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BIOTECHNOLOGICAL POTENTIAL OF LIGNINOLYTIC ENZYMES FOR POLLUTANT BIODEGRADATION IN WATER: FROM TEST-TUBES TO FULL-SCALE ENZYMATIC REACTORS

POTENCIAL BIOTECNOLÓGICO DE LAS ENZIMAS LIGNINOLÍTICAS PARA LA BIODEGRADACIÓN DE CONTAMINANTES EN AGUA: DEL TUBO DE ENSAYO A LOS REACTORES ENZIMÁTICOS A GRAN ESCALA

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Abstract

Population growth, industrialization and the lack of restrictions on the use of recalcitrant compounds have led to the release of large amounts of pollutants. Water resources have been particularly affected by the presence of these compounds because the conventional wastewater treatments are unable to completely remove these pollutants. In fact, certain pharmaceutical products have been detected in drinking water at concentrations as high as 0.3 μg/L. The seriousness of environmental pollution has triggered the development of scientific activities for the prevention and control of water pollution; among them, the application of enzymatic processes is emerging as a promising alternative. The stability of some ligninolytic enzymes and the development of processes for their repeated reuse, have been fundamental to scale-up enzymatic experiments from test-tubes to full-scale reactors. This review shows the key advances for scaling-up the enzymatic degradation processes, the problems that have arisen during the operation of different reactors and the strategies that have served to solve these problems. Finally, the work includes a cost comparison between the utilization of enzymatic treatments and other technologies. The available information indicates that enzymatic treatments have a real potential to compete with other processes in terms of efficiency, costs and environmental impact.

Keywords: Ligninolytic enzymes, wastewater, recalcitrant pollutants, enzymatic reactors, cost analysis, life cycle assessment.

Resumen

El crecimiento poblacional, la industrialización y la falta de restricciones en el uso de compuestos recalcitrantes ha ocasionado la liberación al ambiente de numerosos contaminantes. Los recursos hídricos han sido particularmente afectados debido a que las tecnologías convencionales para su tratamiento son ineficientes. De hecho, se han reportado concentraciones de compuestos farmacéuticos de hasta 0.3 μg/L en agua potable. Lo anterior ha impulsado el desarrollo de actividades científicas para prevenir y remediar dicho problema; al respecto, la aplicación de procesos enzimáticos ha surgido como una estrategia prometedora. El descubrimiento de enzimas ligninolíticas estables y el diseño de mecanismos para su reutilización han permitido el escalamiento de los procesos enzimáticos desde tubos de ensayo hasta reactores a gran escala. En esta revisión se presentan los avances científicos que fueron clave para conseguir el escalamiento de esta tecnología, los problemas surgidos durante la operación de distintos reactores y las estrategias que han servido para darles solución. Además, se expone un análisis comparativo sobre el costo de aplicar un tratamiento enzimático con respecto a otras tecnologías. La información disponible indica que los procesos enzimáticos tienen un potencial real para competir con otros tratamientos en términos de eficiencia, costo e impacto ambiental.

Palabras clave: Enzimas ligninolíticas, agua residual, contaminantes recalcitrantes, reactores enzimáticos, análisis de costos, análisis de ciclo de vida.

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1 Recalcitrant organic pollutants in water

Water pollutants comprise a broad spectrum of chemical compounds from natural and anthropogenic sources. Compounds having anthropogenic origins have spread in the environment as a result of industrial emissions, inefficient or non-existent wastewater treatment processes, inefficient management and disposal of solid waste, accidental spills, production and use of pesticides and massive use of pharmaceuticals and personal care products, among others (Becerril-Bravo, 2009).

Pharmaceuticals and personal care products (PPCPs) have become an important pollution source, since antibiotics, anti-inflammatories, hormonal contraceptives, deodorants, sunblocks, disinfectants, etc., are used on a daily basis and their degradation products are inadvertently discarded (Becerril-Bravo, 2009). The concentration of these compounds in treated effluents and environmental samples varies between pg/L and μg/L, however, even at these concentrations, they can exert highly damaging biological effects (Schwarzenbach *et al*., 2006). As an example, hormones like estrone, 17β-estradiol, and 17α-ethinylestradiol have been found in real effluents at concentrations between 0.29 and 1.52 ng/L and at this level they can exert endocrine disrupting activity (Lloret *et al*., 2013). The exposure to these compounds can disturb the endocrine system of humans and wildlife causing abnormal thyroid function, decreased fertility, decreased hatching success (fish, birds, and turtles), demasculinization and feminization of males (fish, birds, and mammals), defeminization and masculinization of females (fish, gastropods, and birds) and alteration of immune function (birds and mammals) (Kuss *et al*., 2018; Rivas *et al*., 2004; Colborn *et al*., 1993).

Another group of important water pollutants are polycyclic aromatic hydrocarbons (PAHs) and phenolic compounds. The latter and some of its substituted, such as 2,4-dichlorophenol, 2,4,5-trichlorophenol, 3-methoxyphenol, etc. are used on a large scale as wood preservatives, pesticides and precursors of herbicides (Georgieva *et al*., 2010; Duchnowicz *et al*., 2002). The toxicity of phenolic compounds and their derivatives, depends on the specific compound and the exposure levels; just as an example, it has been reported that concentrations between 0.5 and 4 mM of 2,4-dichlorophenol or 2,4,5-trichlorophenol can cause hemolysis, hemoglobin oxidation, and lipid peroxidation in human erythrocytes (Duchnowicz *et al*., 2002). In addition, it is known that these compounds can bind to some enzymes, inhibit redox reactions in cells, cause oxidative stress, metabolic acidosis, renal failure, increased aminotransferase activities, pyrexia, hyperventilation, etc. In addition, phenolic compounds add odour and taste to water, reduce the concentration of dissolved oxygen and some of them are suspected to be carcinogenic (López *et al*., 2011; Bradberry *et al*., 2004; Duchnowicz *et al*., 2002).

On the other hand, PAHs are generated during incomplete combustion of fossil fuels and also in some industrial activities. The environmental impact of these compounds lies in their mutagenic and carcinogenic potential; in addition, due to their hydrophobic nature, they tend to accumulate on soil organic matter, which decreases their bioavailability and degradation by wild microorganisms (Eibes *et al*., 2006). Another class of recalcitrant compounds are synthetic dyes, which are extensively used in textile industries, leather tanning, production of paper and plastics, etc. Dyes can be detected in water even at very low concentrations (5 μg/L) and its presence undoubtedly reduce the amount of sunlight to photosynthetic organisms. The inhibition of photosynthetic processes reduces the production of oxygen and therefore, promotes the establishment of anoxic conditions which is lethal for aquatic life (Wong and Yu, 1999; Champagne and Ramsay, 2010). The previously mentioned pollutants are just an example of the great variety of compounds that are daily produced, used and discharged in the environment. The specific biological effects that these compounds exert can greatly vary depending upon the individual susceptibility, the route and level of exposition, etc. (Duchnowicz *et al*., 2002). These aspects are the subject of another review and therefore will not be thoroughly reviewed in this work; however, in the following sections we have included real information about the occurrence of different pollutants in the environment and different alternatives for its degradation into harmless compounds.

2 Wastewater treatment technologies for the removal of recalcitrant pollutants

The objective of a wastewater treatment is to treat the waste stream to a harmless level for the receiving body (Drinan, 2001). Conventional wastewater treatment processes are based on a combination of physical, chemical and biological methods. These methods are organized in sequential stages commonly recognized as primary, secondary (or biological) and tertiary (or advanced) treatments. Primary treatments remove a portion of the pollutants by physical processes (screening and sedimentation), secondary treatments, also known as biological treatments, use the metabolic activity of a consortia of microorganisms (mostly bacterial and archaeal populations) to decompose organic compounds into carbon dioxide, methane (in anaerobic processes), stable solids, and more organisms. Finally, the advanced processes improve the effluent quality by removing more contaminants than can usually be achieved by primary and secondary treatments, for example, nitrogen, phosphorus and heavy metals; however, not all wastewater effluents are presently being treated beyond secondary treatment (Drinan, 2001).

The most widely used biological treatments for domestic and industrial waste streams are the activated sludge systems, however, the chemical structure of recalcitrant pollutants is so stable, that their degradation efficiency in this kind of processes is extremely variable, going from 0 to 100% depending on the compound (Hollender *et al*., 2009; Luo *et al*., 2014; Stolz, 2001; Drinan, 2001), in addition, for some compounds (for example, diclofenac, galaxolide, tonalide, nonylphenol, etc.) the removal occurs mainly by sorption processes (Luo *et al*., 2014). In fact, the wastewater treatment plants (WWTP) effluents are considered one of the major source of micropollutant release into the environment (Luo *et al*., 2014).

Considering the ecological problems related to the discharge of even trace concentrations of these pollutants, the need to apply a mandatory tertiary treatment is evident. In this regard, the proposed alternatives for tertiary treatments are; 1) Physical methods such as adsorption, filtration, coagulation-flocculation, etc., 2) Oxidation methods like ozonation, Fenton oxidation, photocatalytic oxidation, etc. 3) Use of alternative microorganisms like white-rot fungi, algae or specific bacterial strains and 6) Enzymatic treatments, based on the use of oxidative enzymes like laccases and peroxidases (Holkar *et al*., 2016; López *et al*., 2011). Table 1 shows the main advantages and disadvantages of the use of the most common physical and chemical methods, and also about the use of alternative microorganisms and enzymes.

It is noteworthy from Table 1 that all the actual alternatives for the removal of recalcitrant pollutants have specific advantages and disadvantages, but is also true that the sources of micropollutants are so diverse that a single technology cannot meet the specific needs of each of them. Therefore the study and continuous improvement of all these technologies is equally important; what probably seems invaluable at this moment can be very valuable in due time.

Details about all mentioned technologies is out of the scope of this work, but further information can be found in Mir-Tutusaus *et al*., (2018), Oturan and Aaron, (2014), Luo *et al*., (2014), Hollender *et al*., (2009), Khataee *et al*., (2013), between others. In the following sections this review will focus on the aspects that have led to the development of enzymatic treatments from the first attempts performed in test-tubes to the performance of full-scale enzymatic reactors.

3 Ligninolytic enzymes for the degradation of recalcitrant organic pollutants

3.1 First attempts at test-tube scale

A number of oxidative enzymes from bacteria, fungi and plants have been used in waste treatment applications, however, the ligninolytic enzymes produced by white-rot fungi are probably the most studied with bioremediation purposes, particularly laccases and peroxidases (Martínez-Sánchez *et al*., 2018). White-rot fungi have a unique ligninolytic system that is also useful for the degradation of a wide variety of recalcitrant pollutants; this is due to the relatively nonspecific nature of ligninolytic enzymes and the structural resemblance between these compounds and lignin. Oxidation mechanisms of ligninases is based on the formation of free radicals, which in turn oxidize pure or mixed contaminants (Gasser *et al*., 2014; Mester and Tien, 2000).

First works dealing with the application of ligninases started with small-scale assays performed in test-tubes, Ependorff tubes or even in smaller assays with reaction volumes less than 500 μL. Only few of these studies reported the scaling-up of their experiments to Erlenmeyer flasks or glass beakers. In general, first assays were performed in batch mode, without enzyme recovery and/or recycling, and in most cases the biodegradation reaction was carried out at very controlled conditions, for example, using buffered solutions instead of real wastewater, controlled temperatures instead of ambient temperature, and preventing the presence of enzymatic inhibitors. Some examples of these assays are described below.

3.2 Use of laccases

Laccases are phenol oxidases that catalyze the oxidation of several aromatic compounds using oxygen as electron acceptor and generating water as the final product (Manavalan *et al*., 2015). In addition, when steric hindrances restrict the direct oxidation of a given compound, it can be oxidized by an indirect mechanism based on the generation of small laccase-radicalized mediators. This strategy consists on the addition of low molecular weight compounds that once oxidized by the enzyme, can interact with the bulky substrate targets. Among the compounds that can be used as redox mediators are 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), N-hydroxyacetanilide (NHA), coumaric acid, etc. (Su *et al*., 2018; Camarero *et al*., 2005). The most biotechnologically useful laccases have been isolated mostly from basidiomycetes, this is because the redox potential of fungal laccases (> 0.71 V) is greater than that of plant or bacterial enzymes (0.36-0.46 V) (Bronikowski *et al*., 2017).

In 2000, Abadulla *et al*., reported that laccases of *Trametes hirsuta* successfully degraded triarylmethane, indigoid, azo and anthraquinonic dyes in small-scale reactions (4-mL test-tubes or 300-mL Erlenmmeyer flasks) and reduced their toxicities (based on the oxygen consumption rate of *Pseudomonas putida*) by up to 80% (anthraquinonic dyes). Murugesan *et al*., (2007) studied the decolorization of remazol brilliant blue R (RBBR) and remazol black-5 (RB-5) using crude laccases of *Ganoderma lucidum*; the assays were carried out in 1-mL Ependorff tubes containing 50 mg/L of the selected dye and 20-25 U/mL of laccases dissolved in a buffered solution. Maximum decolorization of RBBR was 40% and it was observed after 2 h incubation; the addition of 1 mM of HBT improved decolorization to 92.4%. RB-5 was not decolorized in the absence of HBT, and maximum decolorization in the presence of the mediator was 77.4% after 2 h. These results showed that dyes exhibit differential susceptibility to degradation. Champagne and Ramsay, (2007) reported that purified laccases of *Trametes versicolor* were able to degrade reactive blue 19 (0.036 mM) at a high extent (90%) and with a maximum decolorization rate of 8.3 μM/U·h; these results were obtained in batch mode and with a total volume reaction of 50 mL. Méndez-Hernández *et al*., (2013) showed that the enzymes of certain microorganisms can resist the inactivation caused by high ionic strength and/or temperature, which was relevant for the application of these enzymes for the treatment of real textile wastewaters. In their work these authors showed that the laccases of *Fomes* sp. EUM1 exhibited a high rate of decolorization of the textile dye Acid Blue 74 (95% in 5 h) and remained stable at 40 °C in the presence of NaCl, 50 mM during all the incubation period; in this case, the assays were performed at a slightly higher scale using a total reaction volume of 250 mL. In addition, tests using *Daphnia magna* revealed the non-toxic nature of the decolourized solution. Later, Zhu *et al*., (2013) showed that pure laccases of *Russula virescens* could degrade not only textile dyes such as RBBR or reactive blue, but also laboratory dyes such as bromothymol blue, malachite green and eriochrome black T. In this case, the treatments consisted of micro-reactions containing 400 μL of the selected dye dissolved in sodium acetate buffer (10 mM, pH 4.5) and 10 μL of purified laccase.

Regarding the use of laccase-mediator systems, two alternatives emerged from the results of different works: 1) the use of synthetic mediators like HBT, ABTS, etc., or 2) the use of natural compounds derived from lignin, such as syringaldehyde, acetosyringone, phenolic aldehydes, etc. Initially, synthetic mediators arose like an excellent alternative to improve laccase performance, however, their high cost and the evidence about their toxicity began to cause concern; it was then proposed their substitution by lignin derivatives, which in some cases resulted as effective as synthetic mediators but without their toxicity problems (Camarero *et al*., 2005). Lloret *et al*., (2010) studied the degradation of different endocrine disrupting chemicals (EDCs) (17β-estradiol, 17α-ethinylestradiol and estrone) by commercial laccases of *Myceliophthora thermophila* with and without the

addition of redox mediators. The authors found the complete removal of 17α-ethinylestradiol and 17β-estradiol after 5 and 3 h, respectively. Estrone was more recalcitrant since it was degraded only 60% after 8 h (pH 5.0), however, this result was significantly improved by the addition of natural or synthetic mediators. The compound was completely removed after 8 h in the presence of violuric acid, 80% degradation was observed by the use of HBT and 70% by using p-coumaric acid or some other natural mediators. Different herbicides and pesticides can also be degraded by laccase-mediator systems (Zhu *et al*., 2013; Pizzul *et al*., 2009). Pizzul *et al*., (2009) showed that laccases of *Trametes versicolor* can be applied in the degradation of glyphosate, a persistent compound widely used as herbicide. Those authors demonstrated that although glyphosate is not a natural substrate for laccases, its biodegradation (41% in 24 h) can be achieved indirectly by the addition of ABTS as redox mediator.

3.3 Use of ligninolytic peroxidases

Ligninolytic peroxidases are hemoproteins that catalyze reactions in the presence of H_2O_2. These enzymes are produced by different microorganisms, however, lignin peroxidases (LiP), manganese peroxidases (MnP) and versatile peroxidases (VP) produced by white-rot fungi are the most studied with respect to their capacity to degrade recalcitrant compounds (Oliveira *et al*., 2016; Eibes *et al*., 2006; Taboada-Puig *et al*., 2011). Lignin peroxidases (LiP) were first described in the ligninolytic fungus *Phanerochaete chysosporium*, but they have been isolated from several other fungi, among them, *Trametes versicolor*, *Phlebia radiata*, *Ganoderma lucidum* and *Bjerkandera* spp. (Oliveira *et al*., 2016; Hofrichter *et al*., 2010). LiP are able to oxidize different water pollutants, including aromatic dyes, EDCs, etc., however, the studies dealing with the use of LiP are much less than those about laccases. Most LiP applied to the degradation of recalcitrant pollutants have been obtained from *Phanerochaete chrysosporium* strains, which produce several LiP isoenzymes. The specificity and kinetic properties of each isoenzyme are variable, however, the majority present high affinity for veratryl alcohol, which explains the use of this compound as redox mediator (Ferreira-Leitao *et al*., 2007, Ollikka *et al*., 1993).

Ferreira-Leitao *et al*., (2003) studied the enzymatic decolorization of methylene blue (MB) by a LiP of *Phanerochaete chrysosporium* and compared the results with the utilization of a horseradish peroxidase (HRP). Reaction mixtures contained 8 μM of MB, 80 μM of H_2O_2 (molar ratio MB:H_2O_2 1:10), and 1.3 μM of HRP or 1.1 μM of LiP. The LiP decolorized the dye significantly faster than the HRP, LiP achieved 80% decolorization in 2 min, while HRP required 42 min to reach 70% decolorization of the dye. In addition, it was observed than under certain conditions LiP can mediate the aromatic ring cleavage of MB. More recently, Mao *et al*., (2009) studied the removal of 17β-estradiol by means of a LiP produced by *P. chrysosporium*; the assays were carried out in test-tubes containing a total volume reaction of 2 mL. After testing different pH conditions and H_2O_2 concentrations the authors observed almost 100% removal by using pH 4.6, H_2O_2 0.1 mM, LiP 0.02 U/mL and 17β-estradiol 10 μM. Analysis of the degradation products of 17β-estradiol by mass spectrometry strongly suggested that the compound tends to polymerize likely via oxidative coupling, which according to the authors is useful to reduce the estrogenicity of the parent compound. More recently, Dong *et al*., (2014) studied the LiP removal of nonylphenol (10 μM) in test-tubes containing a final reaction volume of 2 mL. The authors studied the removal under varying conditions, i.e., different pH, H_2O_2 concentration, temperature and enzyme activity. The best conditions for nonylphenol removal were: H_2O_2 10 μM, pH 4.6, LiP 0.005 U/mL, reaching a maximum removal of 85% in 60 min.

On the other hand, it has also been explored the application of bacterial LiP on the biodegradation of organic pollutants. As an example, Parshetti *et al*., (2012) found that LiP produced by *Kocuria rosea* MTCC 1532 can degrade aromatic dyes belonging to different structural groups, for example, triphenylmethane, azoic, heterocyclic and polymeric derivatives. In this work, small-scale assays were performed in test-tubes containing a final reaction volume of 2.5 mL, pH was adjusted to 7.0 and 0.25 U of bacterial LiP were added as biocatalyst. The purified LiP decolorized methyl orange (100%), cotton blue (60%), methyl violet (80%), amido black (60%), orange HE2R (90%), reactive blue 25 (100%), direct blue 6 (70%), reactive yellow 81 (80%), red HE4B (90%), green HE4B (100%) and reactive green 19 A (60%) in 5 h. However, the intracellular nature of *K. rosea* peroxidases is an important disadvantage, since its extraction and purification processes may represent and extra cost. On the other hand, similar to LiP, the potential of fungal MnP for the removal of recalcitrant pollutants has also been explored.

Interestingly, the catalytic cycle of MnP includes the oxidation of Mn^{2+} to Mn^{3+}, these ions are quite unstable but have an enormous oxidizing potential. In order to stabilize these ions, ligninolytic fungi naturally produce dicarboxylic acids, like oxalate and malonate. These acids form chelate complexes with Mn^{3+} and together they can diffuse in the media and oxidize a wide range of aromatic compounds. According to this, if MnP are going to be applied in the oxidation of organic pollutants it would be necessary to add Mn^{2+} and dicarboxylic acids, in addition to H_2O_2. Due to these extra-requirements the study and application of MnP has been limited (Hofrichter *et al*., 2010; Cameron *et al*., 2000; Mester and Tien, 2000). An example of its application is the work published by Eibes *et al*., (2006). In this study the MnP of Bjerkandera sp. BOS55 proved to be useful for the degradation of polycyclic aromatic hydrocarbons (PAHs) due to its stability in the presence of organic solvents. The solubility of PHAs in aqueous media is low, this condition limits their contact with the enzyme and therefore its degradation. In this context, the addition of organic solvents is a useful strategy, however, solvents can inhibit the enzymatic activity, and then it is necessary to identify stable and active enzymes under these conditions. Eibes *et al*., (2006) found that the MnP produced at lab-scale by *Bjerkandera* sp. BOS55 was stable in acetone solutions (36% v/v), being able to remove three PAHs: anthracene, dibenzothiophene and pyrene. These compounds were degraded to a large extent after a short period of time (less than 24 h), and in the case of dibenzothiophene, it was identified a ring cleavage compound (4-methoxybenzoic acid) as biodegradation product. Finally, the third most studied fungal peroxidases are versatile peroxidases (VP); these enzymes were first described in the white-rot basidiomycete *Pleurotus eryngii* (Martínez *et al*., 1996), and its name refers to the singular capability of these enzymes to combine the catalytic properties of LiP and MnP; therefore VP can oxidize de characteristic substrates of both enzymes. The substrate promiscuity of VP is related to their high redox potential (> 1.4 V) and also to the presence of specific catalytic sites for the oxidation of high and low redox potential compounds (Garcia-Ruiz *et al*., 2012).

There are several reports on the application of VP to degrade recalcitrant organic pollutants. As an example, Taboada-Puig *et al*., (2011) assessed the degradation of different EDCs, bisphenol A, nonylphenol, triclosan, 17α-ethinylestradiol and 17β-estradiol (10 mg/L each), using an immobilized VP produced at lab-scale with *Bjerkandera adusta*. Assays were performed in a final volume of 5 mL, at controlled pH values (pH 5.0) and using an enzyme activity of 250 mU. The biocatalyst was able to completely remove all the compounds except triclosan (the residual concentration was 74%), in addition, the estrogenic activity of most pollutants was significantly reduced. Similarly, Eibes *et al*., (2011) studied the removal of a series of pharmaceuticals: antibiotics (sulfamethoxazole), antidepressives (citalopram hydrobromide and fluoxetine hydrochloride), antiepileptics (carbamazepine), anti-inflammatory drugs (diclofenac and naproxen) and estrogen hormones (estrone, 17β-estradiol, 17α-ethinylestradiol) by means of a VP from *Bjerkandera adusta*. The assays were carried out in Erlenmeyer flasks with a total volume reaction of 50 mL. The reaction mixture contained the selected compound to be degraded (2.5 mg/L), the enzyme (10-1000 U/L), Mn^{+2}, (33 μM), sodium malonate (1-10 mM, pH 4.5) and H_2O_2 (added at a rate of 1-10 μM/min. The results showed a significant removal of antibiotics, anti-inflammatories and hormones even at a low enzyme concentration. On the contrary, the antidepressants and the antiepileptic drugs were much more recalcitrant; the maximum degradation achieved for citalopram was 18% and lower than 10% for fluoxetine after 7 h of enzymatic treatment with a high VP concentration (1000 U/L). From the information previously exposed it can be concluded that ligninolytic enzymes are a potential tool for the biodegradation and/or biotransformation of different recalcitrant pollutants in water. The next step in the application of ligninases was their use in small-scale enzymatic reactors; the results of these assays are presented below.

3.4 Lab-scale enzymatic reactors

As previously described, ligninolytic enzymes are particularly useful for the degradation of different types of recalcitrant pollutants, however, all the mentioned results were obtained in small assays carried out under reaction conditions completely different from those prevailing in a real waste streams. Moreover, none of these studies considered the recovery and reuse of enzymes which is an essential requirement for a full-scale application (Nguyen *et al*., 2014). Taking that into consideration, gradually the research work began to focus on designing efficient strategies to recycle the enzymes and also to improve their stability. These efforts led to the development of

the enzymatic reactors.

In general, enzymatic reactors can be classified according to its operation mode (batch-wise semi-continuously, continuously), flow regime (completely mixed or plug flow regime) or geometric configuration: tank reactors (continuous stirred tank reactors, perfusion basket reactors, two-stage reactors) and tubular reactors (fluidized bed reactors, packed bed reactors) (Arca-Ramos *et al*., 2018). The use of enzymes to degrade recalcitrant pollutants has been studied in all these reactors, and in most cases, the retention of the biocatalyst has been successfully achieved by the utilization of an adequate membrane; that is why these systems are known as enzymatic membrane reactors.

The key feature of an enzymatic membrane reactor is its ability to retain the biocatalyst inside the system by means of a membrane (Jochems *et al*., 2011; Nguyen *et al*., 2014). This is the reason why these reactors became very popular, since regardless of the use of free or immobilized enzymes, the retention of the biocatalyst can be effectively achieved by the use of an ultrafiltration module. Enzyme retention can be done basically through two mechanisms; on the first one, the enzyme (free or immobilized) remains suspended on the reaction media and is retained by a semi-permeable membrane with a pore size small enough to stop the biocatalyst washing, these are known as enzymatic reactors with selective membranes. In the second case, the enzymes are immobilized in/on the membrane, which acts as selective barrier and also as support for immobilization. These systems are known as biocatalytic membrane reactors (Giorno and Drioli, 2000).

3.4.1 Enzymatic reactors with selective membranes

These bioreactors are equipped with an appropriate membrane placed inside an ultrafiltration module. This unit can operate as an external device connected in closed circuit to the reactor tank or remain submerged inside the reactor. In the first case, the effluent is pumped to the filtration module where it is separated into two streams the permeate, containing the compounds that pass through the membrane, and the retentate containing the enzyme that would be recirculated to the tank (Fig. 1A). In the second case, the enzyme is dissolved or suspended in the reaction medium and washing is prevented by a membrane module located inside the reaction tank (Fig. 1B) (Arca-Ramos *et al*., 2017).

The feasibility of these systems to remove recalcitrant compounds has been tested in laboratory scale reactors with working volumes smaller than 2 L. Lloret *et al*., (2012) studied the continuous degradation of two estrogens, estrone and estradiol, in an enzymatic reactor with selective membrane using commercial laccases from *Myceliophthora thermophila*. The studied system consisted of a stirred tank reactor of 250 mL coupled to an external ultrafiltration module. The filtration module consisted on a polyethersulfone membrane (Prep / Scale-TFF Millipore, nominal molecular weight cutoff of 10 kDa) connected to the reactor through PTFE tubing. The influent containing the estrogen mixture (4 mg/L of each compound in phosphate buffer pH 7.0, 100 mM) was continuously fed by a peristaltic pump at a rate of 1 mg/L·h; laccases were added in a single dose (500 U/L) at the beginning of the experiment. The effluent from the reactor was conducted to the ultrafiltration module by a second pump, where the retentate flow was recirculated to the reaction tank, and the permeate flow was used for the analytic assays. The highest removal rates at steady state conditions were up to 95% for estrone and practically 100% for estradiol. Laccases remained stable after 10 h of continuous operation and the estrogenic activity of the effluent was reduced by 97%.

In a subsequent study, the authors reported the performance of a similar system, but at a higher scale (2 L) and during a longer operation time (4 days) (Lloret *et al*., 2013). This reactor was used for the treatment of a real effluent obtained from the outlet of the secondary clarifier of a wastewater treatment plant (WWTP). The concentration of estrone, 17β-estradiol, and 17α-ethinylestradiol were in the range of 0.29 and 1.52 ng/L; the reactor was fed at a flow rate of 8.3 mL/min and laccases were added at an initial activity of 100 U/L. Estrone was removed by 98% with an elimination rate of 0.37 mg/L·h, while 17β-estradiol and 17α-ethinylestradiol were completely degraded.

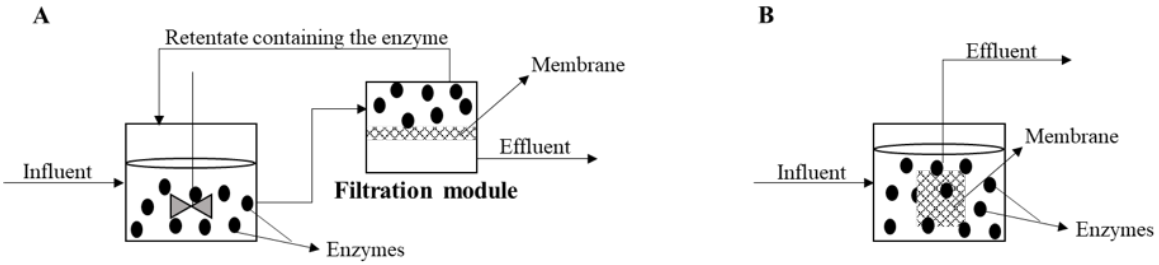

Fig. 1. Enzymatic reactors with selective membranes. A) External filtration module, B) Internal filtration module.

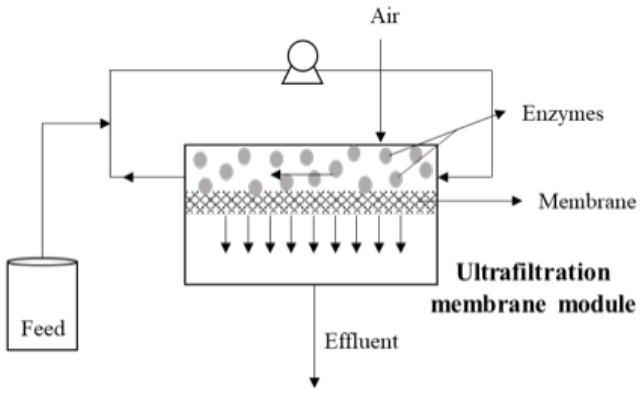

Fig. 2. Schematic diagram of the tangential flow enzymatic membrane reactor (Modified from Chhabra *et al*., 2009).

The enzyme retained 80% of its initial activity after 100 h and it was observed a significant reduction (84%) on the estrogenic activity of the effluent. The formation of dimeric products of 17β-estradiol and 17α-ethinylestradiol was demonstrated by liquid chromatography atmospheric pressure chemical ionization (LC-APCI) and confirmed by determination of accurate masses through liquid chromatography electrospray time-of-flight mass spectrometry (LC-ESI-TOF) (Lloret *et al*., 2013).

Nguyen *et al*., (2014) studied the degradation of diclofenac and bisphenol A in a reactor of 1.5 L equipped with an internal ultrafiltration module. The membrane was made of polyacrylonitrile and it had a molecular weight cutoff of 6 kDa. The operating conditions were: TRH; 8 h, feeding rate; 570 and 480 μg/L·d for bisphenol A and diclofenac, respectively, pH; 7.0 and temperature: 28° C. The recombinant laccase of *Myceliophthora thermophila* expressed in *Aspergillus oryzae* was used as biocatalyst; the initial enzymatic activity was 90 μM/min. During the first 72 h it was observed a gradual inactivation of the enzyme, which was attributed to possible inhibitory effects caused by the pollutants and/or to shear stress. In order to maintain the enzymatic activity within a range of 70-100 μM/min, in a subsequent assay small doses of laccase (200 μL) were added to the reactor every 12 h. Under these conditions, bisphenol A was significantly degraded (85 ± 7%), but only 60 ± 6% of diclofenac was removed. Even when diclofenac degradation seems to be low, it is important to mention that the removal of diclofenac in a conventional WWTP using activated sludge is around 25%, in addition, this removal mainly occurs by sorption instead of degradation (Luo *et al*., 2014). Moreover, degradation by laccases was further improved by the addition of syringaldehyde (1.0 mL/min, final concentration 5 mM), which increased diclofenac degradation to 80% and bisphenol A to 95%. Unfortunately, according to the ToxScreen3 assay addition of syringaldehyde significantly increased the toxicity of the treated effluent (Nguyen *et al*., 2014).

The adverse effects of using redox mediators can be overcome if these compounds are removed from treated effluents. Chhabra *et al*., (2009) proposed the recovery and reuse of the redox mediator ABTS used during the enzymatic biodegradation of Acid Violet

17. These authors worked with a tangential flow enzymatic membrane reactor with laccase of Cyathus bulleri (100 U/L) as biocatalyst.

The system consisted on a reaction vessel (250 mL working volume) coupled to a polyacrylonitrile ultrafiltration membrane with a nominal molecular weight cut-off of 20 kDa. The cross flow was maintained using a centrifugal pump at flow rate of 1 L/min. The system was continuously fed for 25 days with a simulated textile effluent containing the dye and ABTS (100 μM). The ABTS was recovered from permeate by precipitation with ammonium sulphate and reused in a subsequent assay (Fig. 2).

A significant and stable decolorization of the dye (95%) was observed during the reactor operation, laccases retained 45% of its initial activity and 70% of the mediator (ABTS) was recovered and successfully reused in a new assay under the same experimental conditions. Genotoxicity effects of the decolorized solution were evaluated using the Ames test and the results showed a significant detoxification of the dye (Chhabra *et al*., 2009). As can be seen, the toxicity problems and also the extra-costs associated to the use of redox mediators can be mitigated by recycling strategies; however, as previously described, the use of semi-permeable membranes can retain the enzymes inside the reactor but cannot contribute to their resistance against inactivation. Enzyme stability is fundamental for the practical application of biocatalysts, since greater stability results in longevity of the immobilized enzyme which translates into low cost contribution of the catalyst in the bioremediation process costs (Nair *et al*., 2013).

In this regard, the immobilization of different enzymes in/on different materials can increase their stability. Nair *et al*., (2013) immobilized the laccases of *Coriolopsis gallica* on mesoporous silica spheres using a two-step adsorption-crosslinking process. After optimizing the immobilization scheme, it was observed a maximum biocatalyst activity of 383 U/g and a significant improvement in enzyme stability. The immobilized laccases showed a significantly higher thermostability than free enzymes extending their half-life from 6.1 to 31.5 h at 55°C, and from 0.6 to 3.9 at 75°C. The authors also studied the application of this biocatalyst to eliminate organic micropollutants in an enzymatic membrane reactor (Amicon stirred ultrafiltration cell). The system was used to treat a real WWTP effluent spiked with bisphenol A, 17-α-ethinylestradiol and diclofenac (final concentration 10 mM each). The reactor was operated at a HRT of 1.25 h and the initial laccase activity was 1000 U/L. An elimination efficiency of 85% was observed for bisphenol A and 17-α-ethinylestradiol along with 30% for diclofenac. The enzymatic activity remained stable during 80 h of operation and the estrogenic activity of the treated effluent was significantly reduced (Nair *et al*., 2013). Catapane *et al*., (2013) used an enzymatic fluidized bed reactor to study the enzymatic bioremediation of two EDCs belonging to the alkylphenols (APs) class. The reactor was fed with 40 mL of a buffered solution containing octylphenol or nonylphenol (0.10 mM) at a recirculation rate of 140 mL/min and oxygen was supplied through an aerator (100 cm^3/min). The laccases of *Trametes versicolor* were immobilized on polyacrylonitrile beads and added as biocatalysts (12 g) (Fig. 3). The catalytic constants K_m and V_{max} and the time stability of the biocatalyst were determined before and after the immobilization process.

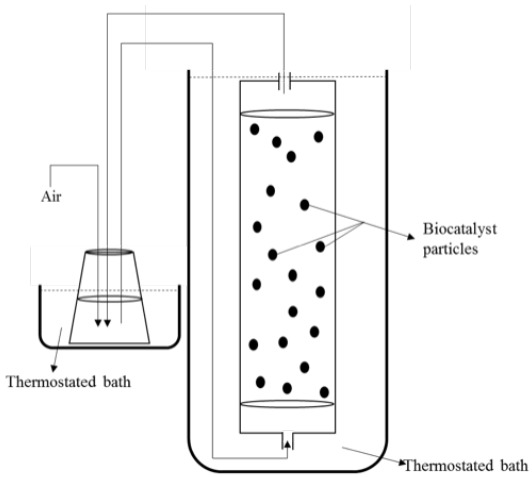

Fig. 3. Schematic diagram of the fluidized bed enzymatic reactor (Modified from Catapane *et al*., 2013).

Immobilized laccases showed a good operational stability, since they retained 95% of its initial activity after 50 days in a solution 1 mM of octylphenol, and 80% after 82 days. On the contrary, free laccases completely lost their activity after 50 days. Regarding the biochemical parameters, immobilization negatively affected the kinetic constants K_m and V_{max} for both pollutants. K_m increased from 0.42 to 0.72 mM, and from 0.7 to 1.11 mM, for nonylphenol and octylphenol, respectively. Similarly, V_{max} decrease from 1.32 to 0.70 μmol/min·mgenzyme, and from 2.5 to 1.25 μmol/min·mgenzyme for nonylphenol and octylphenol, respectively.

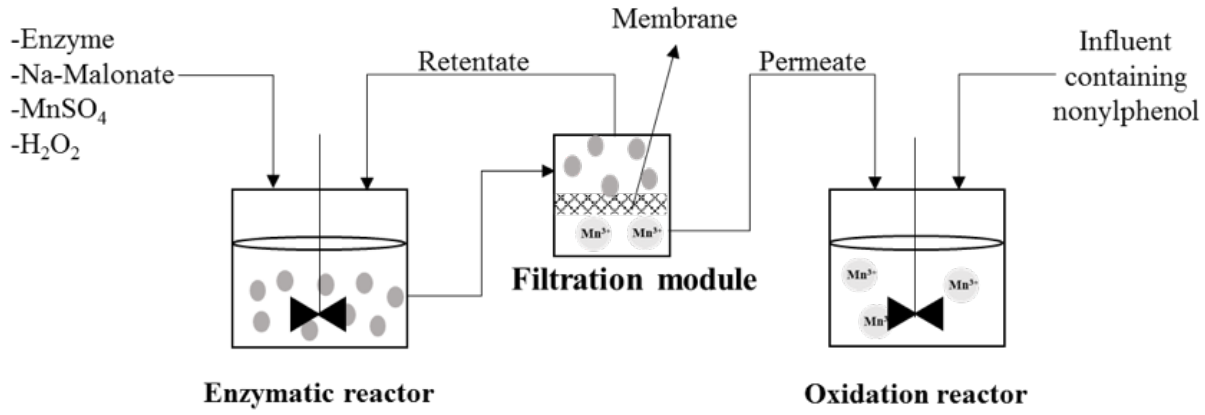

Fig. 4. Schematic diagram of the two-step enzymatic membrane reactor (Modified from Méndez-Hernández *et al*., 2015).

According to these values, the enzyme affinities of the free enzyme are higher than the ones for the immobilized laccases, however, both pollutants were completely degraded after a 90 min treatment in the fluidized bed reactor. Moreover, the estrogenic effects displayed by the nonylphenol and octylphenol solutions disappear after the enzymatic biodegradation (Catapane *et al*., 2013).

Another strategy that can be used to extend the useful life of biocatalysts consists of the compartmentalization of the enzymatic process. Méndez-Hernández *et al*., (2015) studied the bioremediation of a real WWTP effluent spiked with nonylphenol using a two-stage membrane bioreactor. The device consisted of a vessel specifically used for the production of the complex Mn^{3+}-malonate (enzymatic reactor), and a second vessel (oxidation reactor) where this complex was used to oxidize the pollutant. Both reactors were coupled together by an ultrafiltration module containing a polyethersulfone ultrafiltration membrane (molecular cutoff of 10 kDa, Prep/Scale-TFF-1, Millipore) and a VP produced at lab-scale by *Bjerkandera* sp. was used as biocatalyst. The ultrafiltration unit allowed the separation between the enzyme and the Mn^{3+}-malonate complex. The recovered enzyme was recirculated, and the complex was continuously added to the chemical reactor in order to achieve the oxidation of nonylphenol (454 nM).The operational conditions were as follow: 1) Enzymatic reactor. VP: 100 U/L, H_2O_2: 1.8 mL/min (1.5 mM/h), sodium malonate solution and $MnSO_4$: 3.6 mL/min (15 and 1.5 mM/h, respectively), temperature: 30°C, TRH: 50 min, pH: 4.5. 2) Oxidation reactor. Mn^{3+} complex-malonate: 5.4 mL/min, nonylphenol: 6.81 μM/h, TRH 3.6 min (Fig. 4).

The production of the Mn^{3+}-malonate complex in the enzymatic reactor was stable and reached 800-1100 μM/h; this complex practically achieved the total elimination of nonylphenol at TRH as low as 3.6 min.

This result is relevant considering that in conventional WWTPs using activated sludge the reported removal values are around 56% (Luo *et al*., 2014).

Regarding the enzyme stability, at the beginning, it was observed a decrease in the laccase activity, however, the production of the Mn^{3+}-malonate complex was not affected, neither the elimination rate of nonylphenol. Therefore, it was proposed that the decrease in laccase activity could have been due to an enzyme trapping into the membrane more than to an inactivation process (Méndez-Hernández *et al*., 2015; Giorno and Drioli, 2000).

3.4.2 Biocatalytic membrane reactors

As mentioned above, the enzymes can be immobilized on different support materials. The enzymatic reactors containing the enzymes immobilized in/or a membrane are known as biocatalytic membrane reactors, and the membrane itself is usually called as biocatalytic membrane (Jochems *et al*., 2011; Giorno and Drioli, 2000). The enzyme immobilization can be achieved mainly by the following mechanisms:

1. Formation of bonds between the enzyme and the membrane, mainly covalent bonds,
2. Entrapment of the enzyme within the membrane:

- Enzyme incorporation during the membrane manufacturing
- Entrapment of the enzyme within the membrane when an enzyme solution is filtered through it (Jochems *et al*., 2011; Giorno and Drioli, 2000).

The advantages and disadvantages of biocatalytic membranes are close related to those of the enzymes immobilized in any other support. For example, the stability of the enzymes is greater than that of free enzymes, especially the thermostability, the resistance under sudden changes of pH or to the presence of organic solvents. On the other hand, the formation of new bonds between the enzyme and the membrane can negatively affect the kinetic properties of the catalyst then reducing its affinity for the substrate or reducing its maximum reaction rate (Catapane *et al*., 2013; Sathishkumar *et al*., 2012).

In this regard, Sathishkumar *et al*., (2012) found that the laccases of *Pleurotus florida* NCIM 1243 immobilized in poly (lactic-co-glycolic acid) nanofibers presented greater storage, pH and thermal stability than their free counterparts. However, the immobilization negatively altered the constants K_m and V_{max}. The K_m value increased from 422 μM to 809 μM after immobilization, and immobilized laccases reached only 70% of the V_{max} registered for free enzymes. After characterization, it was studied the potential of the biocatalytic membrane for diclofenac degradation, the assays were carried out in batch mode working with a drug concentration of 50 ppm and a laccase activity of 4 U/mL with or without syringaldehyde as redox mediator (250 μM). The biocatalytic membrane completely eliminated diclofenac in the presence or absence of the mediator.

In addition, the membrane was easily reused several times, and although a gradual loss of enzymatic activity was observed, this was compensated by the addition of syringaldehyde (Sathishkumar *et al*., 2012).

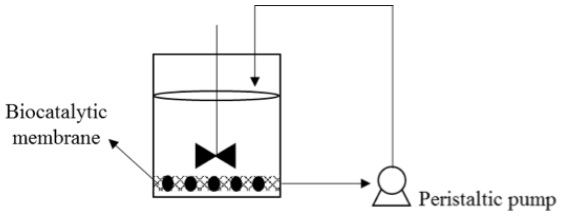

Fig. 5. Schematic diagram of the biocatalytic membrane bioreactor (Modified from Hou *et al*., 2014).

Similar results were observed when immobilizing these type of enzymes in cellulose nanofibers or in nanofibrous membranes composed of chitosan/polyvinyl alcohol (Sathishkumar *et al*., 2014; Xu *et al*., 2013). Once proved the degradation potential of biocatalytic membranes, it was studied the feasibility of including them into enzymatic reactors. Hou *et al*., (2014) studied bisphenol A biodegradation using a biocatalytic membrane reactor operated in batch mode. The system consisted of a small tank (40 mL) equipped with a polyvinylidene fluoride membrane (PVDF) coated with laccases of *Trametes versicolor* . The reactor was connected to a peristaltic pump to achieve constant recirculation of the reaction solution (20 L/m^{3}h) (Fig. 5).

Bisphenol A was degraded 80% after 5 h of operation. In addition, to test the operational stability of the membrane, it was reused for several 24-h cycles feeding a fresh solution of the contaminant at each new cycle. As a result, the reactor was able to remove around 80% of bisphenol A even after 4 cycles of reuse (Hou *et al*., 2014). Subsequently, de Cazes *et al*., (2015) studied the degradation of tetracycline (20 mg/L) in a biocatalytic membrane reactor with tangential flow filtration. The system consisted of a 2 L tank equipped with a gelatin-ceramic membrane grafted with commercial laccases of *Trametes versicolor* . The first degradation assays were carried out for 24 h in batch mode (the retentate flow was continuously recycled and the permeate valve was closed). The best degradation rate (1.39 mg/h) was obtained by using a multichannel membrane of 25 cm length with a pore size of 1.4 μm; in this case the specific degradation rate obtained was 138 mg/L·m^2. Hypothesizing that there would be a better contact between substrates and laccase within the membrane pores, another series of experiments was carried out in batch mode but using tangential flow filtration; in this case, permeate valve was open, and both permeate and retentate were continuously recycled. As expected, degradation rates were enhanced when the tetracycline solution flowed through the membrane pores; the best specific degradation rates (225 mg/L·m^2) were obtained with a permeate flux of 8 L/h·m^2, continuous tangential flow of 10 L/h and in assays of 24 h (de Cazes *et al*., 2015). The authors also studied different variables (membrane pore size, gelatin concentration and laccase concentration) related to the immobilization of the enzymes and its effect on tetracycline removal. Higher tetracycline degradation rates were obtained with membranes of 1.4 μm (pore size) compared to those of 0.2 μm. These results

were in accordance with the spatial distribution of the enzymes on the surface. Laccase-grafted and raw membranes were observed by scanning electron microscopy and it was observed that the membranes with a pore size of 1.4 μm contained enzymes attached no only on the surface but also within the pores; this means that these membranes contained a higher concentration of biocatalytic sites in comparison to the membranes with smaller pores. It was also observed that the increase in gelatin concentration resulted in a higher immobilization yield (between 13 and 15%) and consequently in improved degradation rates (de Cazes *et al*., 2015). Unfortunately, this work does not clearly mention the maximum removal efficiencies achieved by the proposed reactor and no information about the performance of the system in continuous mode (without recirculation) using real wastewater or working with environmental concentrations of the pollutant is presented. Finally, in order to better compare the performance of the reactors previously exposed, Table 2 summarizes the key points of all the assays carried out in continuous mode.

3.5 Pilot and full-scale enzymatic reactors

The first report about the application of ligninolytic enzymes in a pilot-scale reactor was published in 2006 by Soares *et al*., whom studied the treatment of a real wastewater using a stirred-tank reactor installed at a textile company. The wastewater contained a mixture of three different dyes (reactive black 5, reactive red 158 and reactive yellow 27) which accounted for about 85% of all the dyes used at the company. The system consisted of a tank of 1000 L equipped with a marine impeller, pH controller, pH sensor, level sensor and independent pumps for the influent and effluent. As biocatalyst it was used an enzymatic cocktail containing free laccases and peroxidases. The pH of the waste stream was adjusted to 6.4 (optimum pH for the enzymatic cocktail) by the automated addition of acetic acid; the average working temperature was 42.1 °C. The reactor was operated in batch mode with a TRH of 20 min during a 3-month period.

Under these conditions, the extent of decolorization was 91, 78 and 17% for reactive black 5, reactive red 158 and reactive yellow 27, respectively. The treated effluent was tested in relation to its reusability for a washing process. The results showed that there was no significant difference in the washing fastness between dyed cotton clothes that were washed with treated or fresh water (Soares *et al*., 2006). Even though these results were rather positive, it is clear that the authors overlooked the evaluation of some important issues. In particular, it is noticeable that enzyme stability, recovery and recycling were not considered or discussed; similarly, the cost of the proposed treatment was not mentioned and nothing was commented about the possibility of operating the reactor in a continuous mode. More recently, Gasser *et al*., (2014) addressed all these aspects by studying the technical feasibility of a fully automated pilot-scale enzymatic reactor to remove bisphenol A from a real WWTP effluent. The reactor consisted of a 460 L tank equipped with an internal ultrafiltration module (polyether sulfone membrane, pore size: 0.04 μm, 10 m^2 membrane area, BIO-CEL®) and a disk diffuser. The former was used to retain the biocatalyst, and the latter to facilitate hydraulic mixing and prevent membrane fouling. Laccases from *Thielavia* were immobilized onto silica nanoparticles and used as catalysts; a total of 0.5 kg of biocatalyst with a specific activity of 1.23 kU/g were added at the beginning of experimentation. The reactor was continuously fed with the contaminated effluent containing around 0.04-0.67 μg/L of bisphenol A. The system operated for 43 days. The maximum degradation of the pollutant was around 66%. The biocatalyst lost 60% of its initial activity at the beginning of the assay, however, the residual activity remained stable until the process was stopped. The main problems that arose during the operation of the proposed reactor were:

1. Mixing problems: A fraction of the biocatalyst particles settled on the bottom of the reactor reducing their contact with the pollutant and therefore its degradation efficiency.

2. Fluctuations in the input concentration of bisphenol A: Major changes in the input concentration of bisphenol A caused transient fluctuations in the degradation efficiency of the system.

3. When the concentration of bisphenol A was lower than 50 ng/L enzymatic oxidation occur too slow or not at all.

According to the authors the first point can be solved by improving the mixing conditions, which can be achieved by intermittent mechanical agitation or by modifying the aeration conditions. They do not propose any corrective actions to solve the last two issues. However, it would be useful to evaluate more in detail the effect of the input concentration of bisphenol A on its degradation efficiency, as well

as the endocrine disrupting effects of bisphenol A at concentrations below 50 ng/L. Regarding, the treatment costs, it was found that the proposed system can favourably compete with other physical and chemical technologies; more details about this analysis is exposed in the next section (Section 3.6). Finally, Abejón *et al*., (2015a) used a mathematical model to simulate the performance of a full-scale biocatalytic membrane reactor with tangential flow filtration for the degradation of tetracycline. Simulation was based on previous results obtained at lab-scale (de Cazes *et al*., 2015; details about this system were described in Section 3.4.2) and also on the published information about the presence of tetracycline in different types of effluents. The simulation showed that the maximum degradation that could be achieved by the most powerful large-scale system (a reactor with a 39-channel membrane) was 7%. Evidently, this level of removal was insufficient, consequently the authors proposed the use of several reactors connected in series. The performance of these sequential reactors was simulated in three different situations: the treatment of effluents from municipal, hospital and industrial wastewater treatment plants. According to the literature the average concentration of tetracycline in these effluents was 0.282, 0.4 and 11,000 μg/L, respectively. Results showed that in order to attain the fixed discharge concentration of 10-3 μg/L, it was necessary to use an extremely large membrane area (2,708-296,528 m^2). In addition, the number of treatment stages (sequential reactors) required to achieve a significant removal of the pollutant was also very high (122-201 stages), which would result in an equipment of disproportionate dimensions. The authors mentioned that the process effectiveness could be improved by increasing the immobilization capacity of the support or by the use of more active and specific enzymes; however, the potential effect of these modifications was not calculated or discussed. Regarding treatment costs, in a following work the authors reported that the membrane conditioning costs were disproportionate due to the excessive membrane area requirements and the unaffordable enzyme regeneration costs. Even if different reactor features were improved (for example enzymatic kinetics, reactor effective lifetime, regeneration costs, etc.) the specific costs of the treatment (1 €/m^3) were significantly high in comparison with other enzymatic, chemical or physical treatments (Abejón *et al*., 2015b).

3.6 Estimated costs for the enzymatic removal of recalcitrant pollutants

As mentioned in Table 1 a common drawback of many of the existing technologies for the removal of recalcitrant pollutants is their high cost. In the case of enzymatic treatments, enzyme production and immobilization are considered the most expensive operations (López *et al*., 2011).

Most of the works exposed in Section 3.2 were carried out using commercial enzymes (Lloret *et al*., 2012; Lloret *et al*., 2010; de Cazes *et al*., 2015) or enzymes produced at lab-scale (Eibes *et al*., 2006; Taboada-Puig *et al*., 2011; Méndez-Hernández *et al*., 2015) and none of these studies reported the scaling-up of their enzyme production process. Similarly, only few of them studied the pilot or full-scale operation of their lab-scale proposals. This is because the optimization of enzyme production, enzyme immobilization, degradation conditions and scaling-up of all these processes at the same time is difficult. A research group usually focuses only on a small part of the work, which in the end complicates the global analysis of the cost of the proposed processes.

Even with these restrictions, some authors have made good estimations about the cost of their treatment schemes. López *et al*., (2011) calculated the cost of phenol removal by means of a lab-scale enzymatic membrane reactor using free or immobilized MnP. Initially, it was calculated the expenses related to the enzyme production and immobilization. For enzyme production this value was obtained from a submerged fermentation process (SmF) in a 100 L bioreactor. The estimation included: substrates (cheese whey, thiamine, peptone and $MnSO_4$), partial purification (ultrafiltration) and energy consumption (shaker, autoclave, fermenter sterilization and fermentation). The total cost of a 100 L fermentation was calculated in 50.4 USD. Considering that under these conditions the maximum laccase production achieved was 1000 U/L, the cost of 1 kU of MnP was 0.504 USD. Similarly, the production cost of immobilized MnP included: chemicals needed for agarose activation (sodium hydroxide, sodium borohydride, glutaraldehyde, sodium borate, etc.), immobilization capacity of the support (10.4 kU/kg), immobilization yield (50%) and enzyme cost (0.504 USD/kU). The total cost of 1 kg of biocatalyst (immobilized enzyme) was calculated in 858 USD/kg.

Finally, the costs of the treatment included: enzyme production and immobilization, enzyme addition during the treatment and consumption of additional chemicals (Mn^{2+}, sodium oxalate, H_2O_2). The total cost of the treatment was 35.2 and 30,800 USD/kg of removed phenol using free or immobilized enzymes, respectively. Energy consumption and membrane cleaning were not taken into account because these expenses represented less than 5% of total costs.

It is clear that the treatment with immobilized MnP was completely unfeasible, at least using the proposed immobilization technique. On the other hand, in comparison with other alternatives, the cost of the treatment with free enzymes was found to be 5-fold more economic than the use of a UV treatment, but at the same time it was 9-fold more expensive than a Fenton oxidation and 43-fold more expensive than the use of ozonation (López *et al*., 2011). At this point, it is necessary to emphasize that all these costs were calculated from lab-scale assays, therefore, a significant reduction of these values is expected when using full-scale reactors for enzyme production and for the degradation process. In this regard, the authors calculated a 40% reduction in enzyme costs by simply scaling-up the fermentation to a 3000-L fermenter, which in turn would reduce the final cost from 35.2 to 26.4 USD/kg of removed phenol (López *et al*., 2011). Furthermore, the expenses related to the addition of extra-chemicals like $MnSO_4$, sodium oxalate and H_2O_2, which accounted for approximately 40% of the treatment costs, can be completely eliminated by the use of laccases, since these enzymes do not need the addition of any of these chemicals (Gasser *et al*., 2014). In addition, laccase production has been studied and optimized in several works. As an example Osma *et al*., (2011) published an interesting work about the cost of producing laccases under SmF and solid-state fermentation (SSF) using the fungus *Trametes pubescens* and more than 45 different culture media.

As a result it was observed that the cultivation under SSF was 50-fold cheaper than the use of SmF at flask scale. Moreover, the production costs in SSF using small tray bioreactors (area: 20 cm x 15.7 cm, height: 7 cm) was reduced 4-fold in comparison to the use of Erlenmeyer flasks (250 mL). In this case, the final price of laccases was calculated in 0.0004 €/U by using sunflower-seed shells as substrate and Cu^{2+} and tannic acid as inducers; the maximum laccase production obtained in this system was 41,135 U/L.

Similarly in a recent work Postemsky *et al*., (2017) reported high laccase yields in pilot-scale bioreactors using *Ganoderma lucidum* grown in SSF. In this case, the maximum production obtained was 16,442 U/kg of sunflower seed hulls. Regarding the costs of applying laccases, Gasser *et al*., (2014) reported the economic feasibility of removing bisphenol A in a pilot-scale enzymatic membrane reactor using immobilized laccases as biocatalyst. The treatment costs were calculated on the basis of a daily flow of 28,000 m^3/day, an electricity cost of 0.1 €/ kWh and a biocatalyst production cost of 30.3 €/kg. The investment costs included: the reactor vessel and the membrane unit (0.066 €/m^3), while the operational costs corresponded to the energy consumption for filtration (0.001 €/m^3) and aeration (0.030 €/m^3), and the cost of the biocatalyst (0.033 €/m^3). According to this, the total cost of the treatment was calculated in 0.13 €/m^3.

The comparison of this value with that calculated for the use of ozonation (0.078 €/m^3) or powder-activated carbon adsorption (0.144 €/m^3), showed that the costs for the enzymatic treatment were in the same range of these technologies. Moreover, the authors mentioned that the cost of the enzymatic treatment can be reduced to 0.112 €/m^3 by the use of a biocatalyst with a higher specific activity (2.3 kU/g), which would mean a saving of 155,000 € in investments costs (Gasser *et al*., 2014). In addition, it would be interesting to evaluate the operation of this reactor with free laccases.

Overall, the available information indicate that enzymatic treatments have a real potential to compete economically with other technologies. However, in order to reduce the cost of the biocatalyst production and reactor operation the study of these processes must continue.

4 Final considerations

The implementation of a new technology should consider its global sustainability. This aspect can be assessed from three complementary points of view: economic, environmental and social (Romero-Hernandez, 2004). As mentioned in the previous section (Section 3.6), the economic evaluation of enzymatic processes has yielded positive results, however, the information about the environmental and social impact of this kind of treatments is really scarce.

Table 1. Advantages and disadvantages of the use of different technologies for the removal of recalcitrant organic pollutants in water.

Treatment methodology	Principle	Advantages	Disadvantages	References
Coagulation-flocculation	Consist on the neutralization of the electrical charges of colloidal particles through the addition of chemical coagulants; neutralized particles adhere to form large floc particles that readily sediment	Useful for the removal of dispersed dyes	Low efficiency for the removal of vat dyes and endocrine disrupting chemicals Large generation of sludge Introduction of coagulant salts in the aqueous phase	Holkar et al. (2016); Luo *et al*., (2014); Solís-Oba et al., (2009); Drinan, (2001).
Adsorption by activated carbon (AC)	Is based on the attraction and adhesion of pollutants on the surface of activated carbon particles without actually penetrating its internal structure	Medium to high efficiency for the removal of pharmaceuticals, steroid hormones and industrial chemicals Its scaling-up is feasible	Relatively high financial costs Sorption efficiency is reduced in the presence of dissolved organic carbon Removal efficiencies observed at full-scale operation are variable	Luo *et al*., (2014); Grover et al., (2011); Drinan (2001)
Ozonation	Is based on the direct oxidation of pollutants by molecular ozone or indirectly by the formation of hydroxyl radicals	Molecular ozone is a strong oxidant (2.07 V) Medium to high efficiency for the removal of pharmaceuticals, steroid hormones and industrial chemicals. It has disinfection effects Ability to handle fluctuating flow rates and compositions	Ozone must be produced on site Formation of by products Molecular ozone reactions are selective for certain organic compounds (those having nucleophilic moieties)	Luo *et al*., (2014); Ikehata et al., (2006); Hollender et al., (2009); Drinan, (2001)
Advanced oxidation processes (AOPs)	AOPs are based on the *in situ* formation of hydroxyl radicals at a concentration high enough to achieve water decontamination	The oxidation potential of hydroxyl radicals is high (2.80 V) Medium to high efficiency for the removal of pharmaceuticals, steroid hormones and industrial chemicals Ability to handle fluctuating flow rates and compositions The hydroxyl radicals reactions are non-selective and eventually lead to complete mineralization of pollutants	High energy consumption (2014); Formation of by products Its performance is affected by natural organic matter, pH, alkalinity and temperature Colour can hinder photochemical reactions and may impair the performance of photochemical AOPs Carbonate, natural organic compounds, etc. can act as radical scavengers.	Luo *et al*., Oturan et al., (2014); Ikehata et al. (2006)
Algae	Is based on the consumption and/or adsorption of pollutants by algae biomass	Is useful for the degradation and biosorption of synthetic dyes Biomass can be reused during repetitive decolourisation operations	Formation of by products No information about pilot-scale or full-scale application is available	Khataee et al., (2013)
White-rot fungi	Is based on the enzymatic biodegradation of pollutants via extracellular enzymes or adsorption of pollutants on fungal biomass	Is useful for the degradation of mixtures of pollutants Potentially applicable in on-site treatments (for specific contaminated streams)	Nutrient addition can be needed to support fungal growth Addition of nutrients can increase chemical oxygen demand and nitrogen load in the treated effluent Overgrowth of fungal biomass can cause operational problems Maximum degradation occurs at specific pH values Formation of by products Require high hydraulic retention times (1-3 days) Can be sensitive to shock loads Susceptible to bacteria contamination	Mir-Tutusaus et al., (2018); Martínez-Sánchez et al., (2018); Peralta-Zamora et al., (2003)
Ligninolytic enzymes	In based on the enzymatic biotransformation of	Useful for the degradation of mixtures of pollutants It works under non-sterile conditions Potentially applicable in on-site treatments Enzymatic reactions occur under mild conditions	Maximum degradation occurs at specific pH values Gradual inactivation of enzymes High cost of enzyme production Formation of by products	Mir-Tutusaus et al., (2018) Gasser et al., (2014) Mao *et al*., (2010)

Table 2. Enzymatic reactors for the continuous removal of recalcitrant organic pollutants in water

Pollutants	Enzyme	Duration of the assay	Reactor type	Matrix	Removal (%)	Source
Estrone, estradiol	Free laccases	10 h	Stirred tank reactor coupled to an external ultrafiltration module	Buffered solution	Estrone: 95% Estradiol: 100%	Lloret *et al*., (2012)
Estrone, 17β-estradiol, 17α-ethinylestradiol	Free laccases	4 days	Stirred tank reactor coupled to an external ultrafiltration module	Real effluent from a wastewater treatment plant (WWTP)	Estrone: 98% 17β-estradiol: 100% 17α-ethinylestradiol: 100%	Lloret *et al*., (2013)
Diclofenac, bisphenol A	Free laccases	132 h	Reactor equipped with an internal ultrafiltration module	Buffered solution	Diclofenac: 80% Bisphenol A: 95%	Nguyen *et al*., (2014)
Acid violet 17	Free laccases	25 days	Enzymatic reactor with tangential flow filtration	Simulated textile effluent	Acid violet 17: 95%	Chhabra *et al*., 2009
Bisphenol A, 17α-ethinylestradiol, diclofenac	Immobilized laccases	80 h	Modified Amicon stirred ultrafiltration cell	Real WWTP effluent spiked with bisphenol A, 17α-ethinylestradiol and diclofenac	Bisphenol A: 85% 17α-ethinylestradiol: 85% Diclofenac: 30%	Nair *et al*., (2013)
Nonylphenol	Free versatile peroxidases	8 h	Two-stage membrane bioreactor	Real WWTP effluent spiked with nonylphenol	Nonylphenol: 100%	Méndez-Hernández *et al*., (2015)

Several works refer to the enzymatic treatments as environment friendly processes (Nguyen *et al*., 2014; López *et al*., 2011; Giorno and Drioli, 2000), however in most of them this has not been justified by a formal evaluation. Commonly, environmental impacts are evaluated by the life cycle assessment (LCA) methodology. LCA is a suitable tool to analyse all the environmental burdens related to a product or service during all the stages of its life, from raw materials to waste removal (Klöpffer, 1997). Unfortunately, in the field of wastewater treatment and particularly in the case of tertiary treatments for the removal of recalcitrant pollutants, the LCA methodology has not been applied to evaluate enzymatic treatments. Nonetheless, Gabarell *et al*., (2012) used this tool to evaluate the environmental performance of a treatment using *Trametes versicolor* to degrade a textile dye (Grey Lanaset G), and compared the results with those obtained using physical adsorption in granular activated carbon. The analysis included seven impact categories (climate change, ozone depletion, human toxicity, photochemical oxidant formation, terrestrial acidification, freshwater eutrophication, marine eutrophication, terrestrial ecotoxicity, freshwater ecotoxicity, marine ecotoxicity, metal depletion and fossil depletion) and a flow indicator of the cumulative energy demand.

LCA showed that the biological treatment with *Trametes versicolor* offered environmental advantages in four of the evaluated categories (climate change, ozone depletion, photochemical oxidant formation and cumulative energy demand) in comparison

with the use of granular activated carbon. Energy consumption was the principal contributor to the environmental impact of both treatment methods, and in the biological process, this energy was consumed mainly during sterilization and aeration procedures. Even though the biological treatment with *Trametes versicolor* differs from enzymatic processes, these results can be seen as preliminary indicators of the relative environmental performance of these technologies, since both processes are based in the production and use of ligninolytic enzymes for the degradation of pollutants (Martínez-Sánchez *et al*., 2018). Nonetheless, the life cycle assessment of enzymatic treatments is undoubtedly needed.

Conclusions

The remarkable stability of the enzymes produced by some microorganisms and the development of strategies for their recovery and repeated reuse, have been fundamental to scale-up enzymatic experiments from flask or test-tubes to full-scale enzymatic reactors. The main disadvantage of the use of enzymes for the degradation of pollutants is the cost of the process, since the production of enzymes, their immobilization, retention by membranes and reactor operation must be considered. Most works on enzymatic reactors have not considered the costs analysis, thus, it is difficult to compare the cost of enzymatic processes with other technologies; however, the available information indicates that enzymatic treatments are able to favourably compete with other technologies, i.e., ozonation and adsorption. Finally, it is important to consider that the effluents from WWTP are complex matrices that may contain more than one polluting compound, therefore, the research perspectives should include the evaluation of the global effect of enzymatic processes over these compounds, which can be achieved by the use of advanced multi-residue analysis.

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