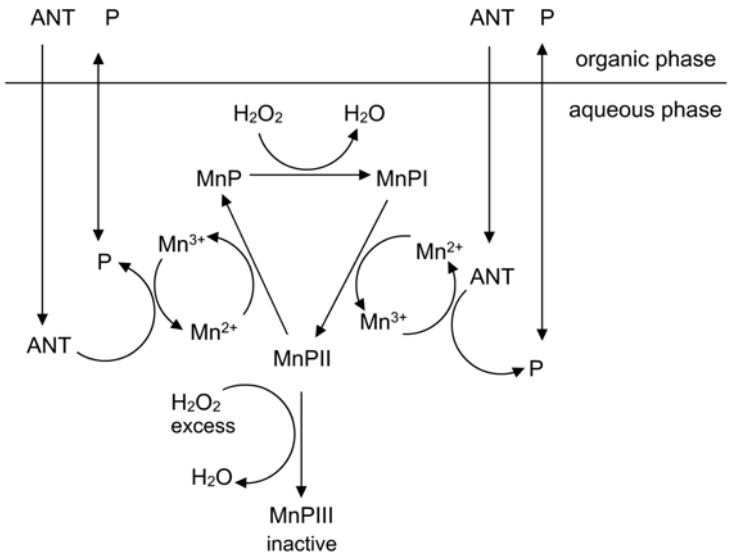

Figure 6-5. Scheme of the transport and enzymatic mechanisms involved in the degradation of anthracene (ANT) by MnP in a TPPB. Mn^{3+} ions formed in the catalytic cycle of MnP oxidize ANT molecules in the aqueous phase to form the products (P) which transfer to the organic phase.

Hydrogen peroxide addition

Hydrogen peroxide, the promoter of the catalytic cycle of MnP, was continuously added by means of a peristaltic pump avoiding high concentration in the reactor, which would cause MnP inactivation. Different hydrogen peroxide addition rates were assayed: 1, 5, 15 and 25 μM/min and anthracene oxidation was evaluated as well as MnP loss rate and efficiency, in terms of anthracene oxidized per unit of activity used. Table 6-2 shows that the higher the hydrogen peroxide addition was, the higher activity loss but not the oxidation rate. H_2O_2 addition rates of 1 and 5 μM/min attained similar efficiencies: 0.047 and 0.046 mg/U, respectively, but anthracene oxidation rate for 1 μM/min was 2.4-fold lower. Therefore, continuous addition of H_2O_2 at controlled flow of 5 μM/min permitted progressive participation of H_2O_2 in the catalytic cycle through suitable regeneration of the oxidized form of the enzyme, minimizing the peroxide dependent inactivation of the peroxidase (Moreira et al. 1997).

Table 6-2. Results of the set of experiments at different addition rates of H_2O_2

H_2O_2 (μmol/L·min)	Degradation rate (mg/L h)	MnP activity loss rate (U/L h)	Efficiency (mg/U)
1	0.16	3.4	0.047
5	0.38	8.4	0.046
15	0.28	17.4	0.016
25	0.27	17.1	0.016

Operational pH was maintained at 4.5 with no significant change during 30 h (Fig. 6-6). At that time, pH started to increase, reaching a maximum of 8 after 70 h for all experiments. The faster the hydrogen peroxide rate was, the faster the inactivation of MnP and the faster pH increased. This pH increase could be related to ammonia liberated due to enzyme proteolysis. Values of pH higher than 6 have been shown to be responsible for marked MnP inactivation (Mielgo et al. 2003). Summarizing, high addition rates of hydrogen peroxide led to high inactivation rate of the enzyme, which led to an increase of pH, which could cause higher MnP deactivation.

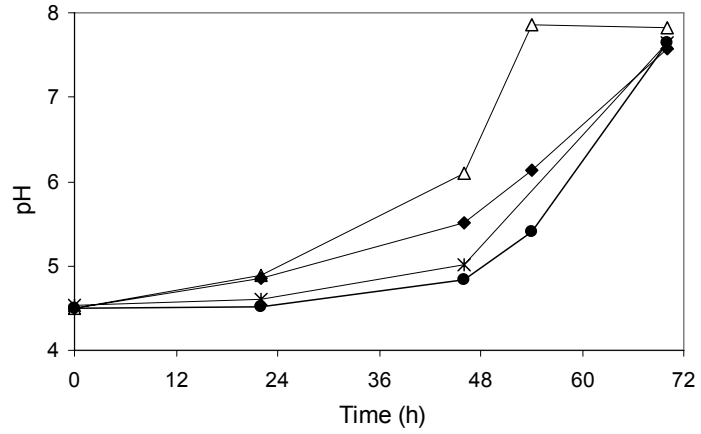

Figure 6-6. Evolution of pH at different hydrogen peroxide addition rates: Δ 25, ♦ 15, ∗ 5 and ● 1 μmol/L min

Sodium malonate concentration and pH control

In order to avoid the detrimental effect of pH increase, sodium malonate was studied as buffering solution, at concentrations ranging from 10 to 66 mM (Table 6-3; experiments 1 to 4).

The increase of the buffer concentration should regulate pH to a larger extent, and hence, activity consumption should be lower. When sodium malonate concentration increased from 50 to 66 mM (experiments 3 and 4), enzymatic loss also increased: from 8.4 to 11.8 U/L h, which was not desirable. Bearing in mind the efficiency, the best values were obtained with 33 or 50 mM malonate (0.046 mg/U). Higher buffer concentrations did not improve enzymatic deactivation and, on the contrary, it caused higher MnP losses. Moreover, pH increased to 8 after 70 h of reaction at the higher concentration of sodium malonate. The lower activity loss was obtained in experiment 1, using 10 mM malonate (6.8 U/L h), although in that case, anthracene oxidation rate was also the lowest (0.29 mg/L h) and removal of anthracene stopped after 47 h of reaction (36% removal) in spite of the presence of enzyme and hydrogen peroxide in the medium.

Table 6-3. Results of the set of experiments at different malonate concentration and pH control

Experiment	pH	Malonate (mM)	Degradation rate (mg/L h)	Activity loss rate (U/L h)	Efficiency (mg/U)
1	Free	10	0.29	6.8	0.042
2	Free	33	0.36	7.7	0.046
3	Free	50	0.38	8.4	0.046
4	Free	66	0.39	11.8	0.033
5	4.51	33	0.37	7.3	0.050
6	4.52	33	0.41	7.5	0.055
7	4.52	10	0.42	5.4	0.079
8	4.52	5	0.42	5.7	0.074

[1] pH controlled with HCl

[2] pH controlled with malonic acid

Organic acids are required in the catalytic cycle of MnP because they facilitate Mn^{3+} release from the active site and also for stabilization of these species in aqueous solution (Banci et al. 1998; Martínez 2002). The concentration of sodium malonate was demonstrated to be decisive for the efficiency of anthracene removal in monophasic reactors (Chapter 3): on the one hand, the oxidation extent was improved, but on the other hand, activity loss also increased.

The following experiments were performed with pH control, and the concentration of malonate was determined in order to check if it was a limiting factor. pH was fixed at 4.5 by adding HCl (1 M) whenever required (Table 6-3 - experiment 5). The operation at fixed pH with HCl led to a slight diminution of MnP consumption rate in comparison with experiment 2, where pH was not controlled (7.3 and 7.7 U/L h, respectively). However, oxidation rate did not undergo great changes (0.37 and 0.36 mg/L h, respectively). Regarding malonate concentration in the reactor, it continuously decreased during the 72 h of reaction at a rate of 39 mmol/h. This fact could be explained by its oxidative decarboxylation by Mn^{3+} (Van Aken and Agathos 2002).

Malonate is an essential compound in the catalytic cycle of MnP, and therefore, its presence on the reaction medium has to be assured, because its deficiency during the process could lead to a rapid decrease of the reaction rate, as happened when the initial concentration of malonate was 10 mM. For that reason, control of pH was carried out by addition of malonic acid: 0.25 M (experiment 6). An increase in the oxidation rate was observed in comparison with experiment 2 (from 0.36 to 0.41 mg/L h) but MnP activity loss remained practically the same (7.6 and 7.5 U/L h, respectively).

Decarboxylation of organic acids generates a carbon dioxide anion radical which permits the endogenous formation of H_2O_2 via Mn^{2+} and a superoxide radical (Van Aken and Agathos 2002). The resulting accumulation of H_2O_2 may explain the greatest activity loss at high concentrations of sodium malonate. Moreover, radical species and peroxides formed during the process are highly reactive and can be, to some extent, used by MnP in an autocatalytic process, which could explain the improvement of the degradation rate (Hofrichter et al. 1998).

Trying to decrease the enzymatic loss, the initial concentration of malonate was reduced to 10 mM, but in this case pH was controlled (experiment 7). As was expected, enzymatic consumption decreased (5.4 U/L h), not only in comparison with experiment 6 but also with experiment 1, where pH was uncontrolled. The oxidation rate, 0.042 mg/L h, was similar to that obtained in experiment 6, but much higher than experiment 2: 1.45-fold. Hence, the efficiency, 0.079 mg/U, was 1.44-times greater than in experiment 6 and 1.88-times higher than experiment 1. Finally, the initial malonate concentration was decreased to 5 mM (experiment 8), but there was no improvement in the efficiency of the system (0.074 U/mg) because MnP consumption did not decrease. Therefore, the conditions selected for the following experiments were: 10 mM malonate, malonic acid as agent for pH control and addition of H_2O_2 at a rate of 5 μmol/L·min.

6.3.3. Optimization of mass transfer

In order to favor transfer of anthracene to the aqueous phase as well as the kinetics of the enzymatic reaction, an enhancement of the interfacial area was evaluated. Equation 6-4 shows that the interfacial area increases with a decrease in the mean drop size and with an increased organic:water ratio. However, it was also described that drop diameters tend to increase with the phase ratio, thus decreasing the interfacial area (Prokop and Erikson, 1972). Therefore, the effect of the fraction of solvent is an important parameter to be analyzed. Moreover, agitation speed directly affects the interfacial area. Since both variables, agitation speed and fraction of solvent, are likely to be co-dependent, a 2^2 experiment design was considered to optimize the system efficiency (Box et al. 1978).

Considering both factors, interfacial surface and enzyme deactivation, the values of agitation considered in the experimental design were 200 and 300 rpm. Moreover, the percentage of silicone oil was assessed at 10 and 30%. Four experiments at the conditions determined by the limits of the range considered as well as two experiments in the centre of the region of interest (250 rpm and 20% silicone oil) were carried out. The anthracene oxidation rate, activity loss rate and efficiency corresponding to each experiment are shown in Table 6-5.

Table 6-5. 2^2 fractional experiment matrix and experimental results

Exp	A	B	Agitation (rpm)	Silicone oil (%)	Degrad (%); (time)	Degrad rate (mg/L h)	MnP deact rate (U/L·h)	Efficiency (mg/U)
1	-1	-1	200	10	92 (72 h)	0.42	5.1	0.083
2	-1	1	200	30	43 (72 h)	0.62	4.5	0.139
3	1	-1	300	10	97 (55 h)	0.61	7.4	0.083
4	1	1	300	30	89 (56 h)	1.76	7.3	0.243
5	0	0	250	20	90 (56h)	1.19	6.8	0.175
6	0	0	250	20	92 (56 h)	1.21	6.4	0.187

It is important to highlight that the increase of either the silicone oil fraction or the agitation rate favor anthracene oxidation rate in a similar extent. However, the increase in agitation led to a marked increase in MnP activity consumption (around 4.8 U/L h for 200 rpm and 7.3 U/L h for 300 rpm). Even so, the highest efficiency was obtained at 300 rpm and 30% silicone oil (exp. 4), where nearly complete oxidation was achieved after 56 h (Fig. 6-7).

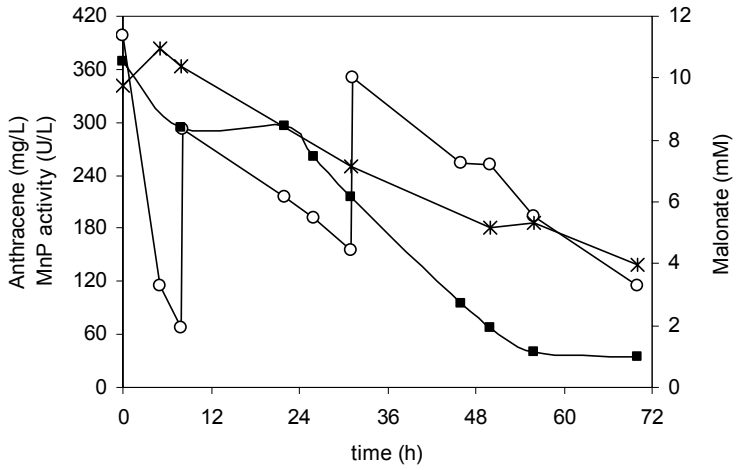

Figure 6-7. Oxidation of anthracene in a TPPB with 30% (v/v) silicone oil, 10 mM malonate, continuous addition of 5 μmol H_2O_2/L min and pH control by addition of malonic acid. Symbols: ■ anthracene concentration in the organic phase, ○ MnP activity and ∗ malonate concentration in the aqueous phase.

The experimental results were adjusted to a response surface defined by equation 6-3.

$$Z_i = b_i + c_i \cdot X + d_i \cdot Y + e_i \cdot X \cdot Y \qquad (6\text{-}3)$$

where "X" and "Y" are the dimensionless agitation rate and silicone oil fraction, respectively, and the subindex (i) indicated the type of objective function (Zi) considered: oxidation rate, activity loss rate or efficiency. The coefficients of the objective functions are shown in Table 6-6. A confidence level of 90-95% was considered to determine the significance of the coefficients. Figure 6-7 shows the response surface for the efficiency.

Table 6-6. Regression coefficients of the 2^2 factorial experimental design

	Constant	Agitation	Silicone oil	Agitation·silicone oil
Degradation rate	0.969	0.333	0.337	0.239
Activity loss rate	6.24	1.26	NS	NS
Efficiency	0.152	0.026	0.054	0.026

NS: no significance

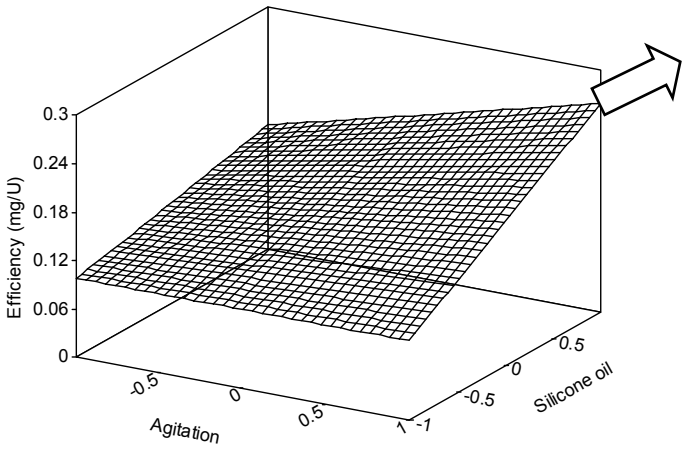

Figure 6-7. Response surface for the efficiency as a function of agitation rate and silicone oil fraction. The arrow represents the path of the steepest ascent.

In the case of oxidation rate, both agitation rate and silicone oil fraction had a similar weight in the equation and the combined effect had 2-thirds of that (coefficients: 0.33, 0.34 and 0.24, respectively). Regarding activity loss rate, only agitation had a significant effect. The increase of the silicone oil volume did not imply a modification of the enzymatic deactivation rate. Moreover, efficiency was mainly dependent on the ratio of the organic and aqueous phases: higher volumes of silicone oil led to higher efficiency values. Both the agitation and the combined effect had similar weight (coefficients: 0.026 and 0.026) and represented around half of the fraction of solvent (0.054).

In order to improve the results in terms of efficiency, additional experiments were carried out on the line representing the steepest ascent of the function on the best point of the surface. The parametric representation of that line is indicated by equation 6-4:

$$\begin{aligned} X &= 0.55 \cdot s + 1 \\ Y &= 0.84 \cdot s + 1 \end{aligned} \qquad (6\text{-}4)$$

where "s" conditions the length of the movement from the base point, in that case (+1,+1).

Although different assays were performed from the base point, considering a golden section protocol (Rudd and Watson 1968), none of them improved the results obtained in experiment number 4, with an agitation rate of 300 rpm and a fraction of silicone oil:aqueous phase of 30%.

Ascón-Cabrera and Lebeault (1993) have studied the influence of the organic phase fraction (8.3 to 83% v/v silicone oil) on the interfacial area and observed maximal values between 20 and 40% and agitation rates between 400 and 700 rpm. The optimal values of the organic phase volume agree with the optimal value obtained in this work: 30% v/v. In our work, agitation rates were not increased to those values, because agitation rates higher than 500 rpm would have a detrimental effect on MnP activity. Shear-induced inactivation of MnP from *Bjerkandera* sp. BOS55 was considered negligible under vigorous magnetic stirring and operational time shorter than 4 days (data not shown), and thus, enzyme inactivation may be caused by dissolved solvent molecules, and/or by contact with the interface (Ross et al. 2000). In the present case, as silicone oil is insoluble in water, the interfacial mechanism is assumed to be the main effect affecting enzyme deactivation. In emulsion reactors the observed rate of enzyme inactivation is function of interfacial tension, liquid density difference, dispersed phase fraction, mixing intensity and reactor geometry (Walstra 1993). In this work the main factor affecting enzyme inactivation was the agitation rate, which increased the interfacial area where the enzymes adsorb and subsequently unfold. The increase in agitation also favored the desorption of the inactivated enzyme from the interface (Baldascini and Janssen 2005).

6.3.4. Process modeling

Process modeling has to take into account the two major aspects involved: i) mass transfer of anthracene and ii) enzymatic kinetics. The coefficients for each mechanism of the proposed model were evaluated.

Mass transfer of anthracene

In biphasic reactors at a specific agitation rate and in absence of the enzyme, mass transfer of anthracene is the only component prevailing (equation 6-5):

$$\frac{dS_w}{dt} = k_L a \cdot (S^* - S_w) \qquad (6\text{-}5)$$

After integration and linearization, equation 6-6 is obtained:

$$\ln(S^* - S_W) = \ln S^* - k_L a \cdot t \qquad (6\text{-}6)$$

Mass transfer coefficients were obtained for agitation speeds ranging from 50 to 350 rpm and fractions of silicone oil from 10 to 30% (v:v). Fitting of the equation 6-6 with the data obtained in the experiment at 50 rpm and 10% silicone oil is shown in Fig. 6-8 and Table 6-7 presents the k_La values at different experimental conditions.

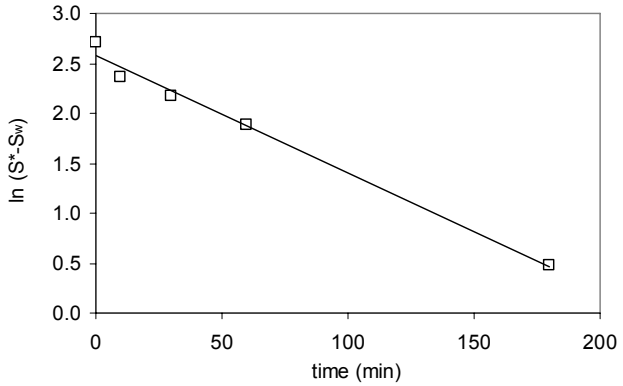

Figure 6-8. Determination of k_La of anthracene in a biphasic reactor with 10% of silicone oil and at 50 rpm.

Table 6-7. Mass transfer coefficients obtained for different conditions of agitation and fraction of silicone oil

Agitation (rpm)	Silicone oil (%)	k_La (min^{-1})	r^2
50	10	0.01	0.99
150	10	0.10	0.99
200	10	0.27	1.00*
250	10	2.99	1.00
350	10	3.29	1.00
50	20	0.02	0.77
150	20	0.36	1.00
200	20	0.30	0.99
250	20	2.26	1.00
50	30	0.12	0.94
150	30	0.36	0.99
200	30	0.68	1.00
250	30	3.14	1.00

* Values of r^2 = 1 corresponded to experiments where the equilibrium was obtained after 1 min of mixing

The data show a great increase of k_La values especially in a short range of agitation speed (200-300 rpm). This effect was more pronounced when working at low fractions of silicone oil. Although mass transfer coefficients were maximized at 250 rpm for all the evaluated proportions of silicone oil, the experimental results of anthracene degradation suggested 300 rpm and 30% silicone oil as the optimal conditions.

The values of k_La were fitted to a surface (r^2 = 0.986) represented in Fig. 6-9 and thus related to the agitation (ω) and fraction of solvent (Φ) through an empiric correlation with five parameters (being f= -0.113, g=0.008, h=3.338, i=230.77 and j=11.859) (equation 6-7).

$$k_La = f + g \times \Phi + h \times \left(0.5 + \frac{\arctan((\omega - i)/j)}{p}\right) \quad (6\text{-}7)$$

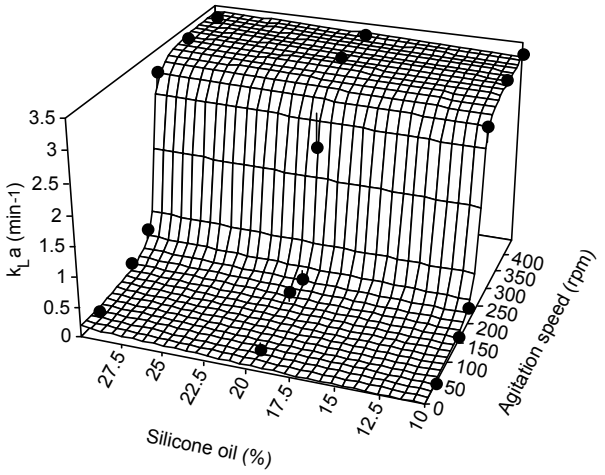

Figure 6-9. Experimental k_La values (●) and surface fitting

The correlation, in principle, is valid for the ranges evaluated: 10-30% silicone oil and 50-350 rpm. However, it could be applicable at higher agitation rates (the coefficient is maximized from 250 rpm) and even at higher fractions of silicone oil, since the surface maintains its tendency (at higher percentages the transition to the maximum k_La is less pronounced). On the other hand, the extrapolation to lower fractions of silicone oil is highly inaccurate, especially at high agitation rates, since the proposed correlation did not take into account that the coefficient diminishes to zero in absence of silicone oil.

In order to obtain the catalytic coefficient both balances in organic and in aqueous phase were considered (equations 6-8 and 6-9):

$$\frac{dS_S}{dt} = -k_L a \cdot \left(\frac{S_S}{k_{SW}} - S_W \right) \cdot \frac{V_W}{V_S} \tag{6-8}$$

$$\frac{dS_W}{dt} = k_L a \cdot \left(\frac{S_S}{k_{SW}} - S_W \right) - (\alpha + \beta \cdot P_W) \cdot S_W \tag{6-9}$$

The kinetic equation was based on the model proposed in Chapter 5, i. e., dependent on the formation of the products. In this case, MnP activity was not taken into account, since pulses were added during the experiments in order to maintain the activity in the range 100-200 U/L. Product concentration in the aqueous medium is obtained by mass balance:

$$P_W = \frac{S_{S0} - S_S - P_S}{V_W / V_S} - S_W \tag{6-10}$$

In order to simplify equation 6-10, the following points were assumed:

i) The concentration of anthracene in water is much lower than the other terms in equation 6-10: $S_W \approx 0$,

ii) The concentration of products in the organic phase is related only to the anthraquinone concentration since it was the only product detected in the solvent. Transfer of anthraquinone to the organic phase was considered to be immediate, and given by its partition coefficient: $P_S = k'_{SW} \cdot P_W$. The value of k'_{SW} was estimated 100, as the ratio between anthraquinone saturation in silicone oil (60 mg/L) and saturation in water (0.6 mg/L).

iii) Once the solvent is saturated with anthraquinone, its concentration does not vary (P_S=60 mg/L). The products formed in this stage are accumulated in the aqueous phase. Therefore, two equations define products concentration, according to equation 6-10 and having into account the previous considerations: one for the first stage where anthraquinone transfers to the organic phase (equation 6-11), and a second equation for the next stage, when products accumulate in the aqueous phase (equation 6-12):

$$P_W = \frac{S_{S0} - S_S}{V_W / V_S + K'_{SW}} \tag{6-11}$$

$$P_W = \frac{S_{S0} - S_S}{V_W / V_S} \tag{6-12}$$

Taking into account that the equilibrium concentration of the substrate in aqueous medium (S*) is given by the anthracene partition coefficient and that the products in the aqueous phase are given by equation 6-11 during the first stage of the process (the solvent is not saturated with anthraquinone: $P_S=K'_{SW}\cdot P_W<60$ mg/L), the resulting equation of the mass balance in the aqueous phase is.

$$\frac{dS_W}{dt} = k_L a\cdot\left(\frac{S_S}{k_{SW}} - S_W\right) - \left(\alpha + \beta\cdot\frac{S_{S0} - S_S}{V_W/V_S + k'_{SW}}\right)\cdot S_W \qquad (6\text{-}13)$$

A steady state might occur for the substrate in the aqueous phase, and the rate of mass transfer and enzymatic reaction can be set identical (Straathof 2003):

$$k_L a\cdot\left(\frac{S_s}{k_{sw}} - S_w\right) = \left(\alpha + \beta\cdot\frac{S_{S0} - S_S}{V_W/V_S + k'_{SW}}\right)\cdot S_W \qquad (6\text{-}14)$$

According to that, the explicit equation for anthracene in the aqueous phase would be:

$$S_w = \frac{k_L a}{k_{sw}\cdot\left(k_L a + \alpha + \beta\cdot\frac{S_{S0} - S_S}{V_W/V_S + k'_{SW}}\right)}\cdot S_S \qquad (6\text{-}15)$$

Substituting in equation 6-8, the equation representing the variation of anthracene in the organic phase for the first stage of the process is given by:

$$\frac{dS_S}{dt} = -\frac{k_L a}{k_{sw}}\cdot\frac{V_w}{V_S}\cdot\left(S_S - \frac{k_L a\cdot S_S}{k_L a + \alpha + \beta\cdot\frac{S_{S0} - S_S}{V_W/V_S + k'_{SW}}}\right) \qquad (6\text{-}16)$$

In the second stage, once the solvent is saturated with anthraquinone, products in the aqueous phase are given by equation 6-11, and the equation which represents the anthracene variation in the organic phase is defined as:

$$\frac{dS_S}{dt} = -\frac{k_L a}{k_{sw}}\cdot\frac{V_w}{V_S}\cdot\left(S_S - \frac{k_L a\cdot S_S}{k_L a + \alpha + \beta\cdot\frac{S_{S0} - S_S - 60}{V_W/V_S}}\right) \qquad (6\text{-}17)$$

The partition coefficient of anthracene in silicone oil had been previously determined (k_{SW}=5012, Table 6-1) and mass transfer coefficient was correlated with the operational parameters, as described above (Equation 6-6). The volume of the aqueous phase increased with time, due to the addition of hydrogen peroxide and this fact was also taken account to calculate $k_L a$.

In order to obtain the profile of anthracene in the organic phase, the equations were solved by using the finite differences numerical method for the conditions of the batch experiments carried out in the experimental design (at different agitations and fraction of solvent). A conditional function was used to select the correct equation, depending on the concentration of the products in the organic phase, which is defined by:

$$P_S = \frac{S_{S0} - S_S}{1 + V_W / (V_S \cdot K'_{SW})} \tag{6-18}$$

If $P_S \leq 60$ mg/L, equation 6-16 will be used to model the process, whereas equation 6-17 will be considered for the other circumstances.

The kinetic constants α and β were estimated by using the method of least squares and their values are shown in Table 6-8. The sequence of the simulation program is shown in Fig. 6-10 and the experimental and simulated data in Fig. 6-11.

Table 6-8. Parameter values of the kinetic model according to an autocatalytic process defined in equation 6-8

α	23.16
β	1.82

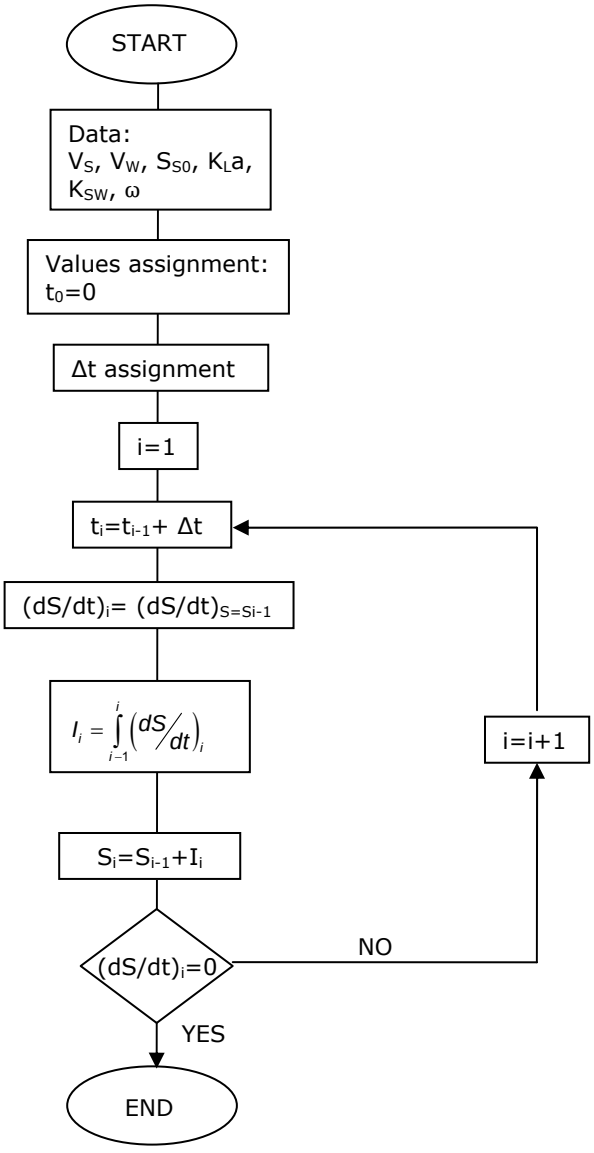

Figure 6-10. Flow chart of the simulation program

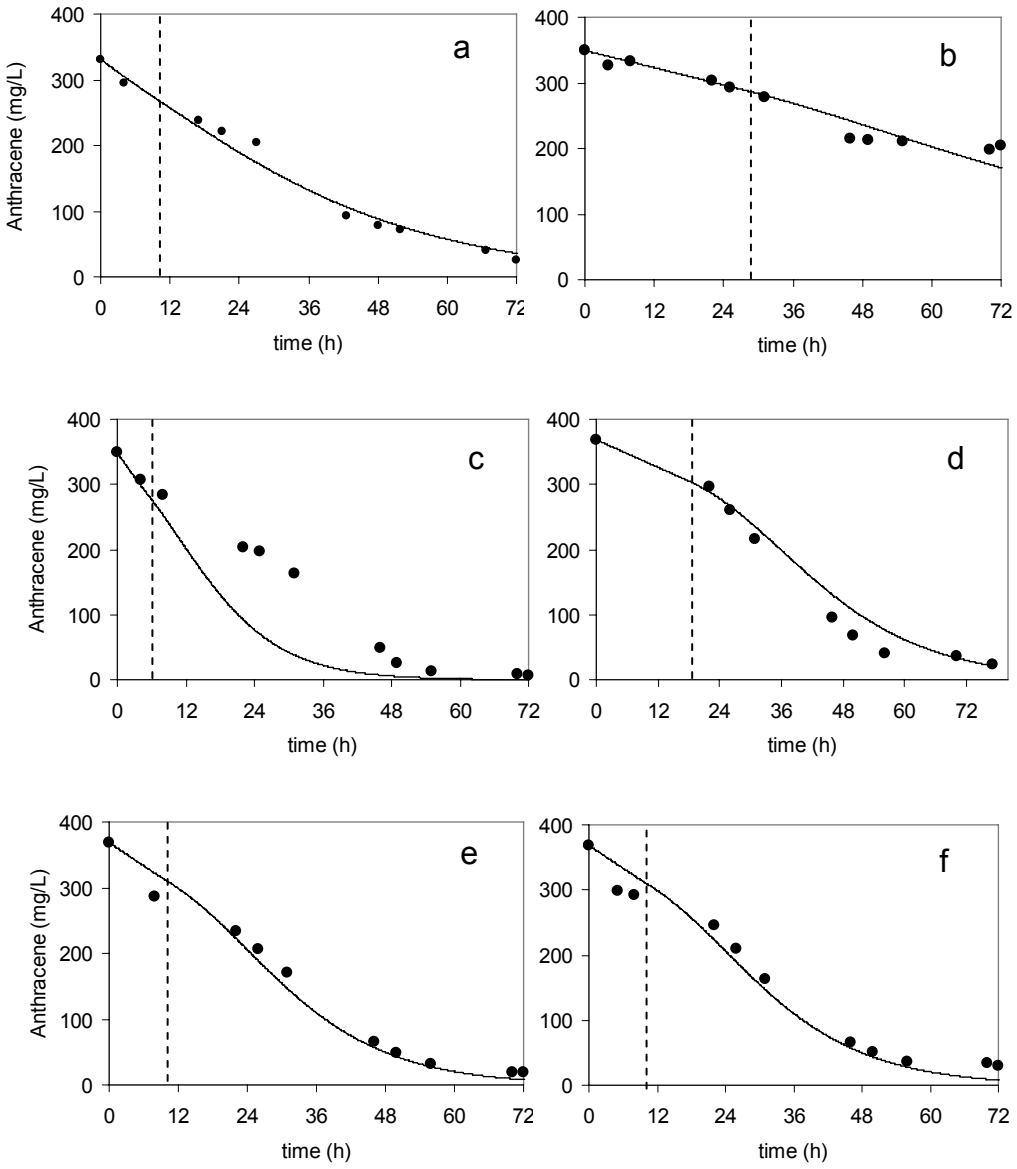

Figure 6-11. Experimental (●) and fitted values (—) obtained from the experimental design: (a) 200 rpm-10% silicone oil, (b) 200 rpm-30% silicone oil, (c) 300 rpm-10% silicone oil, (d) 300 rpm-30% silicone oil, (e-f) 250 rpm-20% silicone oil (duplicates). Dashed lines separate the two stages during the formation of the products.

Fitting of experiment c in Fig. 6-11 does not correlate properly with the experimental data, being a possible explanation based on the mass transfer coefficient used: 211 h^{-1}. The continuous addition of hydrogen peroxide decreased the percentage of silicone oil from 10% to 7%, and the correlation of k_La (Equation 6-7) for this condition is likely not accurate, since it is out of the limits of the study (10 to 30% of silicone oil). It is probable that the real value of k_La is much lower that the predicted, because in the range 10-0% the coefficient reaches zero. In the case of the experiment a, where 10% of silicone oil was also used, the model fitted properly to the data, but in this case the mass transfer coefficient was 10-times lower than in experiment c (21.3 h^{-1}).

The model was also used to fit the data obtained from the experiment at higher fraction of silicone oil (40%) and agitation (375 rpm) (Fig. 6-11). Although the variables are out of the range used for the correlation of k_La, it did not affect the prediction, since the conditions of mass transfer were maximized.

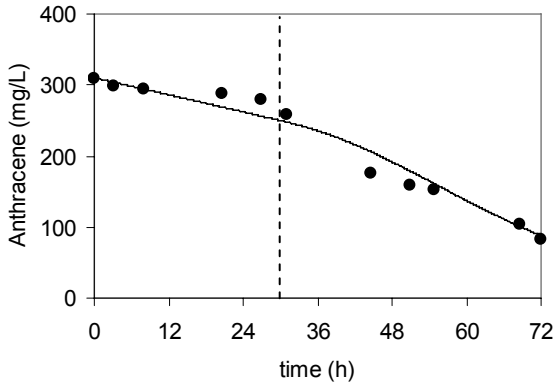

Figure 6-12. Experimental (●) and fitted values (—) obtained from the experiment at 375 rpm-40% silicone oil. Dashed line separates the two stages during the formation of the products.

Apparently, anthracene degradation presents an autocatalytic effect, leading to higher oxidation rates at the end of the reaction. This increase of the degradation rate, which had been observed in monophasic reactors with acetone (Chapter 5), was explained as a catalytic action of the oxidation products formed in the reaction. Anthraquinone (the main oxidation product of the degradation of anthracene by MnP) and, in general, quinones can increase the degradation rates since these substances can play a role as electron carriers (Méndez-Paz et al. 2005). Moreover, the radical species and peroxides formed during the decarboxylation of malonate by Mn^{3+} are highly reactive and can be used by MnP in a partly autocatalytic process (Hofrichter et al. 1998).

In further studies the enzymatic activity should be included in the kinetics of the process to complete the model, as discussed for monophasic reactors in Chapter 5. In the experiments carried out in this chapter, the enzymatic activity was maintained above the threshold value of 100 U/L, thus not having an influence on the degradation. This simplification was considered in order to avoid modeling the enzymatic activity decay, since it is a more complex process than in monophasic reactors.

6.4. Conclusions

The present work was performed to assess the applicability of two-phase enzymatic reactors for degradation of poorly soluble compounds. The organic phase acts as a reservoir of the pollutant, delivering anthracene to the aqueous phase where MnP and the cosubstrates performed the reaction.

By improving the understanding of the main factors affecting the enzymatic oxidation of anthracene, an efficient treatment based on the use of free MnP may be defined. The optimization of these and other parameters for the removal of anthracene in monophasic systems was studied in Chapter 3. There, the most important factors which affected the efficiency of the process were hydrogen peroxide addition rate and concentration of the organic acid (sodium malonate), which were evaluated in the present work. The results obtained here for the optimization of hydrogen peroxide flow rate and the double effect of sodium malonate on the efficiency are in agreement with those obtained in the monophasic system. A new conclusion arisen from this work is the need to control the pH in order to avoid its increase due to enzyme proteolysis.

The availability of poorly soluble compounds is usually a limitation which can be solved favoring mass-transfer rate. This was achieved by increasing the total surface area between the solvent and the aqueous phase by modifying the agitation or the dispersed phase volume. Increasing both factors led to higher values of oxidation rates. Hence, mass transfer processes limited the whole reaction process at the lower values of the conditions studied. Once mass transfer was optimized, the increase of the agitation and the fraction of solvent resulted into lower efficiency, due to the higher enzyme inactivation.

Considering the removal of anthracene by MnP in monophasic systems (with 36% of acetone (v:v) to dissolve 5 mg/L of anthracene) the mean oxidation rate was 0.78 mg/L h and the maximum oxidation rate was 1.35 mg/L·h (Chapter 3) which is below but close to the value obtained for 300 rpm and 30% silicone oil (1.76 mg/L h). In fact, comparing the kinetic coefficients α and β obtained in both reactor configurations, the values estimated in biphasic reactors were more than

100-times higher. In monophasic reactors there were no mass transfer limitations, and the concentration of anthracene, 5 mg/L, was nearly 100-times higher than the aqueous concentration in TPPB. That is the reason for the differences of the values of the kinetic constants, even though the degradation rates were similar. The explanation could lie on the production of the radical species and peroxides mentioned previously. The duration of the experiments in TPPBs was around 10-fold longer than in monophasic reactors; moreover, in the present work there was a continuous addition of malonic acid, and therefore the concentration of decomposition products from the acid was much higher for TPPBs, which could lead to higher oxidation rates. The efficiency of anthracene degradation was increased by 9-fold in biphasic reactor at optimized conditions. This improvement was mainly due to the differences on enzymatic activity loss rate, which was 30 U/L·h in reactors with acetone:water mixtures and only 7 U/L·h in TPPBs.

In order to model the reactor, a study of the mass transfer coefficients was conducted. A sigmoid correlation of the coefficients with the agitation was obtained and the maximum values were obtained at 250 or 300 rpm, independently of the fraction of solvent. Next, a kinetic equation which considered first order with respect to substrate and an autocatalytic effect was applied which resulted in a satisfactory fitting of the data from the experimental design. The kinetic equation is consistent with that derived from monophasic reactors, except that the enzymatic activity term was avoided by maintaining the enzymatic activity above 100 U/L.

Previous studies on non-aqueous enzymatic catalysis have been primarily focused on biotransformation reactions for chemical production or purification, while applications for remediation of environmental pollutants have been largely ignored (Wang et al. 1999). The results achieved show that TPPBs are a promising alternative for the removal of sparingly soluble compounds. The oxidation rate of 1.8 mg/L h obtained here is 3-fold lower than the value obtained in cultures of *Sphingomonas* (Janikowski et al. 2002). But it is worth mentioning that the use of enzymatic reactors is simpler and the operational requirements are lower. Moreover, the reuse of silicone oil and enzyme was shown to be feasible as it was demonstrated in experiments in which the silicone oil depleted in anthracene was separated from the aqueous phase, re-contaminated with the PAH and returned to the aqueous phase in a further batch experiment.

6.5. Nomenclature

a	Interfacial area (dm^2)
α	1st catalytic constant derived from the autocatalytic model (h^{-1})
β	2nd catalytic constant derived from the autocatalytic model ($h^{-1} M^{-1}$)
b	1st coefficient of the response surface (dimensionless)
c	2nd coefficient of the response surface (dimensionless)
d	3rd coefficient of the response surface (dimensionless)
d_{sm}	Sauter mean diameter of drops (dm)
e	4th coefficient of the response surface (dimensionless)
f	1st constant from the sigmoid function (dimensionless)
g	2nd constant from the sigmoid function (dimensionless)
h	3rd constant from the sigmoid function (dimensionless)
i	4th constant from the sigmoid function (dimensionless)
j	5th constant from the sigmoid function (dimensionless)
E	Enzymatic activity in the reactor (U/L)
E_0	Initial enzymatic activity in the reactor (U/L)
E_i	Enzymatic activity in the input flow (U/L)
f	6th catalytic constant from the sigmoid function (L/U)
K_La	Mass transfer coefficient of anthracene (h^{-1})
K_{SW}	Partition coefficient of anthracene in solvent:water (dimensionless)
K'_{SW}	Partition coefficient of anthraquinone in solvent:water (dimensionless)
P_W	Products concentration in the aqueous phase (μM)
P_S	Products concentration in the organic phase (μM)
s	Movement length in the steepest ascent pathway (dimensionless)
S_S	Anthracene concentration in the organic phase (μM)
S_{S0}	Initial anthracene concentration in the organic phase (μM)
S_W	Anthracene concentration in the aqueous phase (μM)
S*	Anthracene concentration in equilibrium conditions (μM)
t	Time (h)
V_S	Volume of the organic phase (L)
V_W	Volume of the aqueous phase (L)
Φ	Solvent proportion (%)
ω	Agitation rate (rpm)
X	Agitation rate (dimensionless)
Y	Silicone oil fraction (dimensionless)
Z	Objective function (dimensionless)

6.6. Acknowledgments

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General conclusions

This thesis contributes to the development of novel technologies for the elimination of poorly soluble recalcitrant compounds. Polycyclic aromatic hydrocarbons (PAHs) were chosen as model compounds due to their toxicity, since many of them are considered as carcinogenic and mutagenic compounds.

The work developed in the present Thesis explores two technologies of innovating character and application to the environmental field. The use of reactors with miscible solvents for degradation of poorly soluble compounds had already been presented by other authors, but those investigations were mainly based on the determination of the substrate oxidized by the enzyme, without taking into account the optimization of the process. The optimization of the anthracene degradation by MnP gave rise to oxidation rates superior to those obtained by other authors. In addition, this technology was applied for the removal of other PAHs with more recalcitrant character, obtaining positive results. Two-phase enzymatic reactors for degradation of compounds with low solubility represent a new configuration, as these compounds had been degraded in two-phase microbial reactors, while enzymatic reactors were only focused in processes for organic compound synthesis. The advantages that this system presents, such as the possibility of solvent and/or enzyme reuse, makes it very attractive for the application to other recalcitrant poorly soluble compounds.

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

I) Monophasic reactors

1. The selection of acetone as miscible solvent for its use in monophasic reactors was based on solubilization capacity and stability of MnP in the mixtures. Acetone at 36% (v:v) increased 140-fold anthracene concentration and during long-term incubations with MnP from *Bjerkandera* at room temperature, it run parallel to the control without solvent.

2. Acetone concentrations higher than 5% (v:v) were demonstrated to be toxic for anaerobic or aerobic populations. Once the enzymatic reactor effluent (36% v:v) is diluted with other streams, acetone may not be toxic and biodegradable by aerobic or anaerobic cultures.

3. Stability studies of MnP from both fungi, *Bjerkandera* sp. BOS55 and *Phanerochaete chrysosporium*, showed that crude from *Bjerkandera* was more resistant to thermal and solvent inactivation than MnP from *P. chrysosporium*.

Therefore, enzymatic crude from *Bjerkandera* was selected for anthracene degradation. Incubations of this crude with mixtures acetone:water in concentrations as higher as 90% (v:v) showed that the enzyme is extremely resistant to acetone.

4. From batch experiments it was concluded that anthracene degradation rate is significantly affected by the concentration of the organic acid: the higher the concentration was, the higher the degradation rate. However the enzyme inactivation is also significantly increased with organic acid concentration. Sodium malonate was the organic acid selected, since it led to the highest efficiency (defined as anthracene degraded per unit of enzyme inactivated).

5. Regarding H_2O_2 addition rate, the range evaluated mainly affected enzyme inactivation rate. An adequate addition of hydrogen peroxide is important in order to reduce MnP inactivation but also to promote a satisfactory conversion.

6. Mn^{2+} concentration has a positive effect on enzymatic stability: the higher concentration, the lower enzymatic inactivation. However, in terms of efficiency its effect was not significant, and the final concentration was selected considering environmental aspects.

7. Inactivation of the enzyme was assayed in different media, concluding that the increase of acetone concentration in a medium containing malonate, hydrogen peroxide and Mn^{2+} has a negative effect on MnP. Taking into account the conclusion 3, this effect is attributed to the formation of MnP inactivating compounds.

8. The environmental factor which mainly affects MnP inactivation rate is temperature. At 40ºC a rapid inactivation of the enzyme occurs. Anthracene degradation is favored in presence of light. Finally, oxygen also favors the degradation rate, suggesting that it can be involved in the degradation mechanism.

9. The optimization of the conditions described above enables to completely degrade anthracene (5 mg/L) in 6 h. In passive aeration, degradation was achieved after 8 h. The only degradation product detected was anthraquinone which represents 50% of the anthracene degraded, this suggesting that other unstable products might be present.

10. Anthracene degradation mechanism was elucidated. Apart from anthraquinone, compounds such as anthrone, dihydroxyanthrone and phthalic acid were detected. In this work, crude MnP led to the ring cleavage of anthracene, traditionally considered as independent from ligninolytic peroxidases.

11. The degradation system was applied to other PAHs, obtaining positive results concerning the oxidation of pyrene and dibenzothiophene. IP values of the other PAHs not degraded were higher than that of chrysene, except for

dibenzothiophene (IP 8.1), suggesting that the IP threshold value of MnP is not definite.

12. An addition of higher enzyme activity was required to complete the degradation of pyrene and dibenzothiophene. Their degradation kinetics are slower than that of anthracene: 12-fold for dibenzothiophene and 34-fold for pyrene.

13. The biomimetic degradation of PAHs by manganese (III) acetate required higher concentration of Mn^{3+} (50-fold the concentration used in *in vitro* experiments), and in the case of pyrene the degradation was not demonstrated.

14. Mechanisms of dibenzothiophene and pyrene degradation are also proposed. In the case of dibenzothiophene, a ring cleavage is observed, as occurred with anthracene. Regarding pyrene, a structure with a hydroxyl radical, 1-hydroxypyrene, was detected, suggesting the direct hydroxylation by $^{\bullet}OH$ radicals during oxidative process.

15. The kinetic model considering an autocatalytic process and first order with respect to substrate accurately predicts anthracene degradation in experiments where MnP is maintained in an adequate range. This speeding up is attributed to the products, mainly quinones, which work as electron carriers.

16. Both experiments from the semi-continuous reactor and the continuous reactor showed that the enzymatic activity influences very much on the degradation extent. The highest degradations of anthracene were attained when the highest MnP activities were present in the reactor. A sigmoid function was included in the model to account for the enzymatic activity, reaching its maximum in the range 100-200 U/L.

17. MnP inactivation was considered as a first order kinetics; however, two periods of inactivation were observed, suggesting a sequential two-step process. The decay constants were similar in all the experiments, except in the continuous reactor which was 4-fold higher.

18. A continuous reactor was operated for 108 h obtaining a 90% of anthracene degradation during the last stage, which was coincident with the highest MnP activities into the reactor.

II) Biphasic reactors

1. Silicone oil was the solvent selected for the experiments in biphasic reactors since it has the lowest log K_{SW} (3.7) of the 15 solvents evaluated and its inactivation effect on MnP at different agitation rates was also the lowest.

2. The optimization of the main factors affecting the catalytic cycle led to similar results than in monophasic reactors. Hydrogen peroxide mainly affects the

inactivation rate and sodium malonate has a double effect: higher concentrations enabled higher degradation rate but lower enzymatic stability.

3. Control of pH is crucial in the operation of biphasic reactors, since pH increased due to the ammonia liberation from the inactive enzyme. Sodium malonate was demonstrated to be oxidized during the reaction. In order to ensure the presence of the organic acid, pH was regulated via malonic acid addition.

4. Agitation and fraction of solvent are important parameters in the operation of biphasic reactors. The agitation optimization consisted in the determination of the agitation speed which enabled the emulsion (~200 rpm) at low enzymatic inactivation. Values as higher as 500 rpm led to a fast MnP inactivation. The system was optimized in terms of efficiency operating at 300 rpm and with 30% of silicone oil (v:v).

5. Mass transfer coefficients (k_La) were determined for the conditions: 50-300 rpm and 10-30% (v:v). K_La increases very much specially in a short range of agitation speed (200-300 rpm). This effect is more pronounced when working at low fractions of silicone oil.

6. The derived kinetic equation, considering first order with respect to substrate and an autocatalytic effect, resulted in satisfactory fitting of data from the experimental design. The kinetic equation was consistent with that applied in monophasic reactors.

Conclusiones generales

Esta Tesis contribuye al desarrollo de nuevas tecnologías para la eliminación de compuestos recalcitrantes de baja solubilidad en agua. Los hidrocarburos aromáticos policíclicos (HAPs) se seleccionaron como compuestos modelo debido a su alta toxicidad, ya que que muchos de ellos tienen propiedades carcinogénicas y mutagénicas.

El trabajo desarrollado en la presente Tesis explora dos tecnologías de carácter innovador y amplia aplicación en el campo medioambiental. El uso de reactores con disolventes miscibles para la degradación de compuestos de baja solubilidad en agua se ha venido desarrollando en los últimos años, dando lugar a diversas publicaciones. Sin embargo esas investigaciones se han basado principalmente en la determinación del substrato oxidado por la enzima, sin tener en cuenta la optimización del proceso. La optimización de la degradación de antraceno por MnP dio lugar a velocidades de oxidación de antraceno superiores a las obtenidas por otros autores. Además, esta tecnología fue aplicada para la eliminación de otros HAPs con carácter más recalcitrante, obteniendo resultados positivos. Los reactores enzimáticos bifásicos para la degradación de compuestos de baja solubilidad representan una configuración innovadora. Los reactores microbianos de dos fases se han venido utilizando para la eliminación de estos compuestos, mientras que los reactores enzimáticos bifásicos han sido enfocados para procesos de síntesis de compuestos orgánicos. Las ventajas que presenta este sistema, tales como la separación y recirculación del disolvente así como la reutilización del enzima, hacen atractivo este sistema para su aplicación en la eliminación de compuestos recalcitrantes de baja solubilidad en agua.

Se han extraído las siguientes conclusiones más específicas de los dos temas principales de la tesis:

I) Reactores monofásicos

1. La selección de acetona como disolvente miscible para su uso en reactores monofásicos se basó en su capacidad de solubilización de antraceno y en la estabilidad de MnP en sus mezclas. La acetona a una concentración de 36 % (v:v) incrementó la concentración de antraceno 140 veces y durante incubaciones de 24 h con MnP de *Bjerkandera* a temperatura ambiente permitió una estabilidad completa de la enzima, similar al control en ausencia de disolvente.

2. Se demostró que concentraciones de acetona superiores al 5 % (v:v) son tóxicas para poblaciones tanto anaerobias como aerobias. Una vez el efluente del reactor enzimático (36 % v:v) se diluye con otras corrientes, la acetona no sería tóxica y podría ser biodegradado por cultivos aerobios o anaerobios.

3. Se estudió la estabilidad de MnP procedente tanto de *Bjerkandera* sp. BOS55 como de *Phanerochaete chrysosporium*, demostrándose que el crudo de *Bjerkandera* fue más resistente a la inactivación térmica y a la causada por el disolvente que el procedente de *P. chrysosporium*. Por consiguiente, el crudo enzimático de *Bjerkandera* fue seleccionado para los experimentos de degradación de antraceno *in vitro*. La alta establidad de MnP alcanzada en incubaciones con mezclas acetona:agua en concentraciones tan altas como 90% (v:V) demostró que la enzima es sumamente resistente a la acetona.

4. La velocidad de degradación de antraceno se ve afectada de forma significativa por la concentración del ácido orgánico: cuanto mayor la concentración, mayor la degradación. Pero de igual modo la inactivación del enzima se ve incrementada por concentraciones altas de ácido orgánico. El ácido orgánico que dio mejores resultados en términos de eficacia (definida como antraceno degradado por enzima desactivada) fue el malonato sódico y a una concentración de 20 mM.

5. Referente a la velocidad de adición H_2O_2, el rango evaluado afectó principalmente a la inactivación de la enzima. Una adición adecuada de peróxido de hidrógeno es importante tanto para reducir la inactivación de MnP como para promover una conversión satisfactoria.

6. La concentración Mn^{+2} tiene un efecto positivo en la estabilidad enzimática: a mayor concentración menor inactivación enzimática. Sin embargo, en términos de eficiencia su efecto no fue muy significativo, y la concentración final se seleccionó considerando aspectos económicos y medioambientales.

7. La inactivación de la enzima fue analizada en diferentes medios, concluyendo que el incremento de la concentración de acetona en presencia de malonato, peróxido de hidrógeno y Mn^{+2} tiene un efecto negativo en la actividad MnP. Teniendo en cuenta la conclusión 3, este efecto es atribuido a la formación de compuestos que inactivan la enzima.

8. La temperatura fue el factor ambiental que afectó en mayor medida a la velocidad de inactivación MnP. A 40ºC la enzima se inactivó de forma casi inmediata. La degradación de antraceno se ve favorecida en presencia de luz. Finalmente, el oxígeno también favorece la velocidad de degradación, lo que sugiere que puede estar involucrado en el mecanismo de degradación.

9. La optimización de las condiciones descritas anteriormente permitió degradar antraceno (5 mg/L) de forma completa tras 6 h. En los experimentos en

ausencia de atmósfera de oxígeno la degradación completa se obtuvo tras 8 h. El único producto de degradación detectado fue antraquinona, representando el 50% del antraceno degradó, lo que sugirió que podrían estar presentes otros productos de degradación más inestables.

10. Se elucidó el mecanismo de degradación de antraceno. Con la excepción de antraquinona, los demás compuestos (antrona, dihidroxiantrona y ácido ftálico) se detectaron en trazas. En este trabajo el crudo MnP dio lugar a la rotura del anillo de antraceno, hecho considerado, tradicionalmente, independiente de las peroxidasas ligninolíticas.

11. Este sistema de degradación en reactores monofásicos fue aplicado a otros HAPs, obteniendo resultados positivos en la oxidación de pireno y dibenzotiofeno. El potencial de ionización de los HAPs no degradados fue más alto que el del criseno, excepto para el dibenzotiofeno (IP 8,1), sugiriendo que el IP límite de MnP no es un valor definitivo.

12. Para la degradación de pireno y dibenzotiofeno fue necesaria una adición de actividad enzimática mayor que en el caso de antraceno y se obtuvieron cinéticas de degradación más lentas: 12 veces inferior para dibenzotiofeno y 34 veces inferior para pireno.

13. La degradación biomimética de HAPs mediante acetato de manganeso (III) requirió concentraciones altas de Mn^{+3} (50 veces la concentración que se usó en los experimentos *in vitro*), y en caso de pireno no se observó degradación.

14. Se propusieron los mecanismos de degradación de dibenzotiofeno y pireno. En el caso de dibenzotiofeno se observó la rotura del anillo aromático, como en el caso de antraceno. En el mecanismo de pireno se determinó una estructura con un radical hydroxilo, 1-hidroxipireno, sugiriendo la hidroxilación directa por radicales $^{\bullet}OH$ durante el proceso oxidativo.

15. El modelo cinético que considera un proceso autocatalítico y primer orden con respecto al substrato predice adecuadamente la degradación de antraceno en los reactores fed-batch donde MnP es mantenido en un rango adecuado. Esta aceleración se atribuye a los productos de degradación, principalmente quinonas, los cuales funcionan como transportadores de electrones.

16. Ambos experimentos del reactor semi-continuo y el reactor continuo demostraron que la actividad enzimática influye en gran medida en la eficacia de degradación. Las mayores degradaciones de antraceno se lograron coincidiendo con las actividades enzimáticas más altas. Se incluyó una función sigmoidea en el modelo para incluir el efecto de la actividad enzimática en la eficacia, alcanzando su máximo en el rango 100-200 U/L.

17. La inactivación MnP fue considerada como una cinética de primer orden; Sin embargo se observaron dos períodos de inactivación, uno más rápido y el siguiente más lento, sugiriendo un proceso secuencial en la desnaturalización de la enzima. Las constantes de desactivación fueron similares en todos los experimentos, excepto en el reactor continuo que fue 4 veces superior.

18. Se operó un reactor en continuo durante 108 h obteniendo un 90% de degradación de antraceno durante la última etapa, coincidente con las actividades MnP más altas en el reactor.

II) Reactores bifásicos

1. El aceite de silicona fue el disolvente seleccionado para los experimentos en reactores bifásicos debido a que presentó el valor mínimo del coeficiente de reparto K_{SW} (3,7) de entre los 15 disolventes evaluados y su efecto sobre la inactivación de la enzima a las diferentes velocidades de agitación fue también el menor.

2. La optimización de los factores principales que afectan al ciclo catalítico condujo a resultados similares a los obtenidos en reactores monofásicos. El peróxido de hidrógeno afectó principalmente a la velocidad de inactivación y el malonato sódico tuvo un efecto doble: concentraciones altas permitieron mayores velocidades de degradación pero también una mayor desactivación enzimática.

3. El control de pH es crucial en la operación de reactores bifásicos, debido a que se produce un aumento del mismo por la liberación de amonio de la enzima inactiva. Se demostró que el malonato sódico se oxida durante la reacción y para asegurar su presencia, el pH se reguló mediante la adición de ácido malónico.

4. La agitación y la fracción de disolvente son parámetros importantes en la operación de reactores bifásicos. La optimización de la velocidad de agitación consistió en la determinación de una velocidad que permitiera la emulsión (~ 200 rpm) y a su vez una desactivación enzimática baja. Velocidades de agitación superiores a 500 rpm dieron lugar a una inactivación inmediata de MnP. La eficacia óptima se obtuvo a 300 rpm y con 30% (v:v) de aceite de silicona.

5. Se determinaron los coeficientes de transferencia de materia (k_La) para las condiciones: 50-300 rpm y 10-30% (v:v). Los valores de K_La aumentaron en gran medida en un rango corto de agitación (200-300 rpm). Este efecto fue más pronunciado al trabajar con fracciones bajas de aceite de silicona.

6. La ecuación cinética fue consistente con la aplicada en reactores monofásicos: primer orden con respecto al substrato y considerando el efecto autocatalítico, resultando en el ajuste satisfactorio de los datos del diseño experimental.

Conclusións xerais

Esta Tese contribúe ao desenvolvemento de novas tecnoloxías para a eliminación de compostos recalcitrantes de baixa solubilidade en auga. Os hidrocarburos aromáticos policíclicos (HAPs) seleccionáronse como compostos modelo debido a alta toxicidade que presentan, xa que que moitos deles teñen propiedades carcinoxénicas e mutaxénicas.

O traballo desenvolvido na presente Tese explora dúas tecnoloxías de carácter innovador e ampla aplicación no campo medioambiental. O uso de reactores con disolventes miscibles para a degradación de compostos de baixa solubilidade en auga veuse desenvolvendo nos últimos anos, dando lugar a diversas publicacións. Con todo, esas investigacións baseáronse principalmente na determinación do substrato oxidado pola enzima, sen ter en conta a optimización do proceso. A optimización da degradación de antraceno por MnP deu lugar a velocidades de oxidación de antraceno superiores ás obtidas por outros autores. Ademais, esta tecnoloxía foi aplicada para a eliminación doutros HAPs con carácter máis recalcitrante, obtendo resultados positivos. Os reactores enzimáticos bifásicos para a degradación de compostos de baixa solubilidade representan unha configuración innovadora. Os reactores microbianos de dúas fases viñéronse utilizando para a eliminación destes compostos, mentres que os reactores enzimáticos bifásicos foron enfocados para procesos de síntese de compostos orgánicos. As vantaxes que presenta este sistema, talles como a separación e recirculación do disolvente así como a reutilización do enzima, fan atractivo este sistema para a súa aplicación na eliminación de compostos recalcitrantes de baixa solubilidade en auga.

Extraéronse as seguintes conclusións máis específicas dos dous temas principais da tese:

I) Reactores monofásicos

1. A selección de acetona como disolvente miscible para o seu uso en reactores monofásicos baseouse na súa capacidade de solubilización de antraceno e na estabilidade de MnP nas súas mesturas. A acetona a unha concentración de 36 % (v:v) incrementou a concentración de antraceno 140 veces e durante incubaciones de 24 h con MnP de *Bjerkandera* a temperatura ambiente permitiu unha estabilidade completa da enzima, similar ao control en ausencia de disolvente.

2. Demostrouse que concentracións de acetona superiores ao 5 % (v:v) son tóxicas para poboacións tanto anaerobias como aerobias. Unha vez o efluente do

reactor enzimático (36% v:v) dilúese con outras correntes, a acetona non sería tóxica e podería ser biodegradada por cultivos aerobios ou anaerobios.

3. Estudouse a estabilidade de MnP procedente tanto de *Bjerkandera* sp. BOS55 como de *Phanerochaete chrysosporium*, demostrándose que o cru de *Bjerkandera* foi máis resistente á inactivación térmica e á causada polo disolvente que o procedente de *P. chrysosporium*. Por conseguinte, o cru enzimático de *Bjerkandera* foi seleccionado para os experimentos de degradación de antraceno *in vitro*. A alta establidade de MnP alcanzada en incubaciones con mesturas acetona:auga en concentracións tan altas como 90% (v:V) demostrou que a enzima é sumamente resistente á acetona.

4. A velocidade de degradación de antraceno vese afectada de forma significativa pola concentración do ácido orgánico: canto maior a concentración, maior a degradación. Pero de igual modo a inactivación do enzima vese incrementada por concentracións altas de ácido orgánico. O ácido orgánico que deu mellores resultados en términos de eficacia (definida como antraceno degradado por enzima desactivada) foi o malonato sódico e a unha concentración de 20 mM.

5. Referente á velocidade de adición de H_2O_2, o rango evaluado afectou principalmente á inactivación da enzima. Unha adición adecuada de peróxido de hidróxeno é importante tanto para reducir a inactivación de MnP como para promover unha conversión satisfactoria.

6. A concentración Mn^{+2} ten un efecto positivo na estabilidade enzimática: a maior concentración menor inactivación enzimática. Con todo, en términos de eficiencia o seu efecto non foi moi significativo, e a concentración final seleccionouse considerando aspectos económicos e medioambientais.

7. A inactivación da enzima foi analizada en diferentes medios, concluíndo que o incremento da concentración de acetona en presenza de malonato, peróxido de hidróxeno e Mn^{+2} ten un efecto negativo na actividade MnP. Tendo en conta a conclusión 3, este efecto é atribuído á formación de compostos que inactivan a enzima.

8. A temperatura foi o factor ambiental que afectou en maior medida á velocidade de inactivación de MnP. A 40ºC a enzima inactivouse de forma case inmediata. A degradación de antraceno vese favorecida en presenza de luz. Finalmente, o osíxeno tamén favorece a velocidade de degradación, o que suxire que pode estar involucrado no mecanismo de degradación.

9. A optimización das condicións descritas anteriormente permitiu degradar antraceno (5 mg/L) de forma completa tras 6 h. Nos experimentos en ausencia de atmosfera de osíxeno a degradación completa obtívose tras 8 h. O único produto de degradación detectado foi antraquinona, representando o 50% do antraceno

degradado, o que suxeriu que poderían estar presentes outros produtos de degradación máis inestables.

10. Elucidouse o mecanismo de degradación de antraceno. Coa excepción de antraquinona, os demais compostos (antrona, dihidroxiantrona e ácido ftálico) detectáronse en trazas. Neste traballo o cru MnP deu lugar á rotura do anel de antraceno, feito tradicionalmente considerado como independente das peroxidasas ligninolíticas.

11. Este sistema de degradación en reactores monofásicos foi aplicado a outros HAPs, obtendo resultados positivos na oxidación de pireno e dibenzotiofeno. O potencial de ionización dos HAPs non degradados foi máis alto que o do criseno, excepto para o dibenzotiofeno (IP 8,1), suxerindo que o IP límite de MnP non é un valor definitivo.

12. Para a degradación de pireno e dibenzotiofeno foi necesaria unha adición de actividade enzimática maior que no caso de antraceno, obténdose cinéticas de degradación máis lentas: 12 veces inferior para dibenzotiofeno e 34 veces inferior para pireno.

13. A degradación biomimética de HAPs mediante acetato de manganeso (III) requiriu concentracións altas de Mn^{+3} (50 veces a concentración que se usou nos experimentos *in vitro*), e no caso de pireno non se observou degradación.

14. Propuxéronse os mecanismos de degradación de dibenzotiofeno e pireno. No caso de dibenzotiofeno observouse a rotura do anel aromático, como no caso de antraceno. No mecanismo de pireno determinouse unha estrutura cun radical hidroxilo, 1-hidroxipireno, suxerindo a hidroxilación directa por radicais $^{\bullet}OH$ durante o proceso oxidativo.

15. O modelo cinético que considera un proceso autocatalítico e de primeira orde con respecto ao substrato predice adecuadamente a degradación de antraceno nos reactores fed-batch onde MnP é mantido nun rango adecuado. Esta aceleración atribúese aos produtos de degradación, principalmente quinonas, os cales funcionan como transportadores de electróns.

16. Ambos experimentos do reactor semi-continuo e o reactor continuo demostraron que a actividade enzimática inflúe en gran medida na eficacia de degradación. As maiores degradaciones de antraceno lográronse coincidindo coas actividades enzimáticas máis altas. Engadiuse unha función sigmoidea no modelo para incluír o efecto da actividade enzimática na eficacia, alcanzando o seu máximo no rango 100-200 U/L.

17. A inactivación MnP foi considerada como unha cinética de primeira orde; Observáronse dous períodos de inactivación, un máis rápido e o seguinte máis

lento, suxerindo un proceso secuencial na desnaturalización da enzima. As constantes de desactivación foron similares en todos os experimentos, excepto no reactor continuo que foi 4 veces superior.

18. Operouse un reactor en continuo durante 108 h obtendo un 90% de degradación de antraceno durante a última etapa, coincidente coas actividades MnP máis altas no reactor.

II) Reactores bifásicos

1. O aceite de silicona foi o disolvente seleccionado para os experimentos en reactores bifásicos debido a que presentou o valor mínimo do coeficiente de reparto K_{SW} (3,7) de entre os 15 disolventes evaluados e o seu efecto sobre a inactivación da enzima ás diferentes velocidades de axitación foi tamén o menor.

2. A optimización dos factores principais que afectan ao ciclo catalítico conduciu a resultados similares aos obtidos en reactores monofásicos. O peróxido de hidróxeno afectou principalmente á velocidade de inactivación e o malonato sódico tivo un efecto dobre: concentracións altas permitiron maiores velocidades de degradación pero tamén unha maior desactivación enzimática.

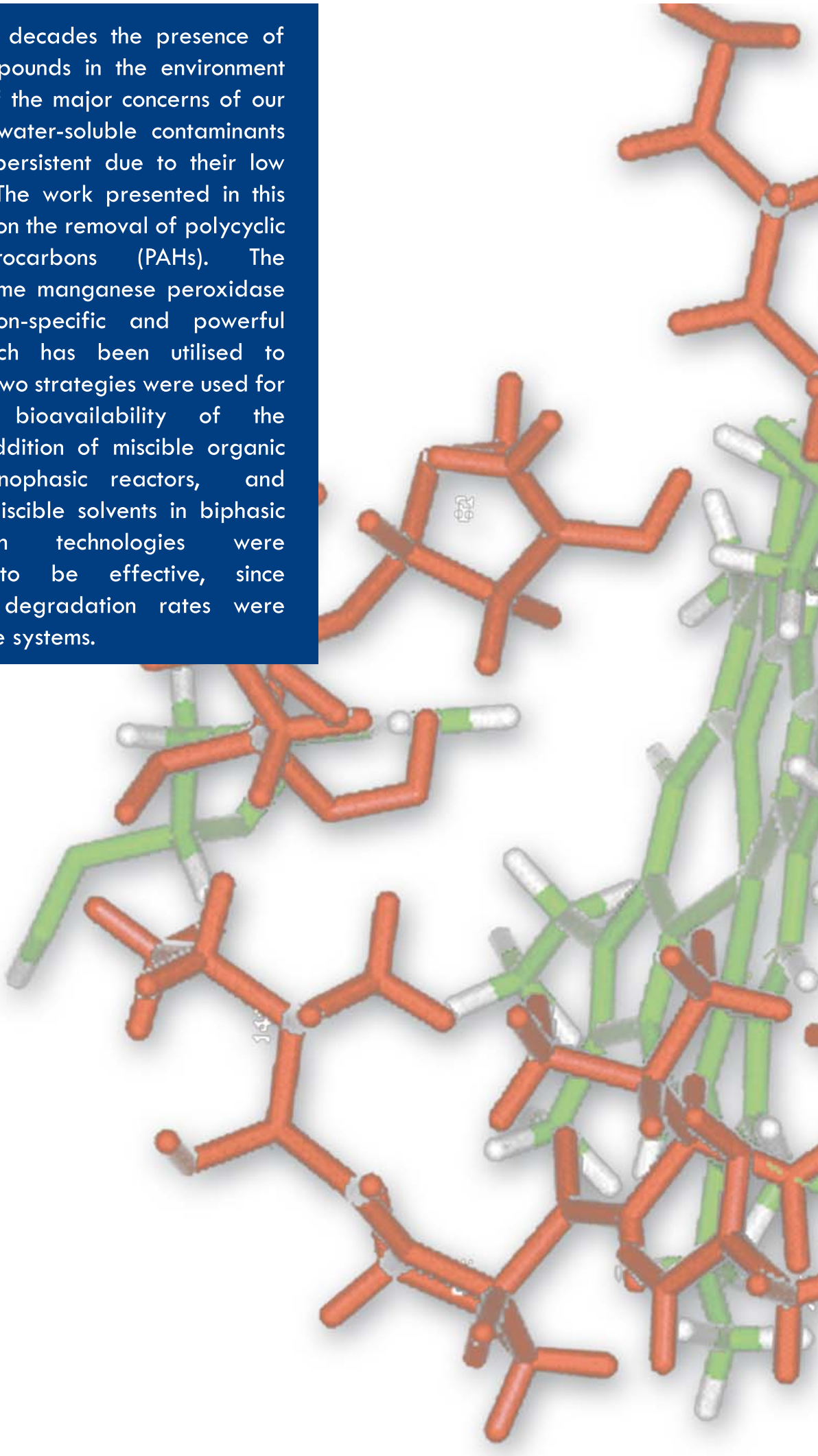
3. O control de pH é crucial na operación de reactores bifásicos, debido a que se produce un aumento do mesmo pola liberación de amonio da enzima inactiva. Demostrouse que o malonato sódico se oxida durante a reacción e para asegurar a súa presenza, o pH regulouse mediante a adición de ácido malónico.

4. A axitación e a fracción de disolvente son parámetros importantes na operación de reactores bifásicos. A optimización da velocidade de axitación consistiu na determinación dunha velocidade que permitise a emulsión (~200 rpm) e á súa vez unha desactivación enzimática baixa. Velocidades de axitación superiores a 500 rpm deron lugar a unha inactivación inmediata de MnP. A eficacia óptima obtívose a 300 rpm e con 30% (v:v) de aceite de silicona.

5. Determináronse os coeficientes de transferencia de materia (k_La) para as condicións: 50-300 rpm e 10-30% (v:v). Os valores de K_La aumentaron en gran medida nun rango curto de axitación (200-300 rpm). Este efecto foi máis pronunciado ao traballar con fraccións baixas de aceite de silicona.

6. A ecuación cinética foi consistente coa aplicada en reactores monofásicos: primeira orde con respecto ao substrato e considerando o efecto autocatalítico, resultando no axuste satisfactorio dos datos do deseño experimental.

Over the last decades the presence of recalcitrant compounds in the environment has been one of the major concerns of our society. Poorly water-soluble contaminants are especially persistent due to their low bioavailability. The work presented in this thesis is focused on the removal of polycyclic aromatic hydrocarbons (PAHs). The ligninolytic enzyme manganese peroxidase (MnP) is a non-specific and powerful biocatalyst which has been utilised to degrade PAHs. Two strategies were used for an increased bioavailability of the contaminants: addition of miscible organic solvents in monophasic reactors, and addition of immiscible solvents in biphasic reactors. Both technologies were demonstrated to be effective, since unprecedented degradation rates were obtained in these systems.



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