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Mass Transfer Enhancement by the Addition of Surfactant in a Two Phase Partitioning Bioreactor for the Degradation of Anthracene by Laccase

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A Two Phase Partitioning Bioreactor (TPPB) was proposed to carry out the degradation of the poorly soluble compound anthracene by laccase from Trametes versicolor. The organic phase consisted of silicone oil saturated with anthracene. The surfactant (Triton X-100) was added to the aqueous phase at concentration above the critical micelle concentration (CMC) to enhance anthracene solubility. The feasibility of reusing the aqueous phase, containing the laccase-mediator system was tested in three consecutive cycles of 24 h with an initial laccase activity of 1200 U/L and 1 mM of the mediator 1-hydroxybenzotriazole (HBT). In the first cycle, total degradation of anthracene took place at a high conversion rate: 16 μmol/L_Rh. The degree of removal achieved in two subsequent cycles with no extra addition of laccase or HBT were 68 % and 40 % for a laccase activity of 150 and 90 U/L in each cycle respectively. These results proved the potential of a TPPB including a surfactant for the removal of poorly soluble compounds by the enzyme laccase and the possibility of exhausting the oxidative potential of the aqueous phase in consecutive cycles to minimize consumption of both laccase and the mediator.

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are chemicals composed of two or more fused aromatic rings, which are widely distributed in the natural environment (Haritash and Kaushik, 2009). These pollutants are released into the environment through the combustion of fossil fuels, waste disposal and accidental spills of petroleum, coal or wood preserving products (Cerniglia, 1993; Pauzi Zakaria et al., 2001). There is toxicological concern about the presence of PAHs in the environment since some of them are known to exert acute toxic effects and/or possess mutagenic, teratogenic or carcinogenic properties. Some PAHs are classified as priority pollutants by the U.S. Environmental Protection Agency (Yuan et al., 2000).
The natural biodegradation of PAHs is restricted by their low water solubility, high hydrophobicity and recalcitrant chemical structure. The use of organic solvents and surfactants has been proposed to increase their apparent solubility in aqueous medium since they can desorb these compounds from soil matrices and facilitate their transfer to aqueous environments where microbes can attack these molecules (Paria, 2008). The two phase partitioning bioreactor (TPPB) concept is based on the use of a water immiscible and biocompatible organic solvent rich in the target compound, in contact with the aqueous phase that contains the biocatalyst (Janikowski et al., 2002). The substrate diffuses from the

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water immiscible phase to the aqueous phase, where enzyme degrades it. Subsequently, substrate delivery is maintained until the pollutant becomes completely depleted. The use of surfactants over the critical micelle concentration (CMC) renders into partial solubilization of PAHs, where the surfactant acts as an emulsifier and the hydrophobic molecules can be incorporated into the micelle core (Edwards et al., 1991).

The combination of a TPPB together with a suitable surfactant may constitute a successful option to overcome problems related to the transfer of the PAH from the organic phase to the aqueous phase (Eibes et al.,2010).

The selection of a suitable biocatalyst for the degradation of PAHs has to account for the high recalcitrance of this type of compounds. White rot fungi are known to degrade a great variety of compounds due to their complex enzymatic system. Enzymatic oxidation of PAHs by these enzymes generates more polar and water soluble metabolites, such as quinones, which could be easier biodegraded by indigenous bacteria present in soils and sediments (Meulenberg et al., 1997).

Laccase (EC.1.10.3.2) is the most widely distributed ligninolytic enzyme among white-rot fungi. It is a multi-copper oxidase that catalyzes the one electron oxidation of substituted phenols, anilines and aromatic thiols to the corresponding radicals with the concomitant reduction of molecular oxygen to water. The capability of laccase from *Trametes versicolor* to degrade anthracene in the presence of ABTS and HBT and natural phenolic mediators has been demonstrated in batch small-scale experiments (Johannes et al., 1996; Cañas et al., 2007). A drawback concerning the use of the laccase-mediator system is the inactivation of the enzyme due to its liability to the free radical attack of redox mediators (Li et al., 1999) and particularly to HBT free radicals (Khlifi et al., 2009). In this sense, the addition of the surfactant Triton X-100 may partially protect laccase from this type of inactivation (Ji et al., 2009).

The objective of this research study is the development of a surfactant assisted biphasic reactor applied for the *in vitro* degradation of anthracene by laccase from *Trametes versicolor*. The enzyme laccase will be present in the aqueous phase with the mediator HBT. The anthracene will be transferred from the organic to the aqueous phase to get oxidised and the main degradation product anthraquinone will be partially transferred to the organic phase according to its partition coefficient. This technology allows working with high concentrations of anthracene and the reuse of the aqueous phase in several batches until exhausting the oxidative potential of the enzymatic system. This approach may lead to a lower demand of laccase and HBT, with the subsequent operational advantages.

2. Materials and methods

2.1 Chemical reagents and enzyme

Anthracene (99 %), Triton X-100 (≥ 98 %) and HBT (99 %) were purchased from Janssen Chimica, Merck and Fluka, respectively. Anthraquinone (97 %), silicone oil 50 cSt, azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (≥ 98 %), organic solvents (HPLC grade) and the commercial laccase from *Trametes versicolor* were purchased from Sigma-Aldrich.

2.2 Determination of enzyme activity

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

2.3 Enzymatic elimination of anthracene

The oxidation of anthracene was carried out at 30 ºC and 250 rpm for 24 h in a two phase partitioning bioreactor considering a conventional configuration of a stirred tank reactor (BIOSTAT®Q reactor, B. Braun-Biotech International, Melsungen, Germany). The total reaction volume was 250 mL with 10 % silicone oil (v:v) previously saturated in anthracene (1800 μM). The aqueous phase consisted of

1200 U/L laccase, 0.1 M sodium acetate buffer at pH 5, 1 mM HBT, 1 % Triton X-100 (v:v). An experiment with no enzyme addition was carried out in parallel as control.
The reusability of the aqueous phase was investigated in two additional batch experiments of 24 h with no extra addition of enzyme or mediator. At the end of each oxidative cycle, the aqueous phase was separated from the exhausted organic phase by decantation and a silicone oil solution freshly spiked with anthracene was added so that the proportion of organic phase was maintained constant: 10 % (v:v). The control assay was subjected to the same procedure.
Before sampling, agitation was stopped for 5 min to equilibrate the system. Samples from both organic and aqueous phase were withdrawn periodically to determine the anthracene and anthraquinone concentrations according to the following procedure: 2 mL of the organic sample were centrifuged for 20 min at 3000 rpm in order to separate tiny aqueous drops and 50 µL of the supernatant were added to a final volume of 5 mL acetonitrile. After 5 min in a vortex, 1 mL of the sample in acetonitrile was analyzed by HPLC for anthracene and anthraquinone determination.
An aliquot of the aqueous phase (1 mL) was taken and laccase activity was determined. Then the sample was frozen after adding 32 µL of 2.5 M HCl to stop the enzymatic reaction. Prior to HPLC analysis, the sample was defrosted and centrifuged for 20 min in order to separate small organic drops.

2.4 High Performance Liquid Chromatography

Anthracene was analyzed by a Jasco XLC HPLC equipped with a diode array detector monitoring the absorbance at 254 nm in a 4.6·150 mm Kinetex reverse phase column (2.6 µm C18 100Å) and a ChromNav data processor. The injection volume was set at 25 µL and the isocratic eluent (80 % acetonitrile and 20 % water) was pumped at a rate of 1.3 mL/min.
Anthraquinone was determined using a 4.6·200 mm Spherisorb ODS2 reverse phase column (5 µm; Waters). The injection volume was 100 µL and the isocratic eluent (80 % acetonitrile and 20 % water) was pumped at a rate of 1 mL/min.

3. Results and discussion

3.1 Anthracene degradation in the first batch

The use of surfactants and organic solvents constitutes a suitable option to enhance the solubilization of organic compounds such as anthracene, whose aqueous solubility is only 0.080 mg/L at 30 ºC (Eibes et al., 2010). With the aim of performing the enzymatic oxidation of this poorly soluble PAH, a TPPB with silicone oil acting as the reservoir of anthracene and surfactant Triton X-100 in a concentration above CMC was used.
Silicone oil was chosen as the solvent due to reported successful implementation of silicone-based TPPBs (Bouchez et al., 1997; Mahanty et al., 2008; Marcoux et al., 2000) and remarkable good features in terms of hydrophobicity. This solvent was previously used for the *in vitro* degradation of anthracene by the ligninolytic enzyme MnP (Eibes et al., 2007) in a TPPB without surfactant. In that system, anthracene was depleted after 56 h with an oxidation rate of 9.9 µmol/L_Rh at optimal operational conditions. Since anthracene is not a substrate for laccase (Johannes et al., 1996), the synthetic mediator HBT was used to increase the oxidation potential of the enzyme.
Figure 1 shows the profile of anthracene and laccase activity during the three consecutive cycles of the TPPB. In the first batch, when starting from an initial activity of 1200 U/L, 90 % of total anthracene was removed after 10 h. The rate of oxidation, calculated from the slope of this interval, resulted in 16 µmol/L_Rh. No anthracene was detected after 24 h, which meant an elimination of 181 µmol/L_R. The oxidation rate of anthracene in the present system was higher than that reported by Eibes et al. (2007), despite the lower oxidation potential of laccase-HBT respect to MnP. These satisfactory results are consequence of the addition of Triton X-100, which enhanced anthracene solubility in the aqueous phase and mass transfer from the organic to the aqueous phase. Whereas below the CMC, surfactants exist as monomers having only minimal effects on the solubilization of polyaromatics, when the surfactant concentration exceeds the CMC, micellar solubilization occurs. Then the aqueous solubility of organic is enhanced by the incorporation of hydrophobic molecules into surfactant micelles (Edwards et al., 1991). Thus, the anthracene concentration in the aqueous phase was approximately

8.5 mg/L, more than 100-fold the saturation value in the absence of surfactant. Increasing the solubility of anthracene in the aqueous phase resulted in a large enhancement of the bioavailability of this compound for laccase.

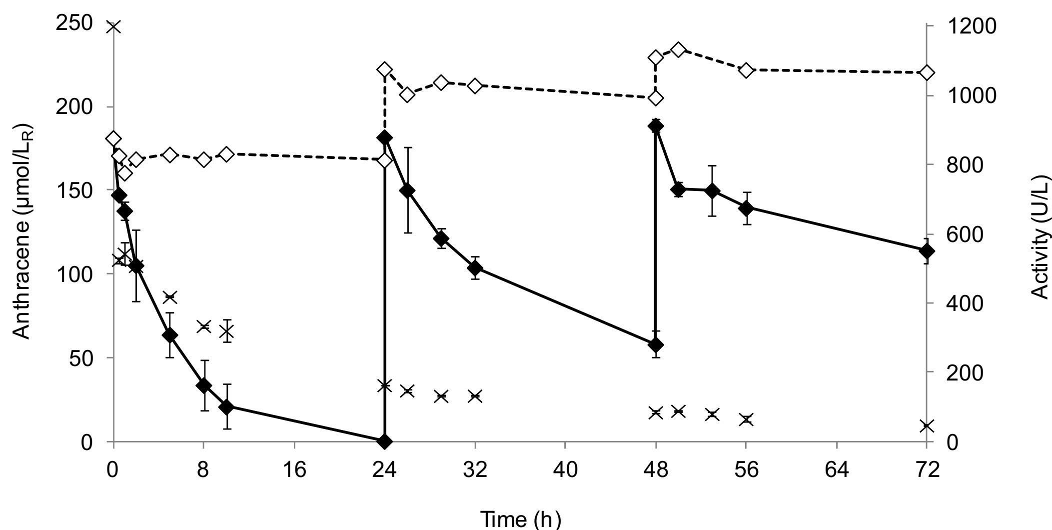

Figure 1: Anthracene concentration and laccase activity (X) during the three consecutive cycles of the TPPB with the laccase-mediator system (—◆—) and the control assay (···◇···). Bars represent standard deviation in two independent experiments.

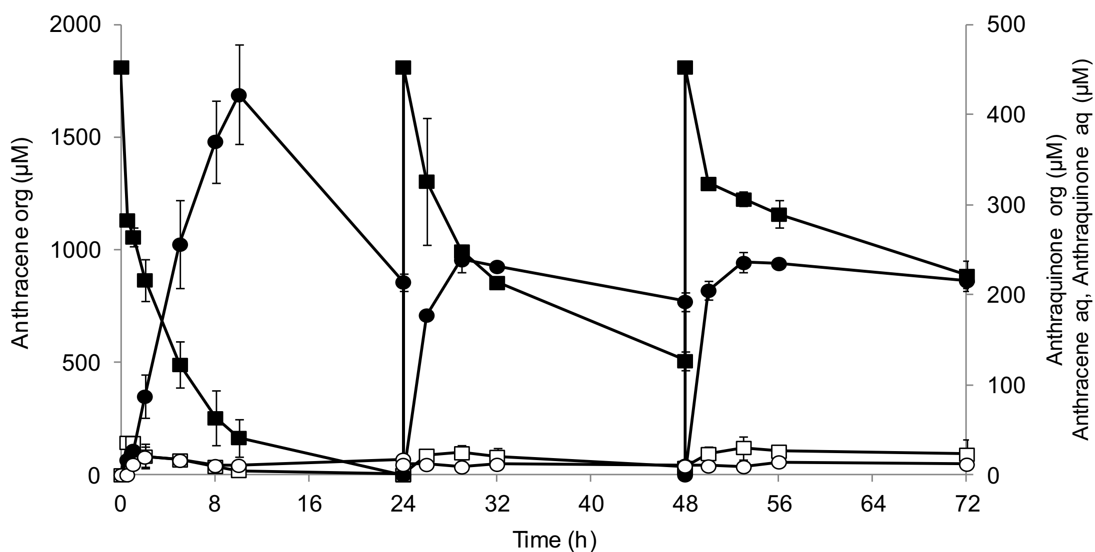

Figure 2: Anthracene and anthraquinone concentrations in each phase, anthracene in the organic solvent (—■—) anthracene in the aqueous phase (—□—), anthraquinone in the organic solvent (—●—), anthraquinone in the aqueous phase (—○—) during the three consecutive cycles of the TPPB.

Another advantage that arises from the use of surfactant consists of the positive effect on enzyme. Such effect had been observed in a BPA-conversion system by laccase (Ji et al., 2009). The presence of this non ionic surfactant in the medium is believed to prevent the laccase inactivation induced by the intermediate free radical attack. Moreover, Triton X-100 helps the enzyme to be in the optimal folding state which is beneficial for its stabilization. The presence of Triton X-100 0.25 CMC has been also demonstrated to reduce VP inactivation with decay coefficients about 1.5 times lower than in its absence (Eibes et al., 2010).
Anthraquinone was obtained as the main product (Figure 2), but in a lower concentration than that predicted by the stoichiometry, which is in agreement with the results reported by Collins et al. (1996), who identified anthraquinone as the major end product of anthracene oxidation by laccase in presence of ABTS as mediator. Moreover, after 10 h of enzymatic treatment, the removal of anthraquinone was observed. In an attempt to identify the degradation products of anthraquinone, standards of phthalic acid, the likely oxidation product of the quinone, were monitored during the experiments. However, no conclusive results were obtained.
Regarding enzyme activity (Figure 1), laccase deactivation occurred in two stages, an initial period of rapid deactivation followed by a period of slow decay. This same behavior had been observed in a previous study carried out by our group to evaluate the deactivation of laccase in the presence of chemicals required in the catalytic cycle of the enzyme (data not shown). It was proved that even in absence of anthracene or HBT, the deactivation always occurred in a two stage period, although the deactivation rate of each period depended on the composition of the medium.

3.2 Anthracene degradation in successive cycles of reusing aqueous phase

The reuse of the aqueous phase containing the laccase-HBT system to degrade anthracene for two additional cycles with no extra addition of enzyme or mediator was demonstrated. The degrees of elimination were 68 % and 40 % for initial laccase activities of 150 and 90 U/L in each cycle respectively. The rate of anthracene oxidation resulted in 9.7 and 6.2 µmol/L_Rh, lower than in the first batch.
The decrease in the reaction rate could be consequence of several reasons, including the lower concentration of enzyme and mediator, the presence of intermediate products that may be more prone than anthracene to be oxidized by laccase-HBT system, or which compete with the mediator to reduce laccase.
Accumulation of anthracene was observed in the reactor used as control throughout the consecutive batchs (Figure 1). Anthraquinone profiles during second and third cycle were similar and characterized by an increase of concentration up to a maximum followed of a slightly decrease as shown in Figure 2. However, the maximum concentration detected was lower than in the first batch.
Regarding laccase activity, whereas in the first batch the inactivation occurred in two stages, in the subsequent cycles a slow and uniform decay took place (Figure 1).

4. Conclusion

A Two Phase Partitioning Bioreactor (TPPB) with silicone oil as organic phase was proposed for the oxidation of the poorly soluble compound anthracene by the laccase-HBT system. The surfactant Triton X-100 was added at a concentration higher than CMC to enhance the apparent solubility of anthracene in the aqueous phase. This system led to the removal of higher loads of anthracene at faster oxidation rates. Moreover, the aqueous phase containing the enzyme could be reused in two additional cycles with no extra addition of laccase or HBT.

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References

Bouchez M., Blanchet D., Besnainou B., Leveau J.Y., Vandecasteele J.P., 1997, Kinetic studies of biodegradation of insoluble compounds by continuous determination of oxygen consumption, J. Appl. Microbiol. 82, 310-316.

Cañas A.I., Alcalde M., Plou F., Martínez M.J., Martínez Á.T., Camarero S., 2007, Transformation of polycyclic aromatic hydrocarbons by laccase is strongly enhanced by phenolic compounds present in soil, Environ. Sci. Technol. 41, 2964-2971.

Cerniglia C.E., 1993, Biodegradation of polycyclic aromatic hydrocarbons, Curr. Opin. Biotechnol. 4, 331-338.

Collins P.J., Kotterman M.J.J., Field J.A., Dobson A.D.W., 1996, Oxidation of anthracene and benzo[a]pyrene by laccases from *Trametes versicolor*, Appl. Environ. Microbiol. 62, 4563-4567.

Edwards D.A., Luthy R.G., Liu Z., 1991, Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions, Environ. Sci. Technol. 25, 127-133.

Eibes G., McCann C., Pedezert A., Moreira M.T., Feijoo G., Lema J.M., 2010, Study of mass transfer and biocatalyst stability for the enzymatic degradation of anthracene in a two-phase partitioning bioreactor, Biochem. Eng. J. 51, 79-85.

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

Haritash A.K., Kaushik C.P., 2009, Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review, J. Hazard. Mater. 169, 1-15.

Janikowski T., Velicogna D., Punt M., Daugulis A.J., 2002, Use of a two-phase partitioning bioreactor for degrading polycyclic aromatic hydrocarbons by a *Sphingomonas sp*, Appl. Microbiol. Biotechnol. 59, 368-376.

Ji G., Zhang H., Huang F., Huang X., 2009, Effects of nonionic surfactant Triton X-100 on the laccase-catalyzed conversion of bisphenol A, J. Environ. Sci. 21, 1486-1490.

Johannes C., Majcherczyk A., Hüttermann A., 1996, Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds, Appl. Microbiol. Biotechnol. 46, 313-317.

Khlifi R., Sayadi S., Belbahri L., Woodward S., Mechichi T., 2009, Effect of HBT on the stability of laccase during the decolourization of textile wastewaters, J. Chem. Technol. Biotechnol. 84, 1828-1833.

Li K., Xu F., Eriksson K.-E.L., 1999, Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound, Appl. Environ. Microbiol. 65, 2654-2660.

Mahanty B., Pakshirajan K., Venkata Dasu V., 2008, Biodegradation of pyrene by *Mycobacterium frederiksbergense* in a two-phase partitioning bioreactor system, Bioresour. Technol. 99, 2694-2698.

Marcoux J., Déziel E., Villemur R., Lépine F., Bisaillon J.G., Beaudet R., 2000, Optimization of high-molecular-weight polycyclic aromatic hydrocarbons' degradation in a two-liquid-phase bioreactor, J. Appl. Microbiol. 88, 655-662.

Meulenberg R., Rijnaarts H.H.M., Doddema H.J., Field J.A., 1997, Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability, FEMS Microbiol. Lett. 152, 45-49.

Paria S., 2008, Surfactant-enhanced remediation of organic contaminated soil and water, Adv. Colloid. Interface Sci. 138, 24-58.

Pauzi Zakaria M., Okuda T., Takada H., 2001, Polycyclic aromatic hydrocarbon (PAHs) and hopanes in stranded tar-balls on the coasts of peninsular Malaysia: Applications of biomarkers for identifying sources of oil pollution, Mar. Pollut. Bull. 42, 1357-1366.

Yuan S.Y., Wei S.H., Chang B.V., 2000, Biodegradation of polycyclic aromatic hydrocarbons by a mixed culture, Chemosphere, 41, 1463-1468.