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Research review paper

Multi-faceted strategy based on enzyme immobilization with reactant adsorption and membrane technology for biocatalytic removal of pollutants: A critical review

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Abstract

In the modern era, the use of sustainable, environmentally friendly alternatives for removal of recalcitrant pollutants in streams resulting from industrial processes is of key importance. In this context, biodegradation of phenolic compounds, pharmaceuticals and dyes in wastewater by using oxidoreductases offers numerous benefits. Tremendous research efforts have been made to develop novel, hybrid strategies for simultaneous immobilization of oxidoreductase and removal of toxic compounds. The use of support materials with the options for combining enzyme immobilization with adsorption technology focused on phenolic pollutants and products of biocatalytic conversion seems to be of particular interest. Application of enzymatic reactors based on immobilized oxidoreductases for coupling enzyme-aided degradation and membrane separation also attract still growing attention. However, prior selection of the most suitable support/sorbent material and/or membrane as well as operational mode and immobilization technique is required in order to achieve high removal efficiency. Thus, in the framework of this review, we present an overview of the impact of support/sorbent material on the catalytic properties of immobilized enzymes and sorption of pollutants as well as parameters of membranes for effective bioconversion and separation. Finally, future perspectives of the use of processes combining enzyme immobilization and sorption technology as well as application of enzymatic reactors for removal of environmental pollutants are discussed.

Keywords: oxidoreductases, enzyme immobilization, support materials, hazardous pollutants removal, pharmaceuticals, enzymatic biodegradation, enzymatic adsorption, enzymatic bioreactors, enzymatic membrane reactors

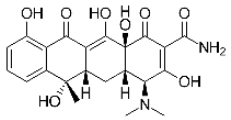
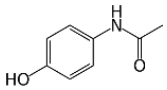
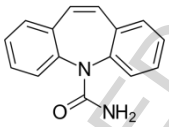
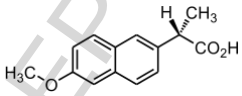
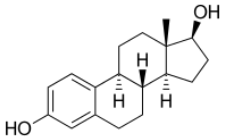
1. Introduction

The total amount of active pharmaceutical ingredients used in the medicine has exceeded 100,000 tons per year (Weber et al., 2016). This number includes antibiotics, anti-inflammatory drugs, pain killers and contraceptive hormones, and it is known that many of these substances are introduced into the environment (Kümmerer, 2009). Another group of pollutants includes dyes, colorants and pigments which are used in various industrial sectors, such as textiles, food and other consumer goods, in the amount of approx. 10^5 tons per year (Vikrant et al., 2018) and are also commonly released into the environment. Consequently, the risk of water pollution with such compounds is high and there are no signs that this problem is decreasing. The reported values of specific phenolic compounds, pharmaceuticals and dyes in aqueous environmental systems such as hospital wastewaters, industrial effluents, surface water, ground water and even seawater are scarce, but usually are within the range from ng/L to µg/L for pharmaceuticals (Heberer, 2002, Cruz-Morato et al., 2014) and to mg/L and even g/L for dyes (Li et al., 2017, Vikrant et al., 2018), with large variations depending on the type of substance, type of effluent and sampling places as well as the type of the water reservoir (ground water, surface waters or wastewaters) which contributes to a global scale of the problem (Table1). These pollutants may negatively affect living organisms as well as the ecosystem, thus there is a need to remove them from the environment in order to ensure a healthier and more sustainable development (Lonappan et al., 2016, Rasheed et al., 2019). Data regarding the type, structure and level of the selected examples of pharmaceutical environmental pollutants collected from various reports was presented in Table 1.

Despite their highly diverse uses, the chemical structures of phenols, dyes and pigments as well as some key antibiotics, pain killers and contraceptive hormones are similar in the sense that the majority of compounds, but not all, contain a phenolic group in their chemical structure (Anku et al., 2017). As discussed below, phenolic compounds and non-phenolic pharmaceuticals are target substrates for several natural enzymes, which is why enzymatic conversion can be crucial for the removal of these diverse compounds from the environment (Ba and Kumar, 2017). Biodegradation with the use of enzymes seems to be a promising strategy to control the level of dyes as well as pharmaceutical pollutants and their derivatives in waters and soils, mainly because enzymes are characterized by distinct selectivity and can catalyze targeted conversion reactions. Inspection of the discussed data infers that the reduction of the quantities of pharmaceutical residue compounds present in the environment,

particularly in wastewaters, is an important environmental challenge (Ba et al., 2013, Barrios-Estrada et al., 2018a, Zdarta et al., 2018a). However, it should be clearly stated that enzymes cannot be used for detoxification of raw wastewater due to the high content of organic and inorganic substances, which should be treated before the recalcitrant phenols or pharmaceuticals. Thus, only properly pretreated wastewater, without interferents and lower concentration of pollutants, can be treated by oxidoreductases in order to remove toxic compounds.

Table 1. Selected examples of the various types of pharmaceutical environmental pollutants*.

Type Compound	Base chemical structure*	Levels reported in environment	References
Antibiotics	Example: Tetracyclin 	Wastewaters: 280–540 ng/L Ground waters: 4.4–9.3 ng/L Surface waters: 5.7–8.7 ng/L	(Javid et al., 2016)
Pain killers	Example: Paracetamol 	Wastewaters: 120–900 µg/L Ground waters: 4.4 ng/L–30 ng/L Surface waters: 0.3–4.5 µg/L	(Cruz-Morato et al., 2014, Sousa et al., 2016, Rivera-Jaimes et al., 2017)
Antidepressants	Example: Carbamazepine 	Wastewaters: 0.5 µg/L Ground waters: N/A Surface waters: up to 1.8 µg/L	(Cruz-Morato et al., 2014, Sousa et al., 2016)
Anti-inflammatories	Example: Naproxen 	Wastewaters: 13 µg/L Ground waters: N/A Surface waters: up to 1.6 µg/L	(Cruz-Morato et al., 2014, Sousa et al., 2016)
Contraceptive hormones/estrogenes	Example: Estradiol 	Wastewaters: 6.2–42.2 ng/L Ground waters: 2.5 ng/L Surface waters: 1–22 ng/L	(Adeel et al., 2017)

* Several other pharmaceuticals *do not* contain a phenolic group, but some of their impurities do e.g. *p*-salicylic acid and 4-hydroxy iso-phthalic acid, which are impurities of acetylsalicylic acid (aspirin). No immediate data are available on their occurrence in the environment.
N/A – not available

Enzymes which belong to the oxidoreductase class (EC 1.x.x.x, where 1 denotes type of the catalytic group and type of catalyzed reaction [redox - oxidation/reduction reactions for oxidoreductases], the second and the third x denote the enzyme's sub-class and sub-sub-class,

respectively, and describe the reaction with respect to the compound, group, bond or product involved in the process, and the final x indicates the position in the sub-sub class and denotes specific metabolites and cofactors involved, such as tyrosinases, laccases and peroxidases) (Table 2) are increasingly investigated as green biocatalysts for the removal of hazardous compounds, including phenol and its derivatives, synthetic and natural dyes, pharmaceuticals and even hormones (Cabana et al., 2009, Bilal et al., 2017a, Skoronski et al., 2017, Bilal et al., 2019a, Costa et al., 2019). The oxidoreductases are generally known for their high specificity constants, however, they vary depending on the type of enzyme, its origin and type of the substrate (Baldrian, 2005). This means that these enzymes can not only efficiently convert the above-mentioned compounds, but also exhibit high affinity to the pollutants. This is particularly important due to the low concentrations of phenols and their derivatives in wastewater. Notably, laccases (EC 1.10.3.2, where 10 denotes acting on diphenols and related substances as donors, 3 denotes reaction with oxygen as acceptor and 2 indicates second place in this sub-sub group), which are produced by both fungi and bacteria, can catalyse the conversion and hence "destruction" of many of the above-mentioned compounds *via* their ability to catalyse the oxidation of phenolic OH-groups during the reduction of oxygen to water (Lloret et al., 2013, Tavares et al., 2017). The main substrates of the laccases include monophenols, polyphenols, methoxy-substituted phenols as well as aromatic amines and diamines. On the other hand, tyrosinases (EC 1.14.18.1, where 14 denotes acting on paired donors, with incorporation or reduction of molecular oxygen, 17 indicates acting with another compound as one donor, and incorporation of one atom of oxygen and 1 indicates first place in this sub-sub group) possess the narrowest substrate specificity. They are mainly active in case of phenols and catechols, if they do not bear electron-withdrawing substituents and O_2 molecules required for catalytic action. However, their mechanism is different compared to that observed for laccases. The oxidation process catalysed by tyrosinases generates ortho-quinones (Land et al., 2003), whereas the conversion with the use of laccases mainly reactive phenoxy radicals as intermediate products (Bronikowski et al., 2017). Regardless, oligomers and polymers are formed as the products of the oxidation of phenolic compounds in case of both enzymes. On the other hand, horseradish peroxidase (EC 1.11.1.7), manganese peroxidase (EC 1.11.1.13), and lignin peroxidase (EC 1.11.1.14) where 11 denotes acting on a peroxide as a separate acceptor, 1 denotes use of peroxides as substrates and 7, 13 and 14 indicate the place of these enzymes in the sub-sub group, are characterized by different substrate specificity. These enzymes exhibit activity towards phenolics, aromatic amines and also non-phenolic compounds with various efficiency and require hydrogen peroxide which is

reduced to water during the catalytic conversion of phenolic compounds. For instance, horseradish peroxidase is a heme-containing enzyme which catalyses the oxidation of phenolic acids, aromatic phenols and non-aromatic amines. Manganese peroxidase can catalyse the oxidation of a wide spectrum of phenolic compounds, different mono- and dimeric phenols, and even dyes. Meanwhile, lignin peroxidase oxidizes a wide range of aromatic phenolic and non-phenolic compounds as well as other organic substances, such as xenobiotics (Bilal et al., 2019b). Nevertheless all of the above-mentioned biocatalysts possess a high biotechnological potential and several reports confirm their ability to catalyse the oxidation of a wide range of hazardous environmental pollutants (Nicolucci et al., 2011, Mukherjee et al., 2013, Le et al., 2016, Bilal et al., 2018a).

Table 2. Oxidoreductase enzymes most frequently used for environmental applications and their selected properties.

Enzyme name	EC number	Sources	Main substrates	pH and temperature optimum	References
Laccase (Lac)	1.10.3.2	fungi, bacteria, plants	monophenols, diphenols, polyphenols, diamines, aromatic amines, N-heterocycles, phenothiazines	pH 3.5–5 temp. 20–25 °C	(Faccio et al., 2016, Senthivelan et al., 2016)
Tyrosinase (Tyr)	1.14.18.1	fungi, bacteria, plants, insects and mammalian issues	phenol, monophenols, bisphenols, multi-substituted phenol derivatives, including chloro- and nitrophenols	pH 5.5–8 temp. 30–40 °C	(Ihekata and Nicell, 2000, Tonin et al., 2012)
Horseradish peroxidase (HRP)	1.11.1.7	roots of horseradish	phenolic acids, aromatic phenols and their derivatives, non-aromatic amines, indoles	pH 7 temp. 25–40 °C	(Bilal et al., 2017b, Bilal et al., 2018b)
Lignin peroxidase (LiP)	1.11.1.14	white-rot fungi and microorganisms	aromatic phenolic and non-phenolic compounds, xenobiotics with redox potential up to 1.4 V	pH around 3 temp. 35–45 °C	(Vialli et al., 1990, Shaheen et al., 2017)
Manganese peroxidase (MnP)	1.11.1.13	white-rot fungi and bacteria	dyes, monomeric and dimeric phenols	pH around 4 temp. 30–40 °C	(Bilal et al., 2017c, Yehia et al., 2017)

However, in order to efficiently exploit enzymes for such purposes – involving low but significant concentrations of the problematic pollutants in aqueous ecosystems – high efficiency and robustness of the enzymatic reaction systems are of key importance. As it was previously mentioned, the concentration of the pharmaceuticals in wastewaters usually does not exceed several μg/L (Table 1) which affects the enzymatic kinetics and conversion rates. In order to enhance the removal efficiencies of phenolic compounds by oxidoreductases, various mediators can be used (Husain and Husain, 2008). There are two possible mechanisms of action of mediators. They may act as electron transfer agents between the

oxidoreductase and the substrate, in case of which the oxidized form of the mediator diffuses from the enzyme catalytic pocket and is able to oxidize the substrates molecules which are inaccessible or too bulky for the enzymes. On the other hand, the mediator may expand the oxidizing capability of the oxidoreductases towards oxidation of non-phenolic compounds at higher redox potential by providing alternative reaction pathways of oxidation (Munk et al., 2018). It should be mentioned that the addition of mediator agents also alters the enzymatic kinetics. However, the Michaelis–Menten kinetic model could be used to estimate the affinity of the oxidoreductases towards different mediators and reaction substrates after the addition of the mediators, and to evaluate the changes in the enzyme kinetics (Lyons, 2003). The most frequently used mediator agents include e.g. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI) or 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO). However, the selection of a suitable mediator is directly associated with the enzyme (redox potential, substrate specificity) and the type of substrate (Baiocco et al., 2003, D'Acunzo et al., 2006). Although the use of mediators affects the costs of the remediation process, their application is constantly growing over recent years due to the significant improvement of the removal efficiencies. Moreover, the use of such compounds may also significantly reduce the duration of the process which at least partially justifies the costs of the mediators.

It should also be mentioned that use of oxidoreductases has not been scaling up yet due to several obstacles. The most important issue is related e.g. to the market price of the enzymes. According to Sigma-Aldrich web page, the price of the commercially available laccase from *Trametes versicolor* is approximately equal to $1000 per 10 g, however the costs of the mushroom tyrosinase and horseradish peroxidase are higher and amount to approx. $2000 per 1 g and even $15000 per 1 g, respectively (Sigma-Aldrich, 2019a,b,c). Moreover, these enzymes suffer due to insufficient catalytic activity. Thus, it would not be feasible to use the commercially available biocatalysts at an industrial scale. One of the possible solutions to overcome this problem is the on-site production of enzymes and their purification to obtain highly active biocatalysts which results in a reduction of total process costs. Nevertheless, it is difficult to establish the total costs of the enzymes required for wastewater purification as they depend on several factors, such as enzyme dosage, its activity, the type of removed compounds and the required process efficiency (Liu et a., 2016). In addition, other strategies have been employed in order to improve the efficiency of enzymatic removal of hazardous pollutants. The most important one is enzyme immobilization, which significantly improves enzyme stability and reusability. Moreover, optimization of the process conditions,

particularly in terms of pH and temperature, as well as the use of enzymatic reactors and recycling of enzymes should be highlighted and considered as promising to obtain high process efficiency (Østergaard, et al., 2015, Aguilar et al., 2018). Enzyme immobilization facilitates the recycling and reusability of enzymes which allows to reduce the cost of the process even by up to 50% (Jørgensen and Pinelo, 2017).

A notable number of scientific and technical (engineering) reports testing novel enzyme immobilization strategies and reaction designs are available, thus indicating that bioremediation *via* enzymatic technology can be a new step forward which provides alternative strategies and materials involving e.g. adsorption technology (Hai et al., 2007, Su et al., 2016, Bilal et al., 2018c). Enzyme immobilization technology is crucial for environmental technology to maximize the reuse of enzymes in order to provide maximal biocatalytic productivity and hence lowest possible enzyme costs (Jesionowski et al., 2014, Zucca et al., 2014, Bilal and Iqbal, 2019). Immobilization of the enzymes provides long-term usability and high biological activity and effectivity of the immobilized oxidoreductases for the removal of pharmaceutical products (Zdarta et al., 2018c) and other environmentally undesirable phenolic compounds such as colorants (Ahmad et al., 2015). However, the immobilization process also possess drawbacks, e.g. its costs, required equipment as well as conformational restrictions of the immobilized enzyme which lead to a decrease of catalytic properties (Bilal et al., 2018d). In addition, there is a need to find suitable support material, characterized by operational resistance and the presence of functional moieties which facilitate stable attachment of the biomolecules. Moreover, it should be emphasized that only a proper immobilization allows for enzyme stabilization and retention of high catalytic properties by the biomolecules (Adeel et al., 2018). From this point of view, the particular interest is oriented to multipoint enzyme immobilization which may improve the enzyme activity, specificity or selectivity or even purify the enzyme (Bilal et al., 2019c). This technique facilitates stable binding of the enzyme which reduces enzyme leakage and enhances the catalytic activity (Mateo et al., 2007). Finally, oriented immobilization contributes to the fact that immobilized biomolecules are deposited onto the surface in such way that their active sites are accessible for the substrate molecules. This is critical for the oxidoreductases, as their active site require a proper enzyme-support connection to facilitate the efficient transfer or uptake of the electrons to or from the support (Hernandez and Fernandez-Lafuente, 2011). Nevertheless, enzyme immobilization combined with substrate adsorption technology as well as the use of enzymatic bioreactors appear to be a particularly promising strategy.

Simultaneous enzymatic biodegradation with adsorption or separation enhance the removal efficiency of pollutants using oxidoreductase-based systems at an industrial and environmentally-relevant scale during bioremediation, and maximize the effectiveness of the process combinations (Martínez-Hernández et al., 2016). The combination of conversion and removal results in numerous advantages, including increased effectiveness of remediation, reduced time of the process by avoiding product accumulation and potential inhibition and, in turn, cleaner effluents; sorption is notably relevant as a new technology in such reactions due to the sorption of products of enzymatic conversion. Additionally, by means of simultaneous sorption and biodegradation, easier operational control of the process and cost reduction can be achieved, which is important for high biocatalytic productivity and thus competitive applications of immobilized enzymes (Homem and Santos, 2011).

Furthermore, precise catalysis of low substrate levels and handling of large volumes of effluents are required in order to efficiently convert and remove environmental pollutants as these compounds are usually present in dilute levels of μg/L. From this point of view, membranes-based technologies are very suitable and of particular interest. However, the use of enzymatic bioreactor systems involving immobilized enzymes has been found to work effectively rather than removal of pollutants by simple filtration, such as micro- or nanofiltration (Rondon et al., 2015). The greatest advantage of bioreactors with immobilized enzymes is the fact that biodegradation and removal processes can occur separately, simultaneously or consecutively, depending on the pollutant and the process requirements (Li et al., 2007). Moreover, through careful process control, parameters such as pH and temperature can be properly adjusted and maintained to ensure constant operational conditions (Al-Khalid and El-Naas, 2012). These facts contribute to a more effective and less expensive enzyme-catalysed biodegradation of hazardous pollutants.

In this article, we review the current state of knowledge concerning the application of simultaneous adsorption/enzymatic biodegradation processes *via* employment of enzymatic bioreactors for the removal of hazardous compounds. Various types of degradation processes, types of reactors and their operating modes which allow to achieve the highest removal efficiency, and properties of support material for enzyme immobilization for such processes are highlighted and discussed. It is also emphasized how the immobilization technique and operational parameters of the process affect the degradation of toxic compounds. Possible directions and future trends for development of advanced methods for removal of persistent pollutants by immobilized enzymes are also presented and discussed.

2. Simultaneous enzymatic biodegradation and adsorption of environmental pollutants

As it is well known, immobilized oxidoreductases are commonly used to degrade numerous hazardous pollutants present in the environment. Nevertheless, it should be emphasized, that attempts are currently made to combine biodegradation and adsorption methods in order to develop even more efficient and economically justified strategies for the degradation of hazardous compounds (Fig. 1). This solution concerns the use of the same material as support for enzyme adsorption and/or covalent immobilization, which improves the stability and reusability of the biocatalyst, in order to achieve higher bioconversion rates and simultaneous physical sorption of selected undesired compounds, which enhances the total removal effectivity (Antecka et al., 2018).

Figure 1. Schematic diagram of simultaneous enzyme immobilization and sorption/biodegradation processes of environmental pollutants.

Although the biodegradation of hazardous compounds with the use of enzymes is generally highly efficient, it also possesses some limitations as well. This is due to the fact that enzymes exhibit high catalytic properties only in narrow ranges of temperature and pH, incomplete removal or degradation of pollutants is observed. Moreover, the formation of oligomeric and polymeric products with high molecular weight may block the active sites of the biomolecules and thus reduce the remediation ability of the enzyme, as it has been reported in case of biodegradation of various bisphenols (Gasser et al., 2014).

In contrast, adsorption is much less sensitive to changes of the process conditions and can occur over much wider pH and temperature ranges, even when the enzyme has lost its catalytic properties. The adsorption process is also very useful when biodegradation of a solution with a high concentration of toxic compounds is carried out. Since enzymes are highly efficient, mainly in lower concentration ranges, on the one hand high concentrations may increase conversion rates, but on the other the high quantity of pollutants extends the biodegradation time, which in turn increases the costs of the process. Adsorption strongly enhances the elimination of hazardous compounds and allows to attain high removal efficiencies at a shorter time (Chung et al., 2003). It should also be noted that adsorption of the pollutants may occur in two ways:

(i) by adsorption of the hazardous pollutants (enzyme substrate),

(ii) by adsorption of the products of enzymatic conversion (biodegradation) (Imran et al., 2012).

In the first case, adsorption not only increases the final efficiency of the removal of toxic compounds, but also ensures the continuous supply of substrates for high enzyme activity. However, adsorption is also used for elimination of the biodegradation products. The compounds formed after enzymatic treatment are usually less toxic, but are still undesired in the reaction mixture. They can be removed by using appropriate sorbents, making the solution even more environmentally friendly (Castellana and Loffredo, 2014). It should be added that sorption processes are generally less selective than enzymatic biodegradation, which corresponds to the fact that a wider range of pollutants can be removed. However, the reusability of the support material for the immobilization of a new batch of enzyme is greatly limited by the fact that various toxic compounds are adsorbed on its surface, which causes fouling due to the affinity towards both the immobilized enzyme and pollutants to be adsorbed. Fouling of the support may lead to a significant loss of enzyme activity as well changes in the physicochemical properties of the support. Furthermore, when pollutants are adsorbed onto the surface of the matrix, active sites of the enzyme molecules might be inhibited, which results in a decrease of enzymatic activity. In addition, fouling of the support may also negatively affect the structure of the enzyme, leading to its inactivation. In fact, fouling of the support is one of the most important reasons which limits the practical application of the simultaneous sorption/enzymatic degradation at a wider scale. Moreover, after adsorption of the pollutants, the availability of the functional groups and porosity of the support decrease and, in consequence, a decrease of sorption capacity is also observed. In this case, the regeneration of the sorbent is expensive and unfavourable in terms of practical applications. This means that the degradation parameters must be carefully controlled, which makes the process more complex and less cost-effective (Zhou and Hartmann, 2013). Nevertheless, in order to retain high catalytic properties of the immobilized enzyme and good sorption properties of the sorbent, there is a need to regenerate the support. One of the possible solution is selective desorption of the pollutant from the support material (Al-Jabari et al., 2017). In this case, the enzyme remains immobilized onto the support surface. However, this technique possess some limitations, e.g. stable enzyme-support interactions or formation of the multipoint biomolecules attachment are required to avoid enzyme leaching. Moreover, a proper eluent should be selected to remove the pollutants from the support. On the other hand, after inactivation of the immobilized enzyme, mainly due to repeated use and

complete loading of the support with toxic compounds, there is a possibility to elute both the inactive enzyme and adsorbed pollutant. This solution is much easier, as compared to the previous one, however there is a risk that the use of a toxic eluent may partially damage the structure of the support/sorbent material.

The degradation of environmental pollutants by immobilized enzymes combined with adsorption process has become a subject area of interest to many research groups, particularly during the last decade. Nevertheless, we strongly believe that this approach should still be of high interest and further studies should be undertaken to develop this technique. This solution is highly effective due to the sorption of both environmental pollutants as well as products of their enzymatic conversion by the sorbent. These facts facilitate the process feasibility and result in less toxic and less polluted effluents (Shen et al., 2011). Moreover, catalytic properties of the biocatalyst are usually improved as a result of its immobilization. Although the type of the used enzyme is associated with the environmental pollutant which should be removed, the proper selection of the support/sorbent material is the crucial step in this methodology as this material affects both enzyme immobilization and sorption processes. Thus, examples of the materials which are used for both enzyme immobilization and simultaneous adsorption of toxic compounds were presented in Table 3 (to enable clearer understanding, the sorption and biodegradation efficiencies were presented separately).

2.1. Materials used for simultaneous adsorption of pollutants and enzyme immobilization

Several different compounds of inorganic, organic and hybrid/composite origin, characterized by various morphology, porous structure and different features have been applied for simultaneous enzyme immobilization and sorption of pollutants. Selected examples of the above-mentioned materials and removed pollutants were summarized in Table 3. Nevertheless, materials used for concurrent biosorption and biodegradation processes possess some limitations and must fulfil certain criteria in order to become effective support materials for biomolecules and, at the same time, highly efficient sorbents. First of all, such materials must offer high stability and mechanical resistance under harsh reaction conditions (Zdarta et al., 2018b). Furthermore, their porous structure, including pore diameter and surface area, should grant them appreciable affinity not only for effective enzyme immobilization, but also for adsorption of hazardous compounds (Bhatnagar and Sillanpaa, 2010). Moreover, according to Loffredo and Senesi (2006), high contents of carbon and oxygen and a stable chemical structure of the sorbent also increase adsorption efficiency. However, the presence

of many various functional groups on the surface of the material is the most important feature for an effective immobilization of enzymes and sorption of pollutant. This strongly enhances enzyme binding, but also determines the surface properties of the material as a sorbent (Gao et al., 2011). For instance, a significant amount of hydrophilic groups is essential for immobilized oxidoreductases, which exhibit better catalytic activity when supported using hydrophilic supports (Strong and Claus, 2011). Moreover, it should be clearly stated that improvement of enzyme stability and reusability is usually observed after providing covalent bonds between the enzyme and the support. In this case, heterofunctional supports are of particular interest which are defined as materials that possesses several different functional moieties capable to bind the protein (Barbosa et al., 2013, Rodrigues de Melo et al., 2017). Groups which facilitate the formation of the enzyme-support covalent bonds include e.g. glyoxyl, epoxy and divinyl sulfone groups. Although such functional groups might be very useful in enzyme immobilization, they possess several limitations. A two steps immobilization protocol is required for most of these strategies, in which the enzyme is immobilized by adsorption at first followed by formation of covalent interactions at alkaline pH (Santos et al., 2015). This might have negative effect on the protein structure and enzyme activity (Barbosa et al., 2014). The use of glutaraldehyde (GA) is an interesting alternative, as one the most universal and, in fact, commonly used surface modifying agents. Techniques in which GA is used are simple, efficient, relatively cheap and are among the most frequently used in enzyme immobilization. Although glutaraldehyde reacts mainly with primary amino groups in the enzyme structure, biomolecules might also be bound by reaction of GA with thiols, phenols and imidazoles moieties creating stable, single or multipoint enzyme-support interactions (Fernández-Lorente et al., 2006).

The simultaneous use of a material for enzyme immobilization and for adsorption also means that the functional groups must not only be compatible with chemical groups of the biomolecule, but should also exhibit affinity to the pollutant or to the products of its bioremediation. Due to the variation in the structure and chemistry of the immobilized and/or adsorbed molecules, there is a great diversity of interactions formed between the attached enzyme and support as well as the hazardous pollutants and sorbent. In general, hazardous compounds may be attached to the surface of sorbents by means of several complex mechanisms, such as surface adsorption, ion exchange, complexation (coordination) or chelation (Crini, 2006). On the other hand, enzymes are linked with the matrix mainly by adsorption and covalent bonds, however, immobilization by entrapment and encapsulation has also been reported (Koyani and Vazquez-Duhalt, 2016, Bilal et al., 2017c). Nevertheless, the

immobilization of the enzyme onto the surface of the support (adsorption and covalent immobilization) is particularly desirable for removal of toxic compounds due to reduced diffusional limitations and improved exposure of the biocatalysts active sites for the substrates dissolved in the solution as well as adsorbed on the support material (Bilal et al., 2018e). Thus, the selection of a material with appropriate features plays a key role for effective immobilization and sorption of pollutants. Additionally, the surface properties of the sorbent should be selected in order to minimize the negative impact of the adsorbed compound on the catalytic activity of the biomolecules.

Table 3. Materials of various origin used for simultaneous immobilization of oxidoreductases and adsorption/biodegradation of environmental pollutants.

Adsorption/ immobilization material	Enzyme	Immobilization technique	Pollutants (enzyme substrates)	Process conditions	Sorption	Degradation	Total removal efficiency	Reference
Alumina spherical pellets	Laccase from *Trametes villosa*	Adsorption immobilization	Reactive Black 5	pH 5, 45 °C, 24 h	79%	5%	82%	(Zille et al., 2003)
Alumina pellets	Laccase from *Trametes versicolor*	Covalent immobilization	Reactive Black 5	pH 5, 50 °C, 36 h	<10%	>90%	100%	(Osma et al., 2010)
Multi-walled carbon nanotubes	Laccase from *Trametes versicolor*	Adsorption immobilization	Bisphenol A,	pH 7, 25 °C, 24 h	9%	71%	80%	(Pang et al., 2015)
			Catechol		5%	85%	90%	
Epoxy-functionalized silica	Laccase from *Myceliophthora thermophila*	Covalent immobilization	Phenol,	pH 4.5, 25 °C, 24 h	10%	70%	80%	(Mohammadi et al., 2018)
			p-Chlorophenol		<5%	60%	60%	
Hollow mesoporous carbon spheres	Laccase from *Trametes versicolor*	Covalent immobilization	Tetracycline	pH 5, 25 °C, 3 h	77%	55%	100%	(Shao et al., 2019)
Potato dextrose agar	Laccase from *Pleurotus ostreatus*	Adsorption immobilization	Municipal landfill leachate	30 °C, 20 days	65%	15%	>75%	(Loffredo et al., 2014)
Chitosan film	Tyrosinase from mushroom	Covalent immobilization	*p*-Cresol,	pH 7, 45 °C, 1 h	92%	100%	92%	(Yamada et al., 2005)
			m-Cresol,		94%	100%	94%	
			Catechol,		100%	98%	98%	
			p-Chlorophenol,		99%	100%	99%	
			m-Chlorophenol		68%	72%	70%	

Cross-linked chitosan beads	Laccase from *Trametes versicolor*	Covalent immobilization	Sulfur Blue 15,	pH 6.5, 30 °C,	20%	65%	(Nguyen et al., 2016a)
					82%		
			Sulfur Brown GD		20%	50%	
					71%		
Chitosan beads	Manganese peroxidase	Entrapment	Textile effluent	pH 4.5, 5 h	20%	90%	(Bilal et al., 2016)
					97%		
Polyacrylonitrile	Horseradish peroxidase	Covalent immobilization	Phenol	pH 6, 25 °C	<10%	>90%	(Wang et al., 2016)
					95%		
Chitosan/Fe membrane	Laccase from *Myrothecium verrucaria* I-5	Adsorption immobilization	Acid Red 73,	pH 7, 28 °C, 12 h	30%	70%	(Wen et al., 2015)
					100%		
			Acid Blue 113		25%	70%	
					95%		
Chitosan/Diaion WK-20 (cation exchange resin)	Tyrosinase	Covalent immobilization	Phenol,	pH 7, 25 °C, 2 h	100%	100%	(Wada et al., 1993)
					100%		
			p-Chlorophenol,		100%	100%	
					100%		
			p-Methoxyphenol,		100%	100%	
					100%		
			p-Cresol,		100%	100%	
					100%		
			Catechol		85%	90%	
					90%		
Chitosan/Diaion WK-10 beads	Tyrosinase from mushroom	Covalent immobilization	*p*-Cresol,	25 °C, 4 h	65%	98%	(Tamura et al., 2010)
					100%		
			4-*n*-Nonylphenol,		50%	99%	
					96%		
			4-*sec*-Butylphenol		60%	96%	
					100%		
Chitosan/alginate beads	Tyrosinase from *Agaricus bisporus*	Entrapment	Phenol	25 °C, 4 h	30%	85%	(Ensuncho et al., 2005)
					92%		
Cellulose/cellulose fibril/maleic anhydride	Laccase	Covalent immobilization	Chlorinated biphenyl	pH 4, 25 °C, 3 h	45%	40%	(Li et al., 2018)
					85%		
Polyacrylonitrile/ montmorillonite/ graphene oxide nanofibers	Laccase from *Trametes versicolor*	Covalent immobilization	Catechol	pH 4, 25 °C	60%	35%	(Wang et al., 2014)
					39%		

Poly(vinyl alcohol)/ poly(acrylic acid)/SiO_2	Horseradish peroxidase	Covalent immobilization	Paracetamol	pH 3, 25 °C, 1.5 h	15%	83%	(Xu et al., 2015)
					98%		
Polyvinyl alcohol/ halloysite beads	Laccase from Aspergillus sp.	Covalent immobilization	Reactive blue	pH 5, 25 °C, 8 h	16%	74%	(Chao et al., 2018)
					90%		
Poly(D,L-lactide-co-glycolide)/ multi-walled carbon nanotubes	Laccase from *Trametes versicolor*	Encapsulation	Bisphenol A	pH 5, 25 °C, 5 h	10%	85%	(Dai et al., 2016)
					95%		
Poly(acrylic acid)/SiO_2 nanofibrous membranes	Laccase from white-rot fun	Covalent immobilization	Triclosan	pH 4, 30 °C, 2 h	45%	20%	Xu et al., 2014
					65%		
Poly(D,L-lactide-co-glycolide) /polyethylene glycol/poly(*p*-phenylene oxide) fibers	Horseradish peroxidase	Encapsulation	Pentachlorophenol	pH 3, 25 °C, 2 h	55%	30%	(Niu et al., 2013)
					85%		

2.1.1. Inorganic materials

A wide variety of different inorganic materials has been used for simultaneous removal of hazardous pollutants by both immobilized enzymes and by adsorption. Organic oxides including e.g. silica, titanium, alumina and iron oxides have been used for the immobilization of oxidoreductases and selective sorption of pollutants such as synthetic and natural dyes, phenolic compounds and antibiotics due to the presence of many functional moieties, mainly hydroxyl groups, as well as well a defined porous structure, good sorption properties and high stability, in (Champagne and Ramsay, 2007, Kim et al., 2011, Yu et al., 2015). There are also other inorganic materials, such as minerals and carbon-based materials, which offer high stability and good sorption properties that can be successfully applied for pollutant removal by enzymatic oxidation and subsequent adsorption (Choi et al., 2008, Ding et al., 2016). An interesting example of the utilization of inorganic oxides (alumina) was reported by Zille et al. and Osma et al. Laccase was immobilized by adsorption onto spherical alumina pellets or by covalent binding onto Al_2O_3 pellets, respectively, and used for the decolorization of industrial effluents containing Reactive Black 5. It was found that in both cases the decolorization occurred due to two processes: adsorption of the dye on the support material and its degradation by the laccase. However, an interesting phenomenon was observed: when the biomolecule was attached to the matrix *via* weak adsorption interactions, approx. 80% of the dye was removed due to the adsorption process and only 4% was degraded by the immobilized laccase (Zille et al., 2003). On the other hand, covalent binding of the biomolecules completely reversed these proportions: in this case over 90% of the Reactive Black 5 was biodegraded and less than 10% was adsorbed onto the alumina pellets. These observations may be explained by the type of the interactions formed between the enzyme and support. Covalent immobilization of the enzyme and the usually accompanying multipoint attachment of the biocatalysts resulted in the saturation of the majority of active sites on the surface of the support. This limited the number of free active sites available for the dye molecules and, as a consequence, the sorption capacity of the material with immobilized enzyme towards the dye was decreased (Osma et al., 2010). Furthermore covalent binding of the laccase increased its stability and enzyme leakage was strongly reduced, which also enhanced the enzymatic removal of the RB5 dye. Nevertheless, under optimal conditions, the biodegradation process was highly efficient in both batch and continuous modes, indicating that such biocatalytic systems may be suitable for potential implementation at an industrial scale.

It can be briefly summarize that use of the inorganic materials as support for simultaneous immobilization and removal of pollutants allows to achieve high removal efficiencies which exceed 90%. A wide range of toxic compounds may be removed, including dyes, phenols and antibiotics. However, enzymatic conversion is the main mechanism of degradation of phenols, as the adsorption process enhances the total removal efficiency. For this technique, mainly laccases are immobilized using adsorption or covalent binding which facilitate the activity of the used biocatalytic systems. Moreover, enzymes immobilized onto inorganic materials are characterized by improved stability and exceptional operational and mechanical resistance.

Simultaneous enzyme immobilization and adsorption of toxic pollutants using inorganic materials is facilitated due to the presence of numerous functional groups, mainly hydroxyl, extraordinary stability and their mechanical resistance. Using these materials, enzymes are immobilized mainly by adsorption which protects their catalytic properties due to the fact that the three-dimensional structure of the biocatalysts is unaltered. However, due to the multipoint attachment, elution of the enzyme molecules from the support is restricted. Moreover, due to the presence of many functional moieties, the formation of the covalent bonds also cannot be excluded, which additionally reduces enzyme leakage. Also effective sorption of both environmental pollutants and products of their bioconversion is enhanced by the presence of several functional groups. It should be emphasized that among inorganic materials, inorganic oxides should be of particular interest for researchers and industrial applications. This is mainly due to their well-developed surface area and porous structure (numerous micropores and macropores) as well as particle sizes, defined morphology and exceptional stability. These features are directly associated with the properties of the naturally occurring inorganic oxides and the methodology of their synthesis which allows for the production of materials with desired physico-chemical characteristics. Moreover, it should be added that such materials are frequently and are easy to obtain, which makes them relatively cheap. Furthermore, the use of inorganic oxides for simultaneous enzyme immobilization and pollutants adsorption results in high removal efficiencies. It should also be added that inorganic oxides exhibit supplementary properties, such as magnetic properties in case of magnetite or photocatalytic properties in case of titanium, which, respectively, facilitate rapid separation of the material after the process by using external magnetic field or may enhance the removal efficiency by photocatalysis.

2.1.2. Organic materials

Aside from many inorganic materials, biopolymers and synthetic polymers are also used for simultaneous enzyme immobilization and sorption of hazardous compounds. The presence of numerous of chemical moieties, such as: –OH, $–NH_2$, C=O and COOH, in their structure enhances efficient immobilization of biocatalysts and sorption of environmental pollutants from water solutions. Biopolymers of different origin, abundant in nature, such as agar, starch and carrageenan, are used for the simultaneous biodegradation and adsorption of toxic compounds mainly due to their good sorption properties and relatively low cost (Costa and Reis, 2004, Srinivasan and Viraraghavan, 2010, Loffredo et al., 2014). Generally, due to the presence of functional moieties and their natural origin, most of these materials exhibit rather high affinity to peptides (Bilal and Iqbal, 2019). Moreover, due to their negligible negative effect on the biocatalysts, biopolymeric supports improve the operational and storage stability of the immobilized enzymes and prolong their catalytic activity which in consequence enhances the practical applications of the produced biocatalytic systems. The most commonly used biopolymer is chitosan, in a variety of forms and sizes. For instance, Nguyen et al. used chitosan beads crosslinked by glutaraldehyde for the removal of sulfur dyes (Sulfur Blue 15 and Sulfur Brown GD) from a water solution through biosorption and subsequent degradation by immobilized laccase. Enzymatic biodegradation was the leading mechanism of pollutant removal. Even at low laccase concentration, over 70 and 80% of Sulfur Brown GD and Sulfur Blue 15, respectively was removed at pH 6.5 from the dye solution at a concentration of 200 mg/L. However, when a mixture of dyes was treated under the same conditions, the efficiency of removal of each dye significantly decreased (Nguyen et al., 2016a). This is associated with the fact that dye molecules compete with each other for access to the active sites of enzymes and not every dye molecule could be converted due to their overcrowding. That is the main limitation of the presented solution and more enzymes should be immobilized to increase the amount of catalytic active sites in order to overcome this problem. In another study, chitosan film was used as a support for covalent immobilization of mushroom tyrosinase. The produced biocatalytic system was applied for the biodegradation of phenol derivatives from artificial wastewater and subsequent sorption of quinone derivatives formed after tyrosinase-catalyzed oxidation of toxic compounds. It was found that pH 7 and a temperature of 45 °C were the optimal conditions for both enzymatic biodegradation and quinone adsorption, which additionally improved the efficiency of the process. After biodegradation and subsequent sorption of products of phenol bioconversion, over 90% of *p*-cresol, *m*-cresol, catechol and *p*-chlorophenol were removed by this procedure (Yamada et al., 2005). Apart from polymers of

natural origin, synthetic polymers are also used for simultaneous biodegradation and adsorption of hazardous compounds. Monomers commonly applied in enzyme immobilization, including polystyrene and polyvinyl alcohol, have also been used for the production of materials for both attachment of laccases or tyrosinases and the sorption of dyes or other phenolic derivatives in one process (Leidig et al., 1999, Zhang et al., 2014). Wang et al. investigated the use of polyacrylonitrile membranes for the immobilization of horseradish peroxidase and further degradation of phenol. The immobilized peroxidase was additionally crosslinked with glutaraldehyde, which on the one hand reduced the elution of the biomolecules from the matrix, but on the other hand significantly decreased the number of chemical moieties able to adsorb phenol. The removal process could be divided into two steps: (i) adsorption of phenol on the surface and in pores of the membrane to increase its availability to the immobilized enzyme and (ii) enzymatic conversion. Nevertheless, the main mechanism of removal was enzymatic biodegradation, and ultimately less than 10% of the phenol was removed by sorption. With the use of the described system, almost total removal of phenol from water solutions at concentrations up to 10 mg/L was achieved, which suggests that a polyacrylonitrile membrane with immobilized horseradish peroxidase has promising applications for the removal of phenol from water solutions (Wang et al., 2016).

It can be briefly summarized that the use of organic materials of both synthetic and natural origin, such as polyacrylonitrile or chitosan, for simultaneous enzyme immobilization and adsorption of hazardous compounds, mainly phenol and its derivatives and dyes, allows for their removal from wastewaters with high efficiencies, which usually exceed 90%. Moreover, oxidoreductases retained their high catalytic properties after immobilization as an effect of their attachment to the support, mainly by stable covalent bonds. Due to this fact, enzymatic conversion is the main mechanism which determines the removal processes of the pollutants. Furthermore, a wide range of enzymes, including laccases, tyrosinases and peroxidases, can be immobilized by adsorption, covalent binding and even encapsulation using polymeric supports. Nevertheless, in our opinion, biopolymers such as chitosan, are more suitable for application in simultaneous immobilization and sorption due to the presence of many chemical groups, formation of various geometrical shapes (which is of particular interest for application in bioreactors and in continuous processes) as well as biocompatibility and frequent abundance in nature, that makes them renewable and relatively cheap.

2.1.3. Hybrid and composite materials

During recent years, hybrid and composite materials are of particular interest among wide range of supports/sorbents used for enzyme immobilization and simultaneous removal of pollutants. Hybrid materials may be a combination of inorganic-organic, inorganic-inorganic and organic-organic species. The role of their formation results from the possibility to obtain novel materials which combine the properties of both components. Depending on the requirements, the hybrid materials may be synthesized using different methods, and the selection of an appropriate method determines their physicochemical properties, such as morphology and dispersive character, electrokinetic and thermal stability as well as parameters of the porous structure and hydrophilic-hydrophobic nature. A broad spectrum of synthesis methods allows to design hybrid materials characterized by diverse physicochemical and structural parameters suitable for enzyme immobilization and removal of hazardous compounds. Another advantage is associated with the fact that their properties may be freely designed by selection of the components which are included in the hybrid materials as well as by means of further treatment using different surface modifications with multifunctional organic or bioorganic substances. On the other hand, composite materials include at least two components – one of them is dispersed in the second one. The most commonly known composite materials are polymers, metallic and ceramic composites. Compared to hybrid materials, properties of the composite materials are not a sum of the properties of its components. Nevertheless, both types of the above-mentioned materials exhibit unique properties which enable their application as effective supports/sorbents used for enzyme immobilization and simultaneous removal of pollutants.

2.1.3.1. Organic-inorganic hybrid materials

There are numerous reports concerning the use of combinations of precursors of different origin to produce functional hybrid materials with different properties. For example, polyacrylonitrile was combined with a naturally occurring montmorillonite to create nanofibers, which were enriched with graphene oxide to increase electron transfer. This material was used for the immobilization of laccase from *Trametes versicolor*, and then for the removal of catechol (Wang et al., 2014). Although addition of graphene oxide enhanced the catalytic properties of the enzyme, most of the functional groups of the precursors are involved in the formation of a hybrid. In consequence, the amount of immobilized enzyme as well as sorption capacity of the above-mentioned material are limited, which is the main disadvantage of the proposed support/sorbent resulting in relatively low total removal

efficiency. In a similar study by Dai et al. (2016), poly(D,L-lactide-co-glycolide)/multi-walled carbon nanotubes hybrid fibers, produced *via* an electrospinning technique, were used for the encapsulation of laccase. The resulting biocatalytic system with a high electron transfer rate was further used for the biodegradation of bisphenol A from a water solution. The immobilized enzyme exhibited good storage stability and reusability, and was more stable than native laccase even at a temperature of 60 °C. It has been reported that the removal of BPA was mainly due to the enzymatic biodegradation, as adsorption of the pollutant did not exceed 10%. Other synthetic polymers, including poly(vinyl alcohol), poly(acrylic acid) and polyamine, as well as biopolymers such as chitosan or alginate, have also been combined with inorganic precursors such as silica, clays and iron ions to produce stable and efficient materials for enzyme immobilization and for further application of such systems in the biodegradation and simultaneous adsorption of dyes, phenols and pharmaceuticals (Perullini et al., 2010, Xu et al., 2015). The combination of a biopolymer (chitosan) with iron ions and creation of a hybrid membrane was suggested by Wen et al. (2015) for the economical and effective degradation of dyes. The membrane demonstrated excellent sorption capacity with respect to the laccase as well as the dye. The results proved that the dyes can be removed by the enzyme and the membrane synergistically; however, the dyes were first adsorbed on the membrane and then degraded by the enzyme. This solution improved substrate accessibility for the immobilized enzyme which resulted in the total removal of Acid Red 73 and Acid Blue 113 at mild conditions.

2.1.3.2. Organic-organic hybrid materials

Aside from combinations of inorganic and organic precursors, hybrids produced by linking of organic materials are also used. These materials are characterized by biocompatibility and high affinity towards peptides which is highly desirable for effective enzyme immobilization. Moreover, the presence of functional moieties additionally increases the efficiency of sorption properties of both hazardous pollutants and products of their conversion. An interesting example was reported by Ensuncho et al. (2005) regarding the combination of two biopolymers, chitosan and alginate, to form a chitosan matrix crosslinked with glutaraldehyde with an alginate-filled pore space, for entrapment immobilization of tyrosinase from *Agaricus bisporus*. The produced beads presented good mechanical properties and preserved the unique adsorption characteristics of chitosan. The biocatalytic system was used for the removal of phenol from water solution and further sorption of *o*-quinone as the product of enzymatic conversion. This solution allowed to retain good catalytic properties of tyrosinase over

repeated biodegradation cycles, while protecting the immobilized enzyme against inactivation caused by the by-products of bioconversion. With the use of this system, over 90% of the phenol was removed under optimal conditions after four hours of the process. However, adsorption efficiency reached approx. 30% as enzymatic conversion reached over 60%. Materials which combine synthetic polymers and biopolymers are also used for effective simultaneous enzymatic biodegradation and adsorption of pollutants. For instance, chitosan was linked with the weakly acidic cation exchange resins Diaion WK10 and WK20. Tyrosinase was then covalently immobilized on the hybrid material and used for the removal of alkylphenols from aqueous solutions. In both cases the enzyme exhibited its highest catalytic activity in the temperature range of 30–45 °C and the pH range of 7–10. Under such conditions, alkylphenols were effectively removed through quinone oxidation by the immobilized biomolecules with efficiencies equal to 100% (Wada et al., 1995, Tamura et al., 2010). After enzymatic oxidation, subsequent quinone adsorption by the chitosan beads was observed. Moreover, the authors suggested that the removal efficiency would increase with increased quantity of chitosan in the beads due to the fact that quinone adsorption may exceed enzymatic conversion of quinones at a certain point (Tamura et al., 2010). Furthermore, they reported that the total amount of phenol was removed after two hours from the solution by the immobilized tyrosinase and chitosan beads. The presented solution is highly effective and ensures great enzyme reusability due to the combination of cationic resin, responsible for covalent binding of the tyrosinase, and chitosan, which plays a crucial role in adsorption process (Wada et al., 1995). For efficient encapsulation of horseradish peroxidase, the poly(D,L-lactide-co-glycolide)/polyethylene glycol/poly(*p*-phenylene oxide) fibers were produced *via* emulsion electrospinning. Furthermore, adsorption and degradation of pentachlorophenol (PCP) by the immobilized peroxidase was investigated. It was found that the sorption of PCP follows the pseudo-second-order model and its efficiency reached 55%. Additionally, sorption of the pollutant strongly enhanced the efficiency of its removal, due to interactions between the adsorbed pentachlorophenol and immobilized enzyme. A total removal efficiency of over 85% of PCP at the temperature of 25 °C and pH ranging from 2 to 4 was observed. Moreover, after encapsulation by emulsion electrospinning, the operational and storage stability of the immobilized biomolecules were significantly improved. This indicates that the produced biocatalytic system may find practical applications in the biodegradation of PCP from actual wastewaters. However, it should also be underlined that pH of the solution is the factor which limits the application of the above-mentioned system in alkaline conditions, as no adsorption and degradation were observed at pH above 4.7, due to

the deprotonation of PCP, enzyme inactivation and its leakage from the support (Niu et al., 2013).

In order to briefly summarize the presented literature review, we would like to highlight that there is a fairly wide group of support materials of various origin that can be applied for the simultaneous biodegradation and adsorption process; however, inorganic materials characterized by good mechanical and operational properties together with synthetic or natural organic substances known from their biocompatibility, are used most frequently. Aside from operational stability, such materials must also exhibit good sorption properties, a favorable and defined porous structure, and the presence of numerous functional groups for effective enzyme binding and sorption of hazardous pollutants. Over recent years hybrid and/or composite materials are also more and more commonly used for simultaneous immobilization and adsorption, mainly due to their tailor-made properties suitable for both the immobilized enzyme and pollutant or product of its conversion to be adsorbed. Therefore we encourage to carry out even more advanced studies in the topic of application of hybrid/composite materials for oxidoreductase immobilization and toxic compounds sorption, as the development of solutions resulting in high removal rates of dyes or phenolic compounds could be important in terms of environmental protection. However, we would like to emphasize that each of the above-mentioned groups of materials also possesses disadvantages. For instance, most of the organic materials are characterized by low sorption properties due to low porosity and low surface area. On the other hand, the presence of mainly hydroxyl groups on surface of the inorganic materials results in the fact that their functionalization is required to form stable enzyme-matrix interactions. Moreover, hybrid materials, aside from exhibiting tailor-made properties, are relatively expensive to obtain.

This technique dates back to the beginning of the last decade, but a rapidly growing interest in applications of the method has been observed during the last few years. The greatest advantage of this method is the fact that compounds which are resistant to enzymatic degradation are effectively removed from polluted solutions by adsorption. Additionally, due to the complex mechanism of remediation, a very wide group of toxic compounds could be efficiently eliminated from solution, usually with extraordinary efficiencies. However, reusability of the matrix after inactivation of the enzyme is limited, since desorption of the pollutants and regeneration of the sorbent are expensive and unfavorable. Consequently, extensive research is still required to improve the process control and efficiency, and to identify new groups of compounds which may be used for effective simultaneous enzyme

immobilization and pollutant removal, thus increasing the applicability of the method in the bioremediation of toxic compounds from wastewaters and industrial effluents.

3. Enzymatic reactors for removal of environmental pollutants

Due to the increasing amount of pollutants prevalent in the environment, there is still a need to evaluate and develop efficient, eco-friendly and cost-effective solutions for their removal. Aside from simultaneous enzyme immobilization and sorption of toxic compounds, another interesting solution based on the use of enzymes is application of bioreactors for biodegradation and removal of persistent substances from wastewaters and industrial effluents. Enzymatic bioreactors usually offer a very good flow regime, reducing the mass transfer limitations which are commonly observed when heterogeneous catalysts are used (Luckarift, 2008). These features mean that removal and biodegradation processes performed in bioreactors are usually characterized by high selectivity and efficiency. Furthermore, due to the reduced time and energy consumption, processes carried out in bioreactors are more environmentally friendly and cost-effective (Balcao et al., 1996). Additionally, it should be emphasized that the effectivity of an enzymatic bioreactor is improved when immobilized enzymes are used as catalysts, due to their improved stability and reusability compared to free enzymes (Husain, 2017).

Many different configurations of enzymatic reactors for environmental applications have been developed in recent years. In general, these bioreactors may be divided into two groups: (i) enzymatic bioreactors (EBRs) and (ii) enzymatic membrane reactors (EMRs) (Nanba et al., 2007, Rios et al., 2014). In both EBRs and EMRs immobilized laccases, tyrosinases and other oxidoreductases (mainly horseradish peroxidase) are employed for the removal of hazardous compounds. However, there are great differences between these two types. EBRs are frequently used in various operational modes, such as batch reaction or continuous reaction, to increase the efficiency of the biocatalytic process. However, after biocatalytic conversion, an additional step is required, namely the separation of the immobilized enzyme from the reaction mixture. In the EMR, however, the catalytic action is simultaneous with membrane separation of the products from the reaction mixture, which results in high purity of the products and reaction effluents (Sanchez-Marcano and Tsotsis, 2002). Selected examples of the use of EBRs with immobilized oxidoreductases for environmental applications were reviewed in section 3.1, whereas instances of the use of EMRs for biodegradation and removal of hazardous pollutants were discussed in detail in section 3.2.

3.1. Enzymatic bioreactors (EBRs)

An enzymatic bioreactor, in broad terms, is a device in which enzyme-catalyzed transformation of substrates into products occur under mild reaction conditions (Miyazaki and Maeda, 2006). Selection of the reactor type and operational mode is conducted according to the process conditions, enzyme activity and required product purity. Immobilized oxidoreductases are, in general, used in two types of bioreactors: (i) batch reactors and (ii) continuous reactors (Webb et al., 2004, Xue and Woodley, 2012, Barrios-Estrada et al., 2018b) as presented in Fig. 2.

Figure 2. Schematic representation of the batch-mode and continuous-mode enzymatic bioreactors for environmental applications.

Batch reactors used for the removal of environmental pollutants by immobilized oxidoreductases include the stirred-tank reactor, the most frequently used type of enzyme reactor, as well as the fluidized bed reactor. Batch reactors are characterized by simplicity and flexibility of use at an industrial scale, offer easy control of the process and are very useful for slow reactions in a viscous mixture (Darnoko and Cheryan, 2000, Shimada et al., 2002). Furthermore, due to their simple construction and relatively high efficiencies, batch reactors require less capital investment than continuous processes. It should be added that immobilized laccases and tyrosinases applied in batch bioreactors offer very good reusability as they can often be used in more than ten consecutive biodegradation cycles (Srikanlayanukul et al., 2016). On the other hand, the advantage of continuous-mode reactors is that immobilized enzymes are constantly in contact with the stream of substrates, which enhances the activity of the biomolecules. Moreover, the flux of the reaction mixture through the biocatalytic beads can be controlled to meet process requirements (Bolivar et al., 2011). The use of a bioreactor in continuous mode also allows to avoid the usually costly separation of the biocatalyst from

the reaction mixture (Almeida et al., 2003). In consequence, after detoxification by immobilized laccases, effluent streams with a purity of over 95% can be obtained (Palli et al., 2017). Since both batch and continuous processes have advantages in practical use, it is impossible to clearly indicate the best operational mode.

It should be highlighted that the design of an enzymatic bioreactor is a complex engineering task. Nevertheless, the key idea is to design reactors capable of achieving the highest conversion rate and the highest quality of products at the lowest costs. First of all, to ensure high efficiencies of the enzymatic reaction, the bioreactor should provide optimal process conditions for the biomolecules, such as substrate supply, product and by-product removal, controlled pH, temperature and agitation speed (Benz, 2011, Le-Clech et al., 2006). Thus, prior to bioreactor design, a detailed study concerning type of the enzyme and substrate as well as conditions for the highest bioconversion efficiency should be carried out. Furthermore, enzymatic bioreactors should ensure the efficient use of the biomolecules and their substrates, reduce diffusional limitations and exhibit low energy requirements (Pino et al., 2018). Finally, the bioreactors should be characterized by simplicity and optimized volume of the vessel to ensure high rate of substrate conversion per volume of the reactor.

Nevertheless, we would like to point out that selection of the most suitable operational method is usually influenced by several variables, such as enzyme stability, type of support materials, process time and required purity of the solution after the process. However, to increase the efficiency of the processes carried out in enzymatic bioreactors and to enhance the biocatalytic properties of the enzymes, biomolecules in immobilized form are commonly used. Support materials of inorganic, organic and hybrid or composite origin may be used for the immobilization of tyrosinases, laccases and horseradish peroxidase for subsequent use in the biodegradation of hazardous pollutants. Selected examples of biocatalytic systems used in enzymatic bioreactors (EBRs) for environmental applications were summarized in Table 4.

Table 4. Materials of various origin used for immobilization of laccases and tyrosinases for application in enzymatic bioreactors (EBRs) towards biodegradation of various environmental pollutants.

Reactor type	Process conditions	Support material	Enzyme	Immobilization technique	Pollutants (enzyme substrate)	Removal efficiency	Reference
Packed bed reactor	pH 7, 28 °C, 60 days	Granular activated carbon	Laccase from *Aspergillus oryzae*	Adsorption immobilization	Sulfamethoxazole, Carbamazepine, Diclofenac, Bisphenol A	94% 92%, 95%, 97%	(Nguyen et al., 2016b)
Packed bed reactor	pH 4, 40 °C, 30 h	*γ*-Aluminum oxide pellets	Laccase from *Trametes modesta*	Covalent immobilization	Lanaset Blue 2R, Terasil Pink 2GLA, Indigo Carmine, Crystal Violet	100%, 70%, 99%, 97%	(Kandelbauer et al., 2004)
Bioreactor	pH 6.5, 20 °C, 7 h	Aminopropyl-controlled pore glass	Tyrosinase from mushroom	Covalent immobilization	Phenol, *p*-Cresol, Catechol, 4-Methylcatechol, 4-Chlorophenol	60%, 100%, 100%, 100%, 100%	(Girelli et al., 2006)
Core-shell microreactor	pH 6.5, 25 °C, 15 min	Superparamagnetic hydrophobic particles/glass plates	Laccase	Covalent immobilization	Syringaldazine	75%	(Al-Kaidy and Tippkotter, 2016)
Column-packed reactor	pH 5, 28 °C, 5 days	Na-alginate beads	Laccase from *Polyporus rubidus*	Entrapment	Reactive Blue, Remazol Black 5, Reactive Orange, Congo Red	85%, 100%, 90%, 100%	(Dayaram and Dasgupta, 2008)
Fluidized bed bioreactor	pH 5, 20 °C, 100 min	Na-alginate beads	Laccase	Entrapment	Pharmaceuticals	75%	(Du et al., 2013)
Packed bed reactor	30 °C, 6 h	Ca-alginate beads	Laccase from *Streptomyces psammoticus*	Entrapment	Phenol	70%	(Niladevi and Prema 2008)
Fixed bed reactor	pH 4.5, 20 °C, 4 h	Cu-alginate beads	Laccase from *Pleurotus ostreatus*	Entrapment	Remazol Brilliant Blue R	70%	(Palmieri et al., 2005)
Bioreactor	pH 7, 25 °C, 12 h	Cu-alginate beads	Laccase from *Ganoderma* sp. KU-Alk4,	Entrapment	Indigo Carmine, Remazol Brilliant Blue R, Bromophenol Blue, Direct Blue 15	100%, 100%, 65%, 54%	(Teerapatsakul et al., 2017)

Continuous-flow microreactor	pH 5, 30 °C, 1 h	Glutaraldehyde and paraformaldehyde	Laccase from *Trametes versicolor*	Cross-linking	Estrone, 17-*β*-Estradiol, 17-*α*-Ethinylestradiol	100%, 100%, 100%	(Lloret et al., 2013)
Perfusion basket reactor	pH 4.5, 35 °C	Poly(ethylene glycol) and glutaraldehyde	Laccase from *Cirripectes polyzona*	Cross-linking	Nonylphenol, Bisphenol A, Triclosan	95%, 100%, 100%	(Cabana et al., 2009)
Packed bed reactor	25 °C, 5 days	Polyurethane foam cubes	Laccase from *Trametes versicolor*	Covalent immobilization	Remazol Brilliant Blue R, Anthraquinone dye B4, Acid Black 24	100%, 98%, 65%	(Yang et al., 2012)
Packed bed reactor	pH 7, 24 °C, 30 min	Eupergit C 250L	Laccase from *Myceliophthora thermophila*	Covalent immobilization	Estrone, 17-*β*-Estradiol, 17-*α*-Ethinylestradiol	65%, 80%, 80%	(Lloret et al., 2012)
Fixed bed tubular bioreactor	pH 6.5, 25 °C, 60 h	KC-Microperl MP100	Co-immobilized laccase *Pyricularia oryzae* and tyrosinase	Covalent immobilization	*α*-Naphthol, 4-Chlorophenol, 2-Chlorophenol, *p*-Cresol	100%, 66%, 50%, 28%	(Krastanov, 2000)

As is shown in Table 4, materials of both inorganic and organic origin as well as hybrids and composites are used as supports for the immobilization of enzymes for application in bioreactors. These materials must be insoluble in the reaction environment and offer good mechanical and operational stability, with regard to the long duration of the process and its repeatability. Moreover, these supports are notable for their good sorption properties and the presence of many functional groups in their structure, for effective binding of the enzyme molecules to prevent elution of the catalyst from the support during the process (Illaes and Wilson, 2003).

3.1.1. Inorganic materials as support materials in EBRs

Among various inorganic materials, minerals, silicas, inorganic oxides such as γ-aluminum oxide, and carbon-based materials are the most frequently used to produce enzyme-matrix systems for application in EBRs (Kandelbauer et al., 2004). For example, in a study described by Nguyen et al., laccase was immobilized by adsorption on granular activated carbon, known for its good sorption properties, and applied for the degradation of sulfamethoxazole, carbamazepine, diclofenac and bisphenol A in a packed bed column bioreactor. The pollutants were removed with efficiencies of over 90% by enzymatic degradation following their adsorption onto activated carbon. The significantly higher efficiency of bioremoval achieved in the EBR with immobilized biomolecules compared to the EBR with free enzyme is associated with the fact that after enzyme immobilization on carbon material, transfer of electrons between the laccase and adsorbed substrate molecules was enhanced and bioreactor effectivity was improved. Moreover, the system demonstrated exceptional reusability: the immobilized biocatalyst retained its activity even after two months of continuous operation (Nguyen et al., 2016b).

3.1.2. Organic materials as support materials in EBRs

Synthetic polymers and biopolymers such as chitosan are also used, due to their mechanical stability under operational conditions and strong binding of the enzyme to the support. In view of their very good gelation properties and the mechanical resistance of the formed beads, alginates are a commonly used biopolymer (Ganaie et al., 2014). In another study, polyurethane foam cubes were used for covalent immobilization of laccase from *Trametes versicolor* by Yang et al. (2012). This biocatalytic system was used in a packed bed reactor for decolorization of Remazol Brilliant Blue R, Anthraquinone dye B4 and Acid Black 24 from model and industrial wastewaters. The immobilized laccase was used in five sequential

degradation cycles and was able to decolorize all tested dyes efficiently, even at a concentration of 2000 ppm. Furthermore, the duckweed test showed a significant reduction of the toxicity of the effluents after enzymatic treatment. In another study, alginate was additionally cross-linked by $CuSO_4$ to form blue spherical beads with entrapped laccase from *Pleurotus ostreatus*. Copper ions were used not only for the gelation of the biopolymer, but also to enhance the catalytic properties of the immobilized enzyme. A fixed bed reactor with enzymatic beads working in continuous mode was used for decolorization of the anthraquinonic dye. Degradation efficiency of 70% was achieved after four hours of the process at pH 4.5 and temperature of 20 °C. The immobilized laccase exhibited remarkable reusability, achieving a high efficiency of decolorization (over 60%) even after 20 catalytic cycles (Palmieri et al., 2005). Although copper ions improve the activity of the immobilized laccase, the limitation of this solution, resulting in approx. 70% degradation, is associated with fact that formed coordinative bonds distort the enzyme structure and decrease its properties. Moreover, cross-linking of the alginate beads resulted in the occurrence of some diffusional limitations, which could additionally decrease the total removal efficiency.

3.1.3. EBRs operational modes

Aside from type of the support material, the operational mode of the bioreactor also affects its operational effectivity. As has been shown, there are different types of enzymatic bioreactors with immobilized enzymes for environmental applications working in batch and continuous modes. These may be classified as: (i) packed bed reactors, (ii) fixed bed reactors, (iii) fluidized bed reactors and (iv) continuous flow reactors (Williams, 2002). The type of used reactor as well as the support material are selected appropriately to the enzyme and the type of catalytic process. Moreover, the choice of reactor and operational mode is usually intended to increase the operational effectiveness of the equipment and the reaction yield. For example, Krastanov (2000) used a continuous fixed bed tubular bioreactor with laccase from *Pyricularia o*ryzae and mushroom tyrosinase co-immobilized onto KC-Microperl MP100, for a rapid degradation of phenols from aqueous solutions. The bioreactor achieved high operational efficiency. With the use of the described method, phenolic derivatives such as *α*-naphthol or catechin could be totally removed from the mixture after a single pass through the reactor. Moreover, after immobilization, the operational stability of the immobilized enzymes with respect to particular substrate types, such as 2,4-dichloropenol and guaiacol, was significantly improved. In another study by Du et al. (2013), a fluidized bed reactor in which the biocatalytic beads consisted of laccase immobilized by entrapment into sodium alginate

capsules was applied in order to increase the intensity of contact between the enzyme and substrates and to reduce reaction time. It was found that the optimal capacity of the bioreactor was 1.5 L and the following conditions were optimal for the degradation of pharmaceuticals: pH 5, temperature 20 °C and reaction time 100 min. Under these conditions, more than 75% of the pharmaceuticals were removed from wastewater. However, low mediator concentration and insufficient quantity of used laccase should be indicated as the main limitation factors which did not allow to achieve total removal efficiency. As it was mentioned above, it is hard to clearly indicate and suggest a universal and, at the same time, the most suitable operational mode for the removal of all of the environmental pollutants by immobilized oxidoreductases in EBRs. Thus we suggest careful consideration of the type of the enzymatic bioreactor, taking the type and form of the support material with immobilized enzyme into account, to ensure enzymatic activity as high as possible and its contact with stream of the substrates.

3.1.4. Enzyme immobilization techniques in EBRs

Depending on the type and form of support material and the reactor construction, enzymes can be immobilized *via* different techniques to increase the efficiency of the degradation process. However, the main concern is that the immobilized enzyme has to be strongly connected with the support to prevent its elution during repeated reaction cycles and to maintain good catalytic properties. Thus, mainly covalent immobilization as well as entrapment are frequently applied, however, adsorption, encapsulation and even cross-linking, are also used to produce biocatalytic beads for bioreactors (McMorn and Hutchings, 2004). An interesting example was reported by Dayram and Dasgupta (2008) regarding the use of laccase from *Polyporus rubidus* for the degradation of four reactive dyes commonly occurring in effluents. The enzyme was immobilized *via* an entrapment method into calcium alginate beads and used in an enzymatic column-packed reactor. The biocatalytic system demonstrated excellent operational stability, and over 85% of the dyes were removed from wastewater. Additionally, due to the limited interference in the enzyme structure, the systems produced by entrapment exhibited good storage stability as their properties were unaltered after ten days of storage. Thus, immobilization by entrapment may be indicated as a promising way to develop easy and cost-effective methods for production of biocatalytic systems for remediation of hazardous dyes. Moreover, it should be emphasized that with the use of immobilized oxidoreductases as a beads in bioreactors, usually over 80% of toxic compounds can be removed. For instance, phenol and its derivatives, such as *p*-cresol, catechol and 4-methylcatechol, were biodegraded using a bioreactor with mushroom tyrosinase covalently

immobilized onto aminopropyl-controlled pore glass. Under pH 6.5 and at ambient temperature, total removal of phenolic compounds was observed. Additionally, in order to obtain effluent which was as pure as possible after the enzymatic treatment, adsorption of colored quinone-type biodegradation products on a chitosan trap was applied (Girelli et al., 2006). This solution should be indicated as particularly interesting since the pollutants were removed *via* simultaneous enzymatic biodegradation by immobilized enzymes and adsorption by support/sorbent material, which additionally increased the removal efficiency, improved the purity of the effluents and decreased their toxicity. In another study, laccase entrapped into copper alginate beads was used in a bioreactor for remediation of selected commercial aromatic dyes. The immobilized laccase exhibited better stability than the free enzyme and higher efficiency in the removal of various synthetic dyes under non-buffered conditions. The airflow rate was the key parameter affecting degradation time and number of batch runs. An airflow rate of 4 L/min was the most suitable for degradation of Indigo Carmine and Remazol Brilliant Blue R. Under this airflow, the total quantity of the dyes was removed from aqueous solution at pH 7 and temperature of 25 °C (Teerapatsakul et al., 2017).

To summarize the application of EBRs equipment based on the immobilized oxidoreductases for removal of environmental pollutants, it can be concluded that conversion of hazardous compounds can be achieved in a shorter time and under mild conditions. A great advantage of the EBRs is associated with the fact that materials of various origin could be used as a supports for immobilized enzyme. However, substances characterized by high stability and resistance as well as numerous functional groups on their surface, which enable the creation of stable enzyme-support interactions, such as inorganic oxides and synthetic polymers should be of particular interest. As noted above, enzymatic bioreactors can be classified as packed bed, fixed bed and fluidized bed reactors, which can operate in both continuous and batch modes. The selection of the bioreactor's operational mode is usually dictated by the process conditions, the type of pollutant and the form of the immobilized enzyme. Various immobilization techniques can be applied to improve process efficiency and different sizes and forms of the produced biocatalytic beads may be obtained. The form of beads is selected to increase the contact time of the enzyme with substrate molecules and the efficiency of the reaction. It is governed mainly by the operational mode of the process and concentration of the toxic compound. Nevertheless, in our opinion, the spherical beads, usually at nano- or microscale, based on materials characterized by high stability and mechanical resistance should be of particular interest due to their operational stability and low diffusional limitations between the immobilized enzyme and ingredients of the reaction mixture. It should also be

added that the type and form of the support material also affects the type of the immobilization. Although various techniques were applied, we encourage the use of covalent immobilization, as this method enhanced the formation of stable, covalent enzyme-support interactions which prevents enzyme elution and ensures high operational stability and reusability of the formed biocatalytic beads. The foregoing examples have shown that a wide range of toxic organic compounds can be effectively removed with the use of EBRs; however, the type of pollutant and the used biocatalyst strongly affect the bioremediation conditions. In our opinion, future research will focus increasingly on the appropriate selection of the operational mode and the form of the biocatalytic beads in order to maximize the efficiency of the processes carried out in EBRs. As a result, enzymatic bioreactors should be used as effective and efficient tools not only for the remediation of hazardous compounds but also in biocatalytic conversion of biomass or production of biofuels.

3.2. Enzymatic membrane reactors (EMRs)

Among enzymatic bioreactors, in recent years there has been an increasing interest in the use of enzymatic membrane reactors (EMRs) with the immobilized oxidoreductases for the biodegradation of environmental pollutants from wastewaters. The greatest advantage of EMRs is that they combine selective mass transport with simultaneous biocatalytic conversion. Thus, selective removal of products from the reaction mixture is achieved. This increases the efficiency of the process by enhancement of the conversion of product-inhibited molecules and by forcing of thermodynamically unfavourable reactions (Rasera et al., 2009). Enzymatic membrane reactors are applied at an industrial scale mainly for production and bioconversion processes (Agustian et al., 2011); however, in this review, particular attention is paid to the use of EMRs in wastewater treatment as a sustainable, eco-friendly and efficient alternative for the currently used techniques. Moreover, the recent trends towards environmentally friendly technologies make EMRs an attractive solution, because they operate under mild conditions in terms of pH, temperature and pressure, reduce diffusional limitations, and allow for easy separation of by-products. Moreover, they do not require complex equipment or chemical additives (Brindle and Stephenson, 1996). The use of immobilized oxidoreductases in EMRs provides the possibility of application of biocatalytic transformations carried out in bioreactors at a large scale (Busca et al., 2008). Additionally, the high reusability of the immobilized enzymes ensures that EMRs offer excellent operational stability, reusability and high productivity in repeated biodegradation cycles. Depending on the immobilization technique, the form of the immobilized biocatalysts and the

required purity of the products, microfiltration or ultrafiltration membranes can be used in EMRs for applications in wastewater treatments (Prazeres and Cabral, 2001). However, the most frequently used membrane configurations are: (i) flat sheet (frame and plate), (ii) hollow fibers and (iii) tubular (Galucci et al., 2011). In addition, enzymatic membrane reactors can operate in both batch and continuous modes. It should be also strongly emphasized that EMRs use membranes as both a porous separator, to selectively divide components of the reaction mixture, as well as a matrix for enzyme immobilization. This solution allows to reduce operation cost associated with the use of additional support materials and provides an opportunity to design the optimal parameters and duration of the process to achieve high efficiency and productivity (Marshall et al., 1993). Enzymatic membrane reactors with immobilized laccases, tyrosinases and other oxidoreductases "over" and "in" the membranes are nowadays more and more frequently used for the degradation of toxic compounds, mainly with regard to the increased operability of the process and purity of the effluents (Fig. 3). Different types of membranes of various origin as well as various immobilization techniques are used to produce biocatalytic systems for use in EMRs. Selected examples of the application of enzymatic membrane reactors with immobilized oxidoreductases for the bioremoval of environmental pollutants from aqueous solution were summarized in Table 5.

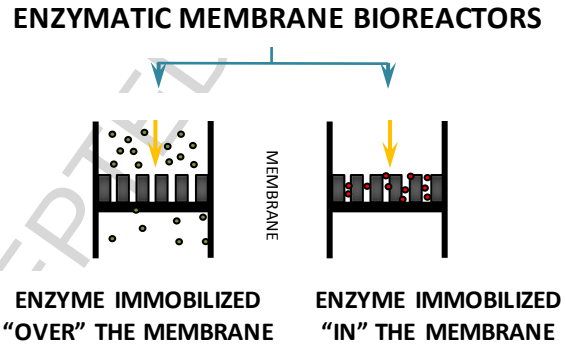


Figure 3. Schematic representation of enzymatic membrane bioreactors for environmental application. Immobilized enzyme "over" the membrane means that enzyme is immobilized on support material and placed in the reactor together with the support (membrane is not a support and acts as a separation unit), whereas in case of enzyme immobilized "in" the membrane, there is no support material, the membrane acts as a support for biomolecules and a separation unit.

Table 5. Reactor operational mode and materials of various origin used for immobilization of laccases, tyrosinases and peroxidases for application in enzymatic membrane reactors for the biodegradation of various environmental pollutants.

Reactor operational mode	Process conditions	Support material	Enzyme	Immobilization technique	Pollutants (enzyme substrate)	Removal efficiency	Reference
Continuous reactor	pH 6, 25 °C	Ceramic membrane	Laccase from *Trametes versicolor*	Covalent immobilization	Tetracycline	>75%	(Abejon et al., 2015)
Continuous reactor	pH 6, 25 °C, 24 h	Ceramic membrane	Laccase from *Trametes versicolor*	Adsorption immobilization	Bisphenol A	97%	(Arca-Ramos et al., 2015)
Continuous reactor	pH 7, 24 h	Ceramic membrane	Laccase	Covalent immobilization	Sulfadiazine, Penicillin G, Doxycycline	99% 94%, 60%,	(Becker et al., 2016)
Fully-recycling continuous reactor	pH 5.5, 22 °C, 48 h	Carbon nanotubes/ poly(vinylidene fluoride) membrane	Laccase from *Trametes versicolor*	Covalent immobilization	Bisphenol A, Carbamazepine, Diclofenac, Clofibric acid, Ibuprofen	90%, 45%, 75%, 40%, 60%	(Ji et al., 2016)
Batch reactor	pH 6, 30 °C, 2 h	Chitosan membrane	Laccase from *Pleurotus ostreatus* 1804	Covalent immobilization	Acid black 10 BX	95%	(Katuri et al., 2009)
Batch reactor	25 °C, 24 h	Gelatin-ceramic membrane	Laccase from *Trametes versicolor*	Covalent immobilization	Tetracycline	87%	(de Cazes et al., 2015)
Batch reactor	pH 7, 25 °C, 48 h	Microporous polypropylene hollow fiber membranes	Co-immobilized laccase from *Rhus vernificera* and horseradish peroxidase	Entrapment	3,4-Dimethylphenols, 4-Ethylphenol, 2-Hydroxy-1,2,3,4-tetrahydronaphthalene, 2-Hydroxy-decahydronaphthalene, 4-Hydroxy-biphenyl	80%, 56%, 87%, 34%, 85%	(Moeder et al., 2004)
Isothermal and non-isothermal continuous reactor	pH 7.5, 25 °C	Nylon membrane	Laccase from *Rhus vernicifera*	Covalent immobilization	Hydroquinone	>80%	(Durante et al., 2004)

Non-isothermal batch reactor	pH 5.5, 25 °C, 1 h	Nylon membrane	Laccase from *Trametes versicolor*	Covalent immobilization	Bisphenol A	85%	(Diano et al., 2007)
Isothermal and non-isothermal batch reactor	30 °C, 1 h	Nylon membrane	Laccase from *Trametes versicolor*	Covalent immobilization	Bisphenol A	>95%	(93) (Mita et al., 2009)
Continuous reactor	pH 5.3, 30 °C, 2 h	Polyethersulfone membrane	Laccase from *Cerrena unicolor*	Adsorption immobilization	Acid Blue 62	100%	(Lewanczuk and Bryjak, 2015)
Stirred tank continuous reactor	pH 5, 26 °C, 100 h	Polyethersulfone membrane	Laccase from *Myceliophthora thermophila*	Covalent immobilization	Estrone, 17-*β*-Estradiol, 17-*α*-Ethinylestradiol	80%, 100%, 100%	(Lloret et al., 2013)
Non-isothermal batch reactor	pH 5.5, 30 °C, 30 min	polypropylene membranes	Laccase from *Trametes versicolor*	Covalent immobilization	Phenol, 3-Methoxyphenol, 4-Acetamidophenol,	100%, 78%, 44%	(Georgieva et al., 2010)
Batch reactor	pH 4, 22 °C, 48 h	Poly(vinylidene fluoride) membrane	Laccase from *Trametes versicolor*	Adsorption immobilization	Bisphenol A	95%	(Jahangiri et al., 2014)
Batch reactor	pH 5, 25 °C, 24 h	Poly(vinylidene fluoride) microfiltration membrane	Laccase from *Trametes versicolor*	Covalent immobilization	*N',N'*-(dimethyl)-*N*-(2-hydroxyphenyl) urea	100%	(Jolivalt et al., 2000)
Fully-recycling batch reactor	pH 7, 30 °C, 3 h	NF270 polyamide membrane	Co-immobilized laccase from *Trametes versicolor* and horseradish peroxidase	Adsorption immobilization	Bisphenol A	95%	(Escalona et al., 2014)

3.2.1. Membranes properties and materials used in EMRs

As it was shown in Table 5, various materials of different origin have been used to prepare membranes with immobilized enzymes for use in enzymatic membrane reactors. The selection of membrane material and its properties, including pore size, the presence of functional groups, hydrophilicity and surface charge, have a crucial effect on the catalytic activity and stability. For instance, membranes with small pores may limit the access of the substrates to the enzymatic active sites or block changes of the laccase conformation after attachment, reducing the efficiency of the entire biocatalytic process (Butterfield et al., 2001). On the other hand, it is known that the use of a permeable membrane enables the integration of the separation process with the chemical reaction. However, from a structural point of view, each pore in the membrane may be considered as a separate microsystem. Thus, the correct deposition of enzyme molecules in a membrane plays a significant role in ensuring the capture of substrate molecules by the immobilized enzymes and increasing the contact time between the substrate and the biocatalyst (Hou et al., 2014), as high permeate flux and, in consequence, shorter contact time may be the limiting factors which significantly reduce the efficiency of the process. The selection of a membrane with properties suitable for the immobilized biomolecules and for the separation of the stream of substrates and products, and stable under operational conditions, provides the possibility of precise control of the process and can minimize losses of substrate and products. Furthermore, an appropriate selection of the membrane can lead to a faster reaction rate and higher yields. It is also possible to achieve lower operational costs and a cleaner products stream (Taboda-Puig et al., 2016).

Nevertheless, membrane fouling is one of the most critical factor influencing efficiency of the EMRs. This is due to the fact that fouling may result in a decrease of the permeate flux and water permeability as well as lead to the changes in membrane selectivity and retention due to deposition of solid molecules onto membrane surface (Guo et al., 2012). There are few main mechanisms of membrane fouling, such as pore blocking, surface adsorption, gel or cake formation as well as inorganic precipitation and biological fouling (Luo et al., 2014a). It should be clearly stated that the fouling phenomenon depends on various factors, however the most important include the type, material and properties of the membrane, process conditions, nature of the solution and interactions between membrane and a solutes (Pino et al., 2018). Enzymes are usually immobilized into pores of the membrane, thus tend to form internal fouling. However, biomolecules might also be deposited onto the surface of the membrane. In this case, fouling is usually observed due to cake layer formation. In order to counter the above-mentioned facts, various strategies have been applied to completely avoid or at least to

minimize the fouling effect. According to the previously published reports, the most promising approach consist of operating in a cross-flow filtration mode and using a properly pretreated solution, without solids (Jørgensen and Pinelo, 2017, Pino et al., 2018).

The materials used as supports (membranes) in EMRs can be classified in three main groups: (i) inorganic (mainly ceramic), (ii) metallic, and (iii) polymeric, however, biopolymeric and hybrid membranes are also used. The applied materials should offer excellent stability and good mechanical resistance to provide operational stability and repeatability for enzymatic bioreactors (Jochems et al., 2011). Also, the presence of many functional groups in the structure of the material is required for effective enzyme binding. For example, the presence of numerous hydroxyl groups on the surface of a ceramic membrane with a mean pore diameter of 1.4 μm was exploited for the covalent immobilization of laccase. The membrane was then used in a continuous enzymatic bioreactor for the degradation of tetracycline, a commonly known antibiotic, in the treatment of effluents from hospital, municipal and industrial wastewater. The results showed that the efficiency of biodegradation strongly depended on the quantity of immobilized laccase. When this quantity was appropriately selected, 75% of the tetracycline was removed under optimal conditions of pH 5 and temperature of 25 °C. This demonstrates that in order to ensure the economic and technical competitiveness of the proposed technique, all parameters must meet the process requirements (Abejon et al., 2015). However, high pore diameter, far exceeding the size of the tetracycline and enzyme molecules, is the factor which limits higher removal efficiency as some of the pollutant molecules pass through the membrane unconverted. Aside from inorganic membranes, some polymeric materials also possess features which make them a suitable barrier for both enzyme immobilization and mixture separation. The use of polyethersulfone, a hydrophilic, water-insoluble polymer with a high quantity of free sulfone groups, enables the formation of effective interactions with an immobilized enzyme. Moreover, this polymer is known for its high resistance to mineral acids, alkalis and electrolytes, at pH ranging from 2 to 13, and is commonly used as a skin layer material for various membranes. A polyethersulfone membrane was used for adsorption immobilization of laccase from *Cerrena unicolor*, and the obtained system was applied for the degradation of Acid Blue 62 dye at he temperature of 30 °C and pH equal to 5.3. After two hours of the process, total decolorization of the solution was observed. With the EMR operating in continuous mode, the immobilized laccase was used for dye removal for four days, achieving over 98% conversion of Acid Blue 62. It should also be noted that the immobilized enzyme was stable over six successive reaction cycles without additional aeration (Lewanczuk and Bryjak, 2015). Nevertheless, at

the membrane selection stage, particular care must be taken to prevent the destruction and decomposition of the immobilized enzyme, so as to maintain the high catalytic activity of the biomolecules during repeated catalytic cycles. For this reason, a microporous polypropylene hollow-fiber membrane was used for entrapment co-immobilization of laccase from *Rhus vernicifera* and horseradish peroxidase. This membrane offers low interference in the structure of the enzyme, and thus the catalytic activity is maintained at a high level. Moreover, the membrane protects the biocatalysts against the negative effects of the reaction conditions by the formation of the shell by the membrane fibers around enzyme molecules, and additionally increases the stability of the entrapped biomolecules. The system was used in a batch reactor for the remediation of selected hydroxylated aromatic compounds. It was demonstrated that after 48 h of the process, the prepared membrane can remove hazardous compounds from aqueous solution with efficiencies of over 80% (Moeder et al., 2004). The drawback of this solution is the fact, that deposition of the enzyme into the membrane fibers is associated with diffusional limitations which decrease the total removal efficiency. Moreover, the use of membranes in bioreactors has a great impact on the permeate flux. This is affected by the quantity of immobilized enzyme, the transmembrane pressure, the axial velocity and the operational mode (He et al., 2017). Usually, substrate particles accumulate on the top of the membrane, which on one hand ensures a supply of new substrate molecules to the immobilized biocatalysts, but on the other hand may create diffusional limitations and, in consequence, decrease the remediation effectivity. This was observed in case of a nylon membrane grafted with glycidyl methacrylate and phenylenediamine, with covalently immobilized laccase from *Trametes versicolor*, in a study by Diano et al. (2007). This biocatalytic system was used for the bioremediation of water polluted with bisphenol A (BPA). Under optimal conditions (pH 5.5 and temperature 25 °C), and after only one hour over 85% of the BPA was remediated. It was also established that the affinity of the immobilized laccase to the BPA molecules increased with an increase of temperature, under non-isothermal conditions. Nevertheless, the formation of a substrate layer on the membrane induced a diffusional resistance, which reduced the catalytic efficiency of the immobilized enzyme.

Simultaneous catalytic action and separation, which is a significant advantage of enzymatic membrane reactors, provides the ability to retain undesired by-products and unreacted substrates above the membrane and to obtain a stream of products characterized by high purity. This is particularly important in the case of laccase enzymes, due to the formation of products with high molecular mass (oligomers and polymers) during the catalytic

transformation of phenolic pollutants (Nazari et al., 2007). The pore size of the membrane must be selected appropriately for the used enzyme and the molecular weight of the undesired compounds. The pore size must be such as to retain the enzyme on the membrane's surface and to prevent it from passing through the membrane, so as to allow the bioreaction. Furthermore, the pores should be large enough to allow the products to pass through and to retain other ingredients of the reaction mixture. For example, commercially available nylon membrane with a pore diameter of 0.2 μm was used for covalent immobilization of laccase from *Trametes versicolor*. The bioremediation of bisphenol A was investigated under isothermal and non-isothermal conditions. After one hour of the process in non-isothermal conditions, over 95% of the BPA was degraded. It was also found that increasing the concentration of bisphenol caused a decrease in the efficiency of the membrane, due to overcrowding of the substrate molecules. Nevertheless, it was shown that the membrane was able to fully retain high-molecular-weight products of the reaction, as these compounds were not detected in the effluent (Mita et al., 2009).

Various materials of different origin have been used for fabrication of membranes which act as a support material for enzyme immobilization and a separation barrier in enzymatic membranes reactors. The production of a membrane based on the selected materials should be simple and such material should be characterized by mechanical resistance to ensure the long-term operational stability of the membrane. From this point of view, in our opinion, synthetic polymers should particularly attract growing attention as to fulfill the above-mentioned requirements and moreover offer the presence of numerous functional groups which enhance enzyme immobilization and may affect selective membrane separation. However, the main drawback of the polymeric materials as membranes is their tendency to undergo fouling, which is highly undesirable as it could affect both the catalytic conversion and flux properties. Aside from membrane materials, another important factor which has to be taken under consideration during membrane selection is its pore size, which on the one hand ensures enzyme retention but on the other hand must ensure high flux-regime to reduce operational time of the process and avoid enzyme inhibition. Membranes with different pore sizes are used as supports in EMRs, however, we strongly believe that the polymeric micro- and ultrafiltration membranes are most suitable for both enzyme immobilization and removal/separation of environmental pollutants, due to their pore size as well as mechanical and operational stability. Moreover, we would like to add that in the recently published studies there is generally little information related to the costs of the membranes for use in EMRs as they depend on membrane material, bioreactor operational mode and energy

demand. Nevertheless, based on the available data, it has been assumed that the total costs of the membrane for use in bioreactor should not exceed 30% of the total costs of bioreactor construction (Young et al., 2013, Lo et al., 2015).

3.2.2. EMRs operational modes

Apart from the type of the support material and the type of membrane used, the configuration of an enzymatic reactor also has a significant impact on its operational effectivity. There are two main types of configuration for EMRs: (i) a reactor integrated with a membrane operation unit and (ii) a reactor with a membrane used as an active catalytic and separation unit (Lopez et al., 2002). Both configurations can operate in batch and continuous mode. In the case of the reactor with a membrane operation unit, immobilized enzymes usually circulate freely in the operational volume of the bioreactor. After the biocatalytic process, immobilized biomolecules are fully retained on the retentate side following membrane filtration and are easy to reuse in the next catalytic cycle. In the case of EMRs, in which the membrane is used as a simultaneous catalytic and separation unit, enzymes are immobilized on the surface of the membrane or are loaded inside its pores. An enzymatic membrane reactor with laccase immobilized onto a ceramic membrane, operating in continuous mode, was designed by Arca-Ramos et al. (2015). The continuous removal of toxic compounds was carried out from synthetic and actual biologically treated wastewaters at pH 6 and temperature of 25 °C, for 48 h. After that time 97% and over 70% of BPA was removed from the model and actual solution, respectively. In contrast, a study by Katuri et al. (2009) present the use of a batch mode reactor with laccase from *Pleurotus ostreatus* 1804 immobilized in a chitosan membrane for decolorization of Acid Black 10 BX. The optimal process parameters for each batch of the degradation cycle were established as follows: pH 6, temperature 30 °C and 2 h process duration. Under these conditions, over 95% of the dye was removed. Moreover, it was found that the EMR with immobilized laccase operating in batch mode was characterized by a short contact time and ensured reusability of the immobilized biocatalyst for a number of cycles. It also exhibited excellent operational stability in repeated applications of the immobilized laccase and achieved high efficiency of dye removal. Although both solutions with enzyme immobilized “over” and “onto/in” membrane are interesting, in order to avoid additional expenses and to simplify the process and the equipment, we encourage to develop solutions which utilize membranes as simultaneous catalytic and separation unit. Moreover, the use of such systems allows to obtain a stream of products characterized by higher purity.

3.2.3. Immobilization techniques for EMRs

In comparison with enzymatic membrane reactors with free biomolecules, EMRs with biocatalysts immobilized in the membrane or onto its surface can significantly improve the reusability of enzymes and limit their inhibition by product molecules. The technique of immobilization, and consequently the form of the immobilized enzyme, used to produce biocatalytic systems for EMRs is determined mainly by the reactor configuration, but increasing the quantity of protein usually increases the catalytic activity until it reaches the maximum, after which the binding of additional amounts of the enzyme does not improve the catalytic properties (Rekuc et al., 2010). Enzyme molecules can be immobilized in or on the membrane *via* covalent bonds as well as by various non-covalent interactions, such as electrostatic or hydrophobic adsorption and hydrogen bonds, or by entrapment (Zhai et al., 2014). Nevertheless, enzymes for use in EMRs are immobilized mainly by covalent binding or adsorption to ensure stable binding and reusability of the biocatalytic system. For example, an efficient technology was developed based on an EMR with laccase from *Myceliophthora thermophila* covalently immobilized with the use of polyethersulfone membrane for continuous removal of estrogenic compounds from wastewaters. The immobilized enzyme enabled the effective conversion of estrone, 17-β-estradiol and 17-α-ethinylestradiol with efficiencies over 80%. Moreover, the immobilized biocatalysts exhibited improved thermal stability and excellent operational stability, allowing the enzymatic membrane reactor to operate effectively for 100 h, which confirms its high productivity and potential as an enzymatic reactor system (Lloret et al., 2013). As immobilization is carried out using covalent binding, a study associated with the optimization of the amount of immobilized biocatalysts and some surface modification of the membrane to increase enzyme-membrane distance should be performed to achieve higher removal efficiency.

However, enzyme immobilization using membranes as support materials is also a promising method for co-immobilization of biocatalysts, because it can be carried out under mild conditions in a single step and the formed interactions maintain the enzymatic activity at a high level (Luo et al., 2014b). The co-immobilization involves the integration of at least two types of enzymes immobilized on the same matrix to increase the catalytic ability of the system for the conversion of compounds (Morthensen et al., 2017). A very good example of this technique was reported by Escalona et al. (2014). Laccase from *Trametes versicolor* and horseradish peroxidase were co-immobilized by adsorption onto the surface of a polyamide NF270 commercial membrane to facilitate the removal of bisphenol A. It was found that over 95% of the BPA was removed after 3 h of the process at the temperature of 30 °C and pH 7.

The EMR coupled with an enzyme recycling system was tested and found to achieve a similar removal efficiency to that of a classic membrane reactor with co-immobilized laccase and horseradish peroxidase. The nanofiltration membrane retained the products of BPA remediation and produced an effluent of high purity. Additionally, only approx. 30% flux decay was observed. The results show that the system is very interesting from the point of view of potential large-scale applications.

Attention should also be paid to the immobilization of enzymes in membranes using a simple and effective method based on membrane fouling. The immobilization technique presented in Fig. 4 is based on adsorption and/or entrapment of the biomolecules in the pores of the membrane and/or on its surface, and is called fouling-induced enzyme immobilization (Luo et al., 2013, 2015).

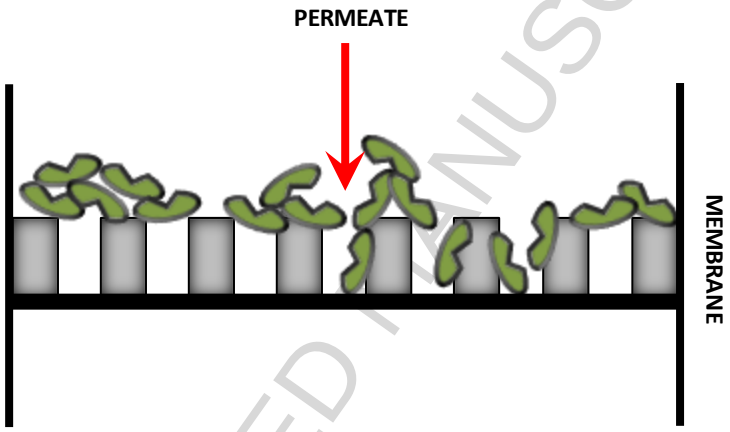


Figure 4. Graphical representation of fouling-induced enzyme immobilization.

Based on the above-mentioned examples, it can be briefly concluded, that enzymatic membranes reactors (EMRs) based on the immobilized oxidoreductases still attract increasing attention for application in conversion and removal of environmental pollutants over recent years. Pure and less toxic effluents can be obtained mainly due to the fact that high bioremoval efficiencies can be achieved under mild conditions and due to simultaneous bioconversion and separation of reaction mixture. This results from the selection of the most suitable operational mode, membrane and technique of the immobilization. As it was presented above, two operational modes are applied, however, in our opinion, a bioreactor with a membrane acting as an biocatalytic and separation unit should be of particular attention. In this solution, there is no need to use additional support material because the membrane is the matrix for the biomolecules. To ensure high operational efficiency of the EMRs, stable and mechanically resistant membranes are required. Attention should be paid to

the polymeric, commercially available membranes due to their availability and possibility of selection of membranes with desired pores size. These membranes also offer the presence of functional groups on their surface and in the pores, which enhance the formation of covalent bonds. Formation of covalent linkage prevents enzyme leakage and usually increases the reusability of the system. Nevertheless, it should be emphasized that after proper selection of operational conditions, various environmental pollutants, such as dyes, phenols, bisphenols and even estrogenes can be removed with efficiencies usually exceeding 90%. It should be added that enzymatic membranes reactors are also used in different branches of life sciences and industry, such as pharmacy, chemical synthesis or biomass conversion. This fact confirms the flexibility and significance of the EMRs-based approaches in their application at the broad industrial scale.

4. General remarks and future perspectives

Environmental pollutants, including a wide range of phenolic compounds as well as natural and synthetic dyes, are produced as waste substances by many branches of industry and consequently these compounds occur in waters and soils. Their removal at an industrial scale, in green and sustainable ways, has become an important problem over recent years, which might be solved by the use of free or immobilized biocatalysts such as laccases or tyrosinases. These enzymes are able to convert numerous pollutants of environmental concern into less toxic derivatives. To achieve high bioconversion efficiency, more advanced solutions, based on immobilized oxidoreductases, have been developed. Thus, in this review, we have presented brief information regarding simultaneous sorption and biodegradation processes as well as the use of bioreactors with immobilized enzymes for bioremediation processes. We have highlighted the advantages of these processes and established that with their use:

(i) bioconversion of environmental pollutants is carried out under mild conditions, without organic solvents, in line with the principles of green chemistry;
(ii) total conversion of hazardous pollutants into less toxic compounds can be achieved;
(iii) biodegradation can be carried out in more efficient and cost-effective ways;
(iv) one-step removal and separation of toxic compounds and their conversion products, to obtain an effluent stream of high purity.

Information concerning support materials, immobilization techniques, and the equipment and operational modes of bioreactors used for the biodegradation of toxic compounds has also been presented which allowed to indicate the most suitable solutions for achievement of high process efficiency. We have also summarized the criteria for the selection of factors which

enable the production of highly stable and highly resistant biocatalytic systems for the removal processes.

Although many methods which apply free or immobilized enzymes for the biodegradation of hazardous compounds have been reported, there is still a need to develop more advanced solutions that increase the efficiency and cost-effectiveness of the removal process. Application of enzymatic bioreactors and simultaneous separation and catalytic conversion to be a promising option for large-scale bioremediation processes under mild conditions in the future appears (meaning that the reactions can be accomplished at the natural water temperature and pH), in accordance with the rules of green chemistry. In contrast to "industrial manufacture" processes, the discussed processes are focused on the removal of undesirable compounds but not necessarily on the production of a commercial product, hence particular attention has to be paid to the operational efficiency and maximal conversion. Thus, further studies to identify the most suitable and most long-term robust carriers for oxidoreductases are required. Future development of support/sorbent materials will be focused on use of microporous nanomaterials and their modification for: (i) targeting enzyme immobilization, to retain high catalytic properties and (ii) selective sorption of pollutants, by ensuring higher affinity of the sorbent to the molecules of toxic compounds. Also hybrid/composite materials will be intensively studied in future due to the possibility of fabrication of tailored support, with desired properties for both enzyme immobilization and adsorption. This will improve the biodegradation efficiency even more and reduce the time and costs of the process. We strongly believe that further study leading to new enzymes development will be carried out, as there is still a need to look for oxidoreductases characterized by high long-term and operational stability. Moreover, the mechanism of catalytic conversion and oxygen/hydrogen peroxide supply for oxidoreductases will be investigated in order to obtain high biocatalysts efficiency. Future development of enzymatic reactors will be focused on new, stable and reusable membranes with numerous functional groups for stable binding of the biocatalysts. In our opinion, the application of the solution using membrane as a support for immobilization of oxidoreductase and, simultaneously, as separation unit will also be more common. This solution enhances the bioconversion efficiency and allows to obtain pure streams of effluents. Moreover, future investigations will focus on the implementations of the developed solutions at large-scale applications for the bioremediation of wastewaters. We hope that this review may provide certain suggestions and ideas for the design and implementation of novel, more efficient solutions for detoxification processes of actual wastewaters with the use of immobilized oxidoreductases.

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