# Free and immobilized biocatalysts for removing micropollutants from water and wastewater: Recent progress and challenges

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## Highlights

- Micropollutant degradation by oxidoreductase enzymes is reviewed
- Free and immobilized enzymes show a great potential for real wastewater treatment.
- Wastewater matrix constituents affect enzymatic biodegradation efficiency.
- Enzymatic membrane reactors facilitate operation ability, and reusability of enzymes.
- Further research is necessary to transfer enzymatic treatment into larger scale.

33 Graphical abstract


34

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**Abstract**

Enzymatic conversion of micropollutants into less-toxic derivatives is an important bioremediation strategy. This paper aims to critically review the progress in water and wastewater treatment by both free and immobilized enzymes presenting this approach as highly efficient and performed under environmentally benign and friendly conditions. The review also summarises the effects of inorganic and organic wastewater matrix constituents on enzymatic activity and degradation efficiency of micropollutants. Finally, application of enzymatic reactors facilitate continuous treatment of wastewater and obtaining of pure final effluents. Of a particular note, enzymatic treatment of micropollutants from wastewater has been mostly reported by laboratory scale studies. Thus, this review also highlights key research gaps of the existing techniques and provides future perspectives to facilitate the transfer of the lab-scale solutions to a larger scale and to improve operationability of biodegradation processes.

**Keywords:** Micropollutants; Bioremediation; Immobilized enzyme; Wastewater; Enzymatic Bioreactor.

## 1. Introduction

The presence of micropollutants in wastewater and fresh water bodies is one of the most significant environmental challenges in all countries around the world. Pollutants such as pharmaceuticals, personal care products, industrial chemicals, and pesticides are omnipresent and it is challenging to remove them with high efficiency in wastewater treatment plants (WWTPs) due to their high stability and resistance to physicochemical and biological degradation. Thus, these micropollutants could easily pass to the surface and ground water having negative impact on the environmental health and human well-being. The long-term and uncontrolled contact with micropollutants could lead to irreversible changes in the environment and ecosystems and could provoke mutations and serious disruptions in living organisms.

Therefore, there is a need to develop and test advanced techniques capable for effective removal of micropollutants from real water solutions. The above-mentioned compounds can be removed by physicochemical methods including adsorption, membrane-based processes, photocatalysis and/or chemical oxidation as well as by biological methods using plants, microbes and enzymes (Cobas et al., 2016; Chaturvedi et al., 2021). Although physicochemical methods can be effective, they are energy-intensive, expensive and can generate toxic by-products. Thus, biological methods are considered as an interesting and promising alternative for removal of wide range of micropollutants usually with high efficiencies and significantly less unintended effect on the environment (Varga et al., 2021).

Biological treatment of micropollutants can be achieved via whole organisms or their selective extract such as enzymes. Application of whole organisms or plants and microbes is usually time-consuming. Furthermore, these organisms are sensible to harsh

77 wastewater conditions and might lost their activity or can be degraded over the process
78 (Azizan et al., 2021). By contrast, enzymes offer high activity and selectivity, quick
79 catalytic action and performed well in biocatalytic conversion of water soluble
80 compounds of various origin, even presented in trace amounts. Enzymes are also less
81 prone to inhibition by substances toxic to living organisms (Feng et al., 2021). Further,
82 comparative environmental assessments show enzymatic treatment of micropollutants
83 as process that mitigate negative effect of the treatment and its final products on
84 environment. Enzymatic treatment requires also less toxic solvents, water and energy.
85 Enzyme could be applied as free (crude enzymes or purified enzymes) and as
86 immobilized biocatalysts, that affect both, bioremediation efficiency and
87 operationability and reusability of the biocatalysts (Shakerian et al., 2020). Further,
88 proper design of the enzymatic system reduce enzyme consumption during the process
89 and facilitate application of an enzymatic reactors for continuous treatment of water
90 solutions. Among six different classes of enzymes, oxidoreductases (EC1) including
91 laccases, tyrosinases and peroxidases as well as hydrolases (EC3) such as lipases are the
92 most commonly used for environmental application. Various oxidoreductases, due to
93 their wide substrate specificity, are capable for treatment of wide range of
94 micropollutants including pharmaceuticals, personal care products, dyes, pesticides,
95 hormones, phenol and its derivates, whereas lipases and other hydrolases could
96 efficiently convert oils, greases or plastics (Zdarta et al., 2018a; Morsi et al., 2020).
97 Recently, various enzymes have been reported to treat water pollutants
98 efficiently. However, most of the currently available studies, have been performed in a
99 laboratory scale or in a pilot scale reactors using model solutions of pollutants and only
100 a few studies show enzymatic treatment of real wastewater. To facilitate widespread

application of biocatalyst and to promote enzymatic treatment for removal of pollutants from water it is required to summarize and discuss available literature reports on this topic, with special attention given to oxidoreductases, as these enzymes exhibit the widest practical application. In this review, for the first time achievements on the enzymatic removal of micropollutants from real and synthetic wastewater and WWTPs effluents by free and immobilized enzymes are summarized and discussed. This paper provides also an overview on the roles and the types of enzymes for wastewater treatment. In the review influence of wastewater matrix constituent on removal efficiency and enzyme activity is systematically discussed. Further, various types of micropollutants and their effect on enzymes as well as different approaches for removal of persistent compounds are highlighted and discussed, followed by a discussion on possible future trends and studies facilitating development of enzyme-based technologies for wastewater treatment. The idea of this review article is also to point out the main challenges in the practical application of enzymatic systems for micropollutants removal to facilitate designing and development of efficient biocatalytic solutions for industrial applications. The knowledge about effect of inhibitory agents on enzyme activity and suitability of selected enzymes for treatment on various micropollutants as well as hints on construction of enzymatic membrane reactors could facilitate practical use of enzymatic treatment around the world. This knowledge is of particular interest for developing countries such as India and China, which environment is polluted by significant amounts of pesticides or dyes. Nevertheless, presented findings could be useful also for a well-developed countries in Europe and North America, where waters are polluted by pharmaceuticals and personal care products. It should be added that recently laccases and peroxidases commercially produced by

MetGen and Novozymes have been applied in WWTPs in Europe to treat textile wastewater and wastewater contains phenolic compounds.

## 2. Micropollutants of concern in wastewater

Micropollutants are omnipresent in wastewater and WWTPs effluents. A significant number of these compounds is out of control due to very limited regulations. Currently there is no legal limits for micropollutants discharge, however some regulations have been introduced. Since 2000 European Union has set up a strategy to define toxic substances and their intermediates in wastewater. In 2015 the list of priority high risk compounds, including hormones, pharmaceuticals, personal care products and food additives, to be observed and neutralized in the near future was published by The European Commission (Barbosa et al., 2016). Since that time, further work has been carried out around the world on new regulations, also concerning the effect of degradation products of pollutants. However, up to date there is still no strict regulations on limits of micropollutants discharge and their presence in WWTPs effluents (Rogowska et al., 2020).

The largest groups of hazardous micropollutants are pharmaceutically active compounds (including various anti-inflammatory substances, pain relief compounds and hormonal contraceptives) and personal care products (e.g. surfactants, antibacterial compounds). The main sources of both pharmaceutical and personal care product pollutants in the environment are domestic wastewater (Asif et al., 2018; Chen et al., 2020). Their concentrations varied from a few ng to mg per litre with potential toxicity impact on aquatic organisms at even at trace levels (Tiwari et al., 2017). For example, Zuccato et al. (2006) observed that a mixture of pharmaceuticals of ibuprofen,

149 ofloxacin, ciprofloxacin, furosemide, and sulfamethoxazole in Italian rivers, elicited a
150 negative impact on human embryonic cells (HEK293) by decrease in proliferation and
151 cell cycle progression. Balbi et al. (2018) determined the effect of diclofenac –
152 nonsteroidal anti-inflammatory drug – exposure dose on the marine bivalve (mollusc)
153 *Mytilus galloprovincialis*. It was shown that 1 µg/L of diclofenac in waters affects gene
154 transcription in the early development stages. The toxicity of other groups of
155 pharmaceuticals, e.g. antidepressants, were investigated by Minguez et al. (2014). They
156 observed that $EC_{50}$ of sertraline and clomipramine for *Daphnia magna* were 1.15 mg/L
157 and 2.74 mg/L respectively. Estrogens are another type of pharmaceutical compounds in
158 wastewater and WWTPs effluents, have been also detected in lagoon pond and sea
159 water, in concentrations exceeding the estrogenic activity threshold of 1 ng/L (Adeel et
160 al., 2017). It was shown that in fish exposed to effluents consist of estrogens, such as
161 17$\beta$-estradiol and 17$\alpha$-ethynylestradiol at concentration 11.1 ng/L and 0.5 ng/L,
162 feminine features were highly developed (Liney et al., 2005, 2006).
163 Personal care products might negatively affect the aquatic system and a range of
164 living organisms. UV filters and stabilizers, present in sunscreens, perfumes, creams, or
165 shampoos, represent such a type of personal care product chemical which could enter
166 the surface and groundwaters from WWTPs or from the sewage network in urban areas.
167 It was shown that their concentration in rivers can reach up to 60 ng/L (Jurado et al.,
168 2014). Ethylhexyl-methoxycinnamate is used as an ultraviolet light absorbant in both
169 cosmetics and personal care products such as sunscreens, and has been shown to have a
170 toxic effect on snails *Potamopyrgus antipodarum* and *Melanoides tuberculata* (Kaiser
171 et al., 2012). Other negative effects of chemicals found in personal care products have
172 been extensively reviewed elsewhere (Schneider et al., 2018),

Another major group of micropollutants is dyes and industrial chemicals from textile industry. Each year, an estimated of over 900 million tons of sewage comes from the textile industry. Textile wastewater contains dyes has strong biological toxicity and the carcinogenic, teratogenic and mutagenic properties (Iark et al., 2019; Guo et al., 2020; Varjani et al., 2020). Notably, dyes have complex chemical structure and are hardly biodegradable in conventional WWTP process. This increases their frequent occurrence in the environment. Dye compounds have potential health impact on human such as tingling and watery eyes or sniffling as well as asthma and allergic skin reactions (Lellis et al., 2019). In addition, it is worth adding that the presence of dyes causes may limit light penetration in the water, which may significantly affect photosynthesis rate of aquatic life (Liu, 2020).

Pesticides also have a significant impact on the natural environment. Annually, hundreds of tons of pesticides are used in horticulture and agriculture (Rodriguez-Liebana et al., 2014) and pollute drinking water resources (Munze et al., 2017). In addition, mixtures of pesticides present in the environment pose problems because of synergistic effect of all of the pollutants (Fernandez-Alba et al., 2001). Low pesticide concentrations might not cause any detectable health problems in humans, but might result in genetic disorders and physiological changes that ultimately affect life expectancy. Symptoms of pesticide poisoning can be divided into the muscarinic syndrome (tearing, sweating, gastrointestinal tone disturbance, nausea, vomiting, diarrhea, urinary incontinence, bronchospasm, miosis, and bradycardia) as well as the nicotine syndrome (tremors, spasms, hypertonicity, hyperreflexia, paralysis, muscle weakness, tachycardia, pallor, hyperglycemia and hypertension). In addition, pesticides can significantly affect the central nervous system, manifested by anxiety, headache,

dizziness, ataxia, sleep and memory disorders, convulsions, and also respiratory depression or coma (Bernardes et al., 2015). It should be emphasized that in groundwater at least 143 different pesticides and over 20 products resulting from their transformation have been found (Aktar et al., 2009). Studies presented by Kock-Schulmeyer et al. (2013) showed that even after wastewater treatment, pesticide concentrations are still high, up to 684 ng/L for diazinon in the WWTP effluent. The compounds that pose the greatest problems include diazinon and diuron, followed by atrazine, simazine, malathion, chlortoluron, terbuthylazine and isoproturon. Therefore, most pesticides compounds are subject to environmental quality standards and analyses.

## 3. Enzyme-mediated bioremediation process

Enzymes the most frequently applied for removal of pollutants are oxidoreductases (EC 1), including laccase (EC 1.10.3.2), tyrosinase (EC 1.10.3.1) and peroxidases (EC 1.11.1.X) such as horseradish peroxidase (EC 1.11.1.7, HRP), chloroperoxidase (EC 1.11.1.10), lignin peroxidases (EC 1.11.1.14, LiP), manganese peroxidase (EC 1.11.1.13, MnP) and/or soybean peroxidase (EC 1.11.1.7, SBP) (Ladole et al., 2020; Pereira et al., 2020). These enzymes catalyze conversion of various aromatic and non-aromatic compounds by a radical-catalyzed oxidation of phenolic moieties of organic compounds with simultaneous reduction of molecular oxygen (laccase, tyrosinase) or hydrogen peroxide (peroxidases) to water. Laccase and tyrosinase due to their low substrate specificity catalyze mainly oxidation of monophenols, diphenols and polyphenols as well as diamines and aromatic amines. However, their catalytic action differ significantly. Laccase generates a free phenoxy radicals during reaction, whereas tyrosinase generates quinones during oxidation as intermediates. Peroxidases are also

capable for conversion of wide range of compounds including various monomeric and dimeric phenols, aromatic phenols and non-phenolic compounds including dyes, pharmaceuticals and even xenobiotics also via free radicals formation as intermediates (Zdarta et al., 2018a). It should be highlighted that although intermediates formed during reaction (i.e. free phenoxy radicals) could be considered as more toxic than initial compounds, these intermediates react with each other immediately. They create various combinations of C–C and C–O bonds leading to formation of dimers, trimers and oligomers of parent compounds as a final products of the reaction (Zdarta et al., 2021). These oligomeric products are known by their significantly lower toxicity as compared to initial micropollutants. Further, they could be easily separated from the post-reaction mixture by simple precipitation and centrifugation, that facilitates obtaining of the pure stream of final solution.

The catalytic activity of these enzymes towards micropollutants depends mainly on source of their origin and extent of glycosylation. Enzymes are mainly produced from fungi, yeast and plants. Animal based enzymes has not been used for micropollutants remediation mainly due to low stability and fast denaturation in the presence of inhibiting compounds (Meng et al., 2017). The glycosylation is an enzymatic process leading to the formation of biopolymers in the enzyme biomolecules affecting enzyme structure, aminoacids arrangements and redox potential (Asif et al., 2017). Redox potential is another important factor affecting enzyme catalytic properties. High redox potential enzyme increase its ability to acquire electron during the oxidation of micropollutants. Amongst the abovementioned enzymes, lignin peroxidase has the highest redox potential of around 1.2 V and might be more frequently used in remediation processes. Recombinant and expressed enzymes have gained considerable

attention as new enzyme source for micropollutant removal. Recombinant technology became a suitable technology to produce enzymes more stable and resistant against inactivation and characterized by wider substrate specificity (Bhatt et al., 2021). Further, recombinant enzymes are capable to generate desire final products and are easier to use, however, due to genetic modification, they are also usually more expensive (Asemoloye et al., 2021). Enzymes that have been applied for bioremediation process are often used as free or immobilised on carriers (Section 3.1 and 3.2).

When considering practical application of enzymes, also economic aspects should be taken into account. The main drawback of the enzymatic treatment of pollutants are costs of the process, particularly costs of the enzyme. Nevertheless, beside costs of the biocatalyst production and purification also money input of enzyme immobilization, when implemented, has to be considered. Further, costs of biodegradation process, including rector costs, required reagents and energy consumption has to be deliberated. Finally, also money input for products separation from post-reaction mixture and cost of their utilization and detoxification should be also taken into account. It is challenging to clearly define the medium costs of the enzymatic treatment, because a numerous of variables and parameters differ from each other in the single process. Nevertheless, some studies reported on economic evaluation of enzymatic treatment of micropollutants. Lopez et al. (2016) reported that overall costs of phenol treatment by free manganese peroxidase are around 15-fold lower as compared to immobilized enzyme, mainly due to loss of enzyme activity upon immobilization. Abejon et al. (2015) presented data showing that laccase treatment of 1 $m^3$ of wastewater containing antibiotics costs around 13 Euro, that is around 20-fold

higher as compared to photo-Fenton or electron beam treatment. However sustainability of the process and low toxicity of final mixtures might overcome this disadvantage.

### 3.1. Application of free enzymes

Free enzymatic system capable for removal of pollutants can be divided into purified (isolated) enzymes and crude enzymes. Crude enzymes from white-rot fungi culture could contain three enzymes (i.e. laccase, LiP and MnP) at different concentration. Although both systems are capable for efficient transformation of hazardous compounds, each of these approaches have advantages and disadvantages. Application of crude enzymes could be cheaper than purified enzymes bypassing the purification process. Crude enzymes could degrade or convert different compounds in wastewater matrix (Lonappan et al., 2018). Crude oxidoreductase enzymes might also contain mediators in the extract, that increase the redox potential of the enzyme and expands the variety of the micropollutants to be converted (Nguyen et al., 2016a). It was reported, that the presence of mediators, such as 1-hydroxybenzotriazole (HBT), syringaldehyde (SA), or 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) might improve the biodegradation rate of laccase even up to 40%, as compared to the purified enzyme (Morsi et al., 2020). On the other hand, crude enzymes usually show lower activity as their free counterparts and might be partially inhibited by the extract constituents. Application of purified enzymes, by contrast, offers several advantages, including targeted and efficient treatment of the pollutants, lower prone to inhibition and high activity over wide range of process conditions (Robinson, 2015). Further, process control and its operation ability are also usually enhanced using purified enzymes due to the familiarity of enzyme optimal conditions, reaction mechanism and limited side reactions (Stadlmair et al., 2018). However, purified enzymes required time-consuming

292 and cost-effective methods of enzyme isolation and purification. For a real wastewater
293 treatment a compromise must be therefore found that depends on costs of the enzymes,
294 their purity and activity as well as capability to convert wide range of hazardous
295 compounds. Recently, for the removal of micropollutants from real wastewater, both
296 purified and crude enzymes were applied. Although study concerning enzymatic
297 treatment of real and/or synthetic wastewater are limited, the most commonly used are
298 oxidoreductases, among which laccases are of the highest interest. The summarized data
299 on removal of micropollutants from real and model wastewater are summarized in Fig.
300 1.

301 **[Figure 1]**

302 Lonappan et al. (2017) used 17.8 U/L of purified white rot fungi *Tremetes*
303 *versicolor* laccase for conversion of diclofenac from synthetic wastewater reaching 98%
304 process efficiency after 24 h treatment. No diclofenac nor its conversion products were
305 detected suggesting hydroxylation and final mineralization as main mechanism of
306 degradation and making this solution promising for reduction of estrogenic activity.
307 Purified laccase from *Trametes versicolor* was used to decompose of 2,4-dinitrophenol
308 with concentration of 1.5 mg/L from synthetic wastewater with 98.5% pollutant removal
309 efficiency after 4 h of the process. Note, however, that free laccase quickly lost its activity
310 in the presence of synthetic wastewater, therefore ABTS was used a mediator to improve
311 removal rate (Rahmani et al., 2020). Recently, a novel research direction was established
312 as enzymes were applied for removal of anticancer drugs. Commercially available,
313 purified laccase from *Tremetes versicolor* at enzyme dosage of 20 U/L was found to be
314 capable for complete degradation of doxorubicin at concentration up to 500 μg/L from
315 model wastewater within 6 h. It was also shown that effective degradation was supported

316 by the significantly lower toxicity of treated mixture due to formation of low-toxic
317 oligomers (Kelbert et al., 2021). Laccase capability towards pollutant removal was also
318 tested in more complex solutions. Purified *Trametes versicolor* laccase was able to
319 remove over 75% of bisphenol A, 17$\alpha$-ethinylestradiol and triclosan from tap water and
320 over 50% of these pollutants from real wastewater, proving negative effect of mainly
321 heavy metal ions on enzyme activity. It was also reported that in wastewater solution, due
322 to slight changes in enzyme microenvironment, its optimum activity was shifted toward
323 neutral pH conditions (pH 6–7) (Maryskova et al., 2021). Spina et al. (2020) used purified
324 *Tarmetes pubescens* laccase at low enzyme dosage for degradation of 15 micropollutants,
325 including pesticides, personal care products and pharmaceuticals from wastewaters
326 collected after the primary sedimentation and the final WWTP effluents, resulting in
327 complete removal of 2,4-dichlorophenol and triclosan and at least 80% removal of half
328 of the analyzed compounds. To improve decolorization efficiency of 18 azo dyes from
329 model wastewater, purified bacterial laccase from *Bacillus subtilis* was supported by the
330 azoreductase PpAzoR from *Pseudomonas putida*. Due to synergistic action of both
331 enzymes decolourization level of model wastewater reached 80% followed by around
332 50% detoxification (Mendes et al., 2011). Cruz-Morato et al. (2013), used crude *Trametes*
333 *versicolor* laccase for degradation of pharmaceuticals in non-sterile urban wastewater.
334 The removal efficiency of 14 different pharmaceuticals was followed, among which
335 ibuprofen, acetaminophen, ketoprofen, azithromycin, propranolol, 2-hydroxyCBD and
336 citalopram were degraded completely at enzyme dosage of 25 U. This study shown that
337 crude laccase is able to convert pharmaceuticals at low concentration from real
338 wastewater samples, however, bacteria, surfactants and organic and inorganic
339 contaminants presented in wastewater matrix affect removal rates of pharmaceuticals due

to inhibition of enzyme activity. Crude *Trametes versicolor* laccase was also tested in pretreatment of hospital effluents consisted of high load of pharmaceutical active compounds and endocrine disruptor compounds (EDCs). Non-sterile treatments resulted in partial or complete removal of 46 compounds followed by the significant reduction of wastewater toxicity. It was also reported that complex mixture of pharmaceuticals affect process time, required to achieve high removal of the pollutants. (Cruz-Morato et al., 2014). Another type of wastewaters, blue wastewater from aircraft, were also subjected to degradation using crude *Trametes versicolor* laccase at 18 U/L dosage. The degradation studies were preceded by optimalization of the laccase fermentation conditions, to produce high laccase activity and to overcome negative effect of wastewater matrix components on enzyme properties. Significant reduction of chemical oxygen demand and increase of biodegradability index of the treated wastewater indicate effective degradation of recalcitrant pollutants and prove that high laccase activity is required to attain high remediation rate of real wastewater (Atilano-Camino et al., 2020). As mentioned earlier, also source of the enzyme might affect its ability to convert micropollutants. Becker et al. (2017) showed that laccase from *Myceliophthora thermophila* is for about 10% more efficient than laccase from *Trametes versicolor* in removing of mixture of hormones and endocrine disrupting compounds and its estrogenic activity from wastewaters even at low enzyme dosage. These differences are probably related to the aminoacids orientation in the active sites of both enzyme that slightly differ from each other. It was also reported that recombinant enzymes could be considered as efficient in removal of micropollutants. Recombinant laccase from *Cerrena* sp. HYB07 was purified and used for decolourization of real textile effluents showing over 60% of process efficiency after 6 days (Yang et al., 2014). Recombinant laccase form *Moniliophthora roreri* showed higher biodegradation

potential towards 17-estradiol, estriol, 17-ethinylestradiol and bisphenol A over wider pH range (from 5 to 7), as compared to free laccase from *Trametes versicolor* (Bronikowski et al., 2017). In addition, recombinant *Agaricus bisporus* tyrosinase in *Saccharomyces cerevisiae* cells showed high activity and great potential towards micropollutants removal indicating also that gene cloning and enzyme recombination provides good potential for protein engineering and functional studies of oxidoreductase enzymes (Lezzi et al., 2012).

Wide range of hydrogen peroxide-dependent peroxidases was found to efficiently convert organic micropollutants. Although most of the studies on this issue were performed using model water solution, some studies report conversion of pharmaceuticals and estrogens from real wastewater matrix using peroxidases of various origin. Auriol et al. (2007; 2008) reported complete degradation of estrone, 17*β*-estradiol and estriol from synthetic wastewater and municipal wastewater within 1 h using horseradish peroxidase. A low enzyme dosage (0.032 U/mL) was required to treat synthetic wastewater. However an enzyme dose of around 10 U/mL was needed to remove pollutants from wastewater due to inhibition effect of real wastewater matrix compounds. In contrast to $O_2$ dependent laccase, HRP is $H_2O_2$ dependent enzyme, therefore proper hydrogen peroxide supply to the enzyme is also of key importance to achieve high removal rate. Na and Lee (2017) determined 20 μM of $H_2O_2$ as optimal concertation leading to elimination of over 90% of 17*β*-estradiol and bisphenol A and 100% of acetaminophen form WWTP effluents at an HRP dosage of 48 U/L. It was also observed that removal of compounds without phenolic moieties in the structure did not exceed 5% indication HRP substrate specificity. A promising and cheaper (Svetozarevic et al., 2021) alternative for horseradish peroxidase seems to be a familiar to HRP enzyme called soybean peroxidase. Crude SBP was capable for over 95% removal of

388 triclosan, nitrophenol and steroidal hormones from municipal wastewater at low enzyme
389 dosage (0.12 U/mL) and at optimized hydrogen peroxide concentration of 80 μM.
390 Further, a matrix effect on enzyme activity was observed in case of real wastewater
391 treatment indicating that use of higher enzyme dosage was required to achieve high
392 removal efficiency (Mashhadi et al., 2019). Also other peroxidases should be
393 considered as available and easy obtainable alternatives for laccase and horseradish
394 peroxidase. Purified chloroperoxidase extracted from *Caldarimyces fumago* at enzyme
395 dosage of 5 U/L showed its robustness as in less than 10 min, diclofenac and naproxen
396 where completely degraded from real wastewater leading to the formation of low-toxic
397 oligomers as the final reaction products (Li et al., 2017). The wide substrate specificity
398 of the peroxidases should also be underlined. Purified peroxidase from *Aspergillus*
399 *oryzae* removed efficiently phenol from industrial wastewater samples within 16 h. It
400 was found however, that in the presence of 2% of formaldehyde almost no phenol
401 removal was observed indicating inhibition of catalytic action of peroxidase (Sukan and
402 Sargin, 2013). Also plant peroxidases are useful for removal of micropollutants.
403 Peroxidase from *Luffa acutangular* showed over 75% removal of azo dye Methyl
404 Orange from liquid effluents under optimized process condition (40 min, pH 3, 30 °C)
405 using 2 mM hydrogen peroxide. Peroxidases extracted from agricultural and bio-wastes
406 demonstrated nice performance in micropollutants removal, however, the removal rate
407 is strongly dependent on pH, temperature, process duration as well as enzyme dosage
408 (Chiong et al., 2016).

409 **3.2. Application of immobilized enzymes**

410 Immobilized enzyme aim to overcome two limitations of the free enzyme approach: i)
411 reusability and ii) stability (Sheldon et al., 2021). Immobilized enzymes could retain

their catalytic properties for long period of time. The supportive structure and protective effect of immobilized materials helps to maintain enzyme properties over a wider range of pH and temperatures conditions (Olcucu et al., 2021). The immobilized enzyme also showed resilient to inhibition effects from different components in real wastewater. Immobilized enzymes facilitate also process operationability and purity of final products (Lassouane et al., 2019). Enzyme reusability and prolongation of enzyme lite-time reduces the operation cost (Basso and Serban, 2019). Type of enzyme, support material and type of immobilization affect final properties of the immobilized biocatalysts. Adsorption, covalent binding, encapsulation and cross-linking are the most frequently used techniques for oxidoreductases immobilization (Aricov et al.,2020; Zhang et al., 2020; Aggarwal et al., 2021), whereas inorganic and hybrid materials were found to be the most suitable support for environmental application (Zdarta et al., 2018a). For the effective wastewater treatment it is essential to produce highly stable systems, resistant to harsh process conditions and inhibition with high activity retention. Thus careful selection of support material and immobilization approach is required. Note also, that proper support selection, for instance use of highly porous material with well-developed surface area, might also facilitate removal of pollutants due simultaneous adsorption and biocatalytic conversion (Zdarta et al., 2019a). Nguyen et al. (2016b) noticed over 90% removal efficiency of bisphenol A, diclofenac, carbamazepine and sulfamethoxazole form wastewaters using laccase immobilized onto granular activated carbon due to biocatalytic conversion supported by adsorption. The percentage of the adsorption in total removal efficiency varied among compounds, however, adsorption was more pronounced (over 50%) in terms of carbamazepine and diclofenac, that are resistant to enzymatic treatment. Naghdi et al. (2017) used 25 mg of

laccase immobilized on carboxyl functionalized nanobiochars for carbamazepine degradation in spiked water and secondary effluent. Around 85% of pollutant degradation was achieved with the contribution of biodegradation higher than 45% and adsorption lower than 35%. A summary of removal efficiency of micropollutants by immobilized enzymes is given in Fig. 2.

**[Figure 2]**

Immobilization approach and type of support materials affect both, enzyme activity and stability of the biosystems. Application of functional materials with desired properties improves also operationability of the immobilized enzymes. For instance magnetic particles, facilitate separation of the immobilized enzyme by external magnetic field (Sadeghzadeh et al., 2020). Functionalized silica magnetic nanoparticles were used as support for deposition of laccase cross-linked enzyme aggregates for removal of pollutants from wastewater. Produced systems containing around 150 mg/g of immobilized laccase allowed removal of around 40% of mefenamic acid and fenofibrate and around 20% of indomethacin and acetaminophen from spiked real secondary effluent (Arca-Ramos et al., 2016), over 90% of mefenamic acid, acetaminophen, diclofenac, carbamazepine and atenodol from municipal and hospital wastewaters (Kumar and Cabana, 2016) and 93% of diclofenac from synthetic wastewater (Primozic et al., 2020) indicating great practical possibility of these systems. In these studies also high reusability potential was highlighted due to possibility of quick biocatalyst separation. To reduce process costs and to reduce diffusional limitations, also cross-linked enzyme aggregates without support material might be applied. Cross-linked enzyme aggregates of laccase from *Trametes versicolor* showed over 85% activity retention and exhibited elimination rate of 90% for carbamazepine

after 72 h treatment and 99% for acetaminophen and mefenamic acid after 24 h treatment from filtered wastewater (Ba et al., 2014).

For the real-life and long-term application of the immobilized enzymes in the wastewater treatment it is essential to create stable enzyme-support bonds and reduce enzyme elution. Thus, covalent immobilization is the most commonly applied using wide range of inorganic and organic supports, usually modified by hydroxyl, amine or carbonyl groups (Zdarta et al., 2018b). Amine functionalized fumed silica nanoparticles witch covalently immobilized laccase of a *Thielavia* genus achieved over 60% of bisphenol A removal from tertiary wastewater treatment. Due to high operational stability, this system shows great scaling-up potential with suggested application in continuous membrane reactor (Gassser et al., 2014). Further, laccase from *Trametes versicolor* immobilized on glycidyl methacrylate modified polyacrylamide-alginate cryogel was used for decolorization of dyes and dephenolization of olive mill wastewater reaching over 80% of Congo Red, Chlorazol Black and Trypan Blue. By contrast, only around 30% decolorization efficiency of Fast Green FCF and Sunset Yellow FCF due to more complicated structure and higher resistance to enzymatic treatment (Yavasser and Karagozler, 2021). Complete decolorization efficiency of batik wastewater was noticed by Yanto et al. (2021) within 1 h, when 1 g of laccase from *Trametes hirsuta* EDN 082 immobilized on light expanded clay aggregate was added to 100 mL of the wastewater solution. Retention of over 95% decolorization efficiency after 4 repeated cycles was also observed. It should be underlined that in these studies limited enzyme elution from the matrix over storage time and repeated use was observed due to stable, covalent binding of the enzyme to the support. Although a numerous of materials were tested for oxidoreductase covalent immobilization, there is

484 a growing need to develop and produce even more advanced tailor-made materials with
485 desired and suitable properties for micropollutants removal. For instance, high stability,
486 mechanical resistance, the presence of numerous of functional groups as well as
487 properties improving direct transfer of the electron to immobilized enzyme are the most
488 important (Alvarado-Ramirez et al., 2021). In this context, poly(lactic-co-glycolic acid)
489 electrospun nanofiber served as support for laccase from *Pleurotus florida*. Support
490 material showed high sorption capacity and allow retention of 83% of activity by
491 laccase. Obtained systems containing over 180 mg of laccase per 1 g of support was
492 also capable for complete removal of diclofenac (Sathishkumar et al., 2012). In another
493 study polyvinyl alcohol/chitosan nanofibrous membrane was enriched with multi-
494 walled carbon nanotubes that facilitate enzyme-substrate electron transfer, to
495 immobilize commercially available laccase (Xu et al., 2015). Both systems showed
496 100% process efficiency in removal of diclofenac. Note however, that this research still
497 need to be developed to facilitate large-scale application as tests were performed in a
498 batch scale using model wastewater mimicking real wastewater.
499 Enzymes might be also co-immobilized to create multienzymatic systems. Due
500 to the use of biocatalysts from various sources, their different substrate specificity and
501 simultaneous action, it is possible to create systems with expanded spectrum of
502 micropollutant removal. Laccase and tyrosinase were co-immobilized using chitosan in
503 the form of combined crosslinked enzyme aggregates and exhibited removal of over
504 95% and 90% of acetaminophen from municipal wastewater and hospital wastewater,
505 respectively (Ba et al., 2014). High redox potential *Trametes versicolor* laccase and a
506 low redox, but low-cost *Myceliophthora thermophila* laccase were co-immobilized
507 using fumed silica nanoparticles. Due to simultaneous action of both enzymes removal

of bisphenol A reached over 99% from secondary effluent from a municipal WWTP. Diclofenac, however, was converted with significantly lower rate (around 40%) indicating resistance of this compound toward enzymatic oxidation under realistic conditions (Arca-Ramos et al., 2016). Redox mediators improve reaction efficiency by facilitation of the electron transfer. A novel approach consisting of co-immobilization of laccase and mediator (ABTS) onto amino-functionalized ionic liquid-modified magnetic chitosan nanoparticles allow removal of over 80% of indole and anthracene and complete removal of bisphenol A and 2,4-dichlorophenol. Further, it was observed that in the presence of co-immobilized mediator, process was twofold more rapid as compared to single immobilized laccase (Qiu et al., 2021).

In order to retain high catalytic activity in environmental processes, adsorption immobilization is applied, where negative effect of immobilization on enzyme structure is limited. Over 200 mg of laccase from *Pleurotus florida* NCIM 1243 was immobilized by adsorption onto 1 g of cellulose nanofibers and was capable for removal of over 90% of Remazol Brilliant Blue R and around 50% of Remazol Black 5 from simulated dyes effluents. Degradation of other dyes was below 20% due to interfering effect of the simulated effluents matrix (Sathishkumar et al., 2014). In another study, laccase adsorbed onto polyacrylonitrile-biochar composite nanofibrous membrane showed over 60% of chlortetracycline removal efficiency from simulated wastewater. In this case, application of mediators might facilitate achievement of higher removal rate (Taheran et al., 2017).

Beside laccases, also immobilized peroxidases are used for removal of micropollutants, mainly due to broader pH and temperature range of these enzymes (Melo et al., 2016). Wang et al. (2016) noticed complete removal of phenol at 1 mM

concentration from synthetic wastewater within 3 h by 50 U/g of horseradish peroxidase immobilized by covalent binding onto acrylonitrile beds presenting also significant reduction of post reaction mixture. Sun et al. (2017) cross-linked laccase aggregates onto $ZnO/SiO_2$ composite support reaching over 80% of Acid Blue 113 and Acid Black 10BX dyes removal. Further, due to additional cross-linking by diethylene glycol diglycidyl ether, immobilized HRP retained over 80% of its activity after 10 repeated uses. Manganese peroxidase at enzyme dosage of around 10 mg, immobilized onto chitosan beads allowed over 70% decolorization of textile industry effluents. Further over 50% reduction of total organic carbon and chemical oxygen demand in the treated effluents was attained. In all these cases however, high removal rate was achieved at relatively high and optimized hydrogen peroxide dosage, underlying strong effect of this co-substrate on reaction efficiency (Bilal et al., 2016). Still growing amounts of micropollutants forces developments on novel, highly effective enzymes capable for treatment of phenolic compounds. Recently, Ariaeenejad et al. (2021) showed complete removal of Methylene Blue dye from wastewater using PersiManXyn1 model enzyme immobilized onto magnetic graphene oxide. Limited negative effect of salts and heavy metal ions on activity of immobilized was also noticed. Further, process might be easily boosted by the addition of $NaBH_4$.

## 4. Impacts of wastewater constitutions on enzymes

Wastewater constitutions include heavy metal ions, salts, organic solvents, organic acids, surfactants, and natural organic matter (NOM) have significant impact on both free and immobilized enzymes (Deska and Kończak 2019; Bilal et al., 2021). They inhibit or improve enzymatic activity and thus, influence micropollutant removal. The

degree of inhibition/activation varies significant upon the wastewater constitution concentrations. In this section, current knowledge on the effect of wastewater matrix constituents on enzyme activity and the removal rate of pollutants are summarized. However, as literature data on real wastewater matrix effect are limited, the discussion delas also with literature reports concerning the use of simulated and/or model wastewaters, for a better understanding of the presented issues. General summary of the effect of various wastewater constituents on removal rate of pollutants is presented in Table 1.

**[Table 1]**

### 4.1. Effect of cations and anions

Results have showed that cations at below 5 μg/L have no or minimal impact on the catalytic activity of enzyme in the bioremediation of micropollutants (Asif et al., 2017). However, the impact increased significantly at high cation levels. Enzyme laccases are sensitive to the presence of cations in wastewater. Cations such as $Hg^{2+}$, $Fe^{2+}$, $Pb^{2+}$ might bind to the Type II and Type III laccase copper sites leading to enzyme inhibition. Further, cations could block the internal laccase electron transfer required to complete oxidation process (Kumar and Srikumar et al., 2012). Yang et al. (2020) reported the suppression of the laccase catalyzed removal of tetrabromo and bisphenol A due to the presence of $Cd^{2+}$, $Mn^{2+}$, and $Co^{2+}$ in model wastewater and its binding to enzyme active sites. By contrast, addition of $Na^{+}$ or $K^{+}$ did not change removal significantly, whereas in the presence of high concentration of $Ca^{2+}$ and $Mg^{2+}$ (about 1–1.5 mM) decrease in reaction rate was noticed. Yadav et al. (2021) observed about 50% increase in the presence of $Co^{2+}$ and about 30% in the presence of $Cu^{2+}$ and $Mn^{2+}$ ions in total removal rate of phenol, 4-chlorophenol and 4-fluorophenol catalyzed by around 25

mg of laccase immobilized using magnetic nanoparticles. Without the presence of copper and cobalt ions only small degradation of substituted phenol occurred. Positive effect of $Cu^{2+}$ and $Ca^{2+}$ ions on removal of triclosan from wastewater due to the promotion of laccase activity by these ions was presented by Sun et al. (2017). By contrast, significant reduction of pharmaceutical removal efficiency was observed in this study in the presence of 10 mM $Al^{3+}$ and $Fe^{2+}$ ions due to the binding of these ions to the active site of the enzyme and blocking of the electron transfer. The thermostable/halotolerant metagenome-derived laccase (PersiLac2) assisted conversion of industrial dyes was boosted of around 50% in the presence of $Cu^{2+}$, $Mg^{2+}$, and around 20% in the presence $K^{+}$ and $Ni^{2+}$ in the model wastewater. It was also stated that in the presence of these ions over 50% decolorization of real textile effluents occurred, whereas without metal ions less than 20% decolorization was noticed (Motamedi et al., 2021). However, increased ionic strength of the solution due to the presence of $Fe^{3+}$ and $Cr^{3+}$ ions increased slightly removal efficiency of phenol from water and wastewater (Wagner and Nicell, 2002). Positive effect of $Cu^{2+}$ and $Fe^{3+}$ ions on activity of HRP immobilized onto magnetic polymethyl methacrylate fibers was also observed by Abdulaal et al. (2020). In addition, the presence of anions such as $Cl^{-}$, $F^{-}$, $SO_4^{2-}$ or $NO_3^{-}$, due to competitive and non-competitive inhibition of anions with enzyme substrate, might reduce removal rate of micropollutants from wastewater. Chlorine ions at concentration below 10 mM has minimal impact of laccase activity. This impact did not cause any reduction in the removal of bisphenol A, diclofenac, and mefenamic. When concentration of $Cl^{-}$ increase to 100 mM, over 30% drop in removal rate of all compounds was observed due to enzyme inhibition (Margot et al., 2013). The strongest inhibitory effect on laccase activity show however hydroxyl anions that binds close to

604 the TI and TII copper ion in laccase active site leading reduction of electron transfer
605 ratio and to enzyme inhibition (Zdarta et al., 2019b). This effect is clearly observed at
606 solutions at higher pH, in which laccase lost its catalytic properties rapidly.

607 **4.2. Effect of organic solvents and organic acids**

608 Organic solvents such as ethanol, methanol, dimethyl sulfoxide or acetone as well as
609 organic acids including formic acid, acetic acid, oxalic acid and citric acid are another
610 group of compounds interfering enzyme assisted micropollutants treatment. These
611 compounds compete with reaction substrate for enzyme active site leading to
612 competitive inhibition (Ademankinwa et al., 2016). Further, solvents and acids might
613 also change the pH of the solution leading to structural aminoacids rearrangements and
614 decreasing enzyme activity (Arregui et al., 2019). Nevertheless, an inhibition effect of
615 organic solvents and acids sternly depends on their concentration. A reduction in
616 removal rate of laccase assisted remediation of triclosan of around 10% in the presence
617 of acetonitrile and of around 5% in the presence of acetone and ethanol was noticed,
618 indicating low negative effect of these solvents (Ramirez-Cavazos et al., 2012). Hou et
619 al. (2014) used 180 mg of laccase immobilized covalently onto 1 g of $TiO_2$
620 nanoparticles and observed only 5% decrease of bisphenol A degradation from synthetic
621 wastewater was observed in the presence of 10% (w/w) of methanol and acetone. These
622 indicates protection effect of support materials and high stability of the immobilized
623 enzymes in the presence of organic solvents. By contrast, Nelson and Anne (2021)
624 observed significant reduction of activity of laccase from *Aureobasidium pullulan* in the
625 presence of 10% acetonitrile (80%) and 10% acetone (50%) due to penetration of
626 enzyme active sites. The reduction of laccase activity results also in lower removal rate
627 of bisphenol. Modification of laccase amino acids residues in the presence of oxalic acid

and citric acid lead to over 90% and 70% enzyme inhibition. Nevertheless, removal of over 70% of Congo Red, Methyl Green and Crystal Violet was attained in the presence of these organic acids, however, extension of the process duration was required to reach high removal values (Kumar et al., 2012). Si et al. (2013) observed no negative effect of ethylenediaminetetraacetic acid (EDTA) in the concentration up to 0.1 mM on the 30 U/L dosage of purified laccase activity in removal of dyes mixture leading to 80% Congo red and 75% of Neutral Red removal. Increase of EDTA concentration up o 1 mM results in only 5% decrease of catalytic activity.

### 4.3. Effect natural organic matter

Natural organic matter and dissolved organic matter are a heterogeneous mixture of high to low molecular weight compounds including humic substances, carbohydrates, lignins, lipids, organic acids and other biological derived products generated at various stages of wastewater treatment (Xiao et al., 2020). It was reported that natural organic matter and dissolved organic matter account for 80% of chemical oxygen demand and around 85% of total organic carbon of the WWTPs effluents (Wang and Chen, 2018). Due to the great variety of various compounds in natural organic matter and dissolved organic matter their effect on enzymatic treatment of micropollutants can be multidimensional. For instance, natural organic matter only slightly inhibit (less than 5%) laccase assisted transformation of 17$\beta$-estradiol. Dimers and oligomers of parent compound were detected as main conversion products indicating that natural organic matter has no effect on final process products (Xia et al., 2014). A significant decrease of triclosan conversion rate in laccase assisted transformation was observed in the presence of natural organic matter. Due to the limitation of self-coupling process of triclosan free radicals, the estrogen-humic acid cross-coupling compounds were

652 determined as the main products (Sun et al., 2016). Around 15% lower conversion of
653 17$\alpha$-ethinylestradiol from wastewater by free horseradish peroxidase at enzyme dosage
654 of 7 U/L was observed in the presence of natural organic matter. Interestingly different
655 mechanism of enzyme inactivation was observed. Low-weight natural organic matter
656 act as a competitive substrates for HRP leading to its inhibition. Whereas high-weight
657 natural organic matter compounds adsorbed 7$\alpha$-ethinylestradiol molecules reducing
658 their availability for laccase (Yang et al., 2018). However, organic matter might consist
659 also natural redox mediators such as syringic acid, syringaldehyde, vanillic acid, or *p*-
660 coumaric acid that boost laccase activity. Shi et al. (2014) observed over 95%
661 conversion rate of laccase catalyzed removal of sulfadiazine, sulfamethazine and
662 sulfamethoxazole in the presence of syringaldehyde, syringic acid and acetosyringone,
663 in less than 10 min at enzyme dosage of 6.7 U/L. The presence of methoxy groups in
664 the mediators structure, which decrease redox potential and increase local density of
665 electrons at phenoxy groups facilitates laccase oxidation of pollutants. Recently, a
666 positive effect of humic acid that is a component of the natural organic matter, on
667 removal rate of micropollutants has also been reported. At low horseradish peroxidase
668 concentration (up to 50 U/L) humic acid slightly facilitate removal of bisphenol A
669 (removal rate of around 90%) from real wastewater due to mitigation of enzyme
670 inactivation. At enzyme concentration exceeding 100 U/L, increase in amount of humic
671 acid provided transformation rate of bisphenol A to 82% (Jiang et al., 2017). By
672 contrast, Sun et al. (2016) noticed about 10% lower removal rate of laccase conversion
673 of 17$\beta$-estradiol in the presence of humic acid. The presence of humic acid affect also
674 mechanism of catalytic conversion of estrogen due to the inhibition of the extent of 17$\beta$-
675 estradiol self-coupling.

676 **4.4. Effect of surface active agents**

677 Surface active agents is a diverse group of compounds with cleaning properties that are
678 also capable to improve compounds solubilization. The levels of surfactants in
679 wastewater vary significantly among type of the pollutant and source of the effluent
680 reaching usually between 0.1 and 1000 μg/L (Berge et al., 2018). Surfactants might be
681 divided into two groups including non-ionic surfactants such as Triton X-100 and ionic
682 surfactants such as sodium dodecyl sulphate or hexadecyltrimethylammonium bromide.
683 The type of the surfactant affect type of its interaction with enzyme. Non-ionic
684 surfactants have less pronounced effect on enzyme structure as only hydrophobic
685 interactions occurred. By contrast, ionic surfactants might interact with charged enzyme
686 molecules leading to more significant enzyme distortions (Azimi et al., 2016). Liu et al.,
687 (2018) reported slight decrease of the indole removal from model wastewater in the
688 presence of Triton X-100 due to changes in the enzyme conformational and affected
689 laccase-phenol interactions. Alshabib and Onaizi (2020) observed around 10% increase
690 of bisphenol A removal efficiency in the presence of 1 ppm Triton X-100 in laccase
691 assisted wastewater treatment at enzyme dosage of 43.6 U/L. Further, in the presence of
692 1 ppm sodium dodecylbenzenesulfonate or hexadecyltrimethylammonium bromide
693 around 10% lower removal rate was observed, indicating negative effect of ionic
694 surfactants on enzyme activity. However, increase of around 15% of bisphenol A
695 removal in laccase treatment of wastewater was observed in the presence of rhamnolipid
696 biosurfactant (Onaizi, 2021). In a continuous study, it was reported that 1 ppm
697 rhamnolipid concentration positively affect also reaction kinetic and increase removal
698 rate even in high salinity level (Onaizi and Alshabib, 2021). Merpol, a non-ionic
699 surfactant presented in textile effluents show no negative effect on the structure of

laccase from *Trametes versicolor* itself. However increase of Merpol concentration, decrease reaction rate and removal efficiency of Reactive Blue 19 form model textile effluents due to enzyme inhibition (Champagne et al., 2012). Summarizing, both denaturation and activation effect of surfactants in wastewater matrix on micropollutants removal was noticed.

## 5. Enzymatic reactor design and scale-up potential

Enzymatic membrane reactor (EMR) utilised membranes which have pore size smaller than the molecular weight of an enzyme to prevent enzyme washout from a continuous operation. In the EMR, enzyme molecules may be freely circulating on the retentate side or immobilized onto the membrane surface or inside its porous structure. There are three common EMR processes in the literature (Fig. 3).

**[Figure 3]**

Laboratory scale EMR has provided promising results (Table 2). Mendoza et al. (2011) investigated the removal of azo dyes applying a laccase/mediator system in an EMR. They used a polysulfonate ultrafiltration (UF) membrane (MWCO: 10 kDa) allowing a complete retention of enzyme within the reactor. Decolourization yields of 52 to 95% depending on the dye type were reported (Mendoza et al. 2011). Chhabra et al. (2009) demonstrated over 95% decolourization of Acid Violet by an EMR. Katuri et al. (2009) explored three different EMR configurations: direct contact EMR (i.e., laccase in suspension), laccase impregnated EMR (i.e., laccase enclosed in a dialysis bag housing a 12 kDa membrane, which prevented laccase diffusion into the buffer solution and allowed dye diffusion into the laccase solution for reaction) and immobilized EMR (laccase immobilized on poly-vinylidene fluoride and chitosan

membranes) for the removal of Acid azo dye. The decolourization efficiency of each reactor configuration depends on enzyme stability and mass transfer limitations between enzyme and substrate. Among the different EMR configurations, immobilization of laccase on poly-vinylidene fluoride and chitosan membranes showed better decolourization efficiency of up to 8 and 21 batches, respectively. This was presumably due to a combination of improved enzyme stability and mass transfer. Lloret et al. (2012b) investigated the removal of two estrogens (i.e., estriol and 17$\beta$-estradiol) by a commercial laccase from *Myceliophthora thermophila* in an EMR equipped with UF membranes with a nominal pore size of 10 kDa. Removal efficiencies between 64–100% were obtained under an enzymatic activity of 500 U/L. The EMR allowed the reuse of the enzyme providing an important cost reduction. Nicolucci et al. (2011) immobilized laccase on polyacrylonitrile beads and submerged the beads in a fluidized bed reactor for the removal of bisphenols (i.e., bisphenol A, bisphenol B and bisphenol F). Complete removal (effluent concentration below detection limit) of all bisphenols was obtained on that study. In a more recent study, Nguyen et al. (2014) used a commercial laccase in a direct contact EMR for the removal of diclofenac and bisphenol A. A stable removal of bisphenol A (>85%) and diclofenac (>65%) was achieved in that study. Asif et al. (2020) utilised high retention nanofiltration membrane for enzyme retention and degradation of 29 emerging trace organic compounds. The NF-EBR could provide higher removal of 19 compounds compared to the ultrafiltration-EMR.

**[Table 2]**

Although the application of membrane help to retain enzyme within the reactor, enzyme denaturation due to various physical and chemical factors during continuous operation necessitates enzyme replenishment. The enzymatic reduction could accelerate

748 in the presence of wastewater constitutions (Section 4). Lopez et al. (2004) reported a
749 27% reduction in enzymatic activity from a single addition after 8 h. Drop in enzymatic
750 activity during EMR operation was also observed by Hata et al. (2010). The authors
751 reported a rapid decrease (70%) in enzymatic activity during the first 4 h of operation.
752 However, a strategy involving addition of laccase and a mediator every 8 h enhanced
753 the performance of carbamazepine removal. Lloret et al. (2012a), however, reported
754 stable enzymatic activity over 10 h of operation of an EMR. Nguyen et al. (2014)
755 observed a complete denaturation of enzymatic activity after 72 h of operation of an
756 EMR with a single addition of enzyme. The authors proposed a strategy to maintain
757 enzymatic activity by addition of 200 μL of the commercial laccase solution per L of
758 the reactor volume every 12 h (equivalent to a laccase dose of 23 mg/L·d).
759 Apart from enzyme reinjection, different methods have been reported to minimize the
760 loss of enzyme during operation of an EMR. For example, ethylenediaminetetraacetic
761 acid and polyethylene glycol, which are believed to possess a protecting role for
762 proteins, especially under oxidative stress, may be added to an EMR (Mendoza et al.
763 2011). The obtained results demonstrated the need for high improvements in the amount
764 of enzyme grafted on the membranes or on enzymatic kinetics to afford the technical
765 and economic competitiveness of the investigated designs and the possibility to be
766 implemented within existing installations (Abejón et al. 2015).
767
768 **6. Challenges and Future Perspectives**
769 This review provides comprehensive understanding of the micropollutants removal by
770 free and immobilized enzymes. However, the data from literature was mainly obtained
771 from studies using a synthetic wastewater (i.e. micropollutants in ultrapure water).

772 Before the implementation of free or immobilized enzyme for wastewater treatment
773 processes, performance assessment using real wastewater would be required. Impact of
774 wastewater constitutions on enzyme stability and performance highlighted the challenge
775 in its application in practice. Biodegradation of enzyme by bacteria in the wastewater
776 may be significant and has also not been studied in the literature. Given the impact of
777 wastewater constitutions and enzyme degradation, enzyme treatment of micropollutants
778 should be applied at tertiary stage or as polishing steps in the wastewater treatment
779 process. On the other hand, preliminary wastewater treatment to remove inhibitors and
780 further application of enzymatic treatment before wastewater enter to WWTP might
781 also results in high biodegradation rate of micropollutants.

782 Immobilization of enzymes on carries appears to be a potential method to retain
783 and increase enzyme stability compared to free enzyme applications. Nevertheless,
784 immobilized enzymes also accompany with enzyme activity loss. The inclusion of
785 enzyme immobilization materials and process would be minimal to overall cost.
786 Advanced materials such as graphene oxide and metal organic framework composites
787 have showed high efficiency on enzyme binding and stability compared to traditional
788 carbon-based materials. Immobilization provides also protection of enzyme structure
789 against inactivation and facilitate enzyme reusability that reduce the overall process
790 costs. Finally, oriented enzyme immobilization could significantly reduce the inhibitory
791 effect of wastewater matrix compounds improving enzyme activity at real process
792 conditions.

793 Enzymatic membrane reactors are promising to improve removal efficiency.
794 EMRs enhance process operationability and facilitate continuous wastewater treatment
795 reducing time and costs of the process. Further, EMRs equipped with membranes with

immobilized enzymes, beside efficient biodegradation of micropollutants, allows also simultaneous membrane separation of process products to obtain final stream at high purity and low toxicity.

Enzyme based wastewater treatment has gain traction with some commercially available enzymes. However, its cost is still a high margin, hinder their industry uptake. Future studies should include life-cycle assessment and techno-economic assessment to comprehensively reveal the enzyme based wastewater treatment feasibility.

## 7. Conclusions

Enzymatic treatment based on free and immobilized biocatalysts is a useful technique for the biodegradation of micropollutants including pharmaceuticals, steroid hormones, personal care products, pesticides, and industrial dyes. Enzymatic processes can be achieved under benign and environmentally friendly conditions. The effectiveness for removing of micropollutants from industrial and municipal wastewater has been demonstrated in numerous laboratory scale studies. Despite large scale applications in other fields, the deployment of enzymatic biodegradation for wastewater treatment is still limited. Further research is necessary to improve enzyme catalytic properties, develop multi-enzymatic systems, and integrate enzymatic treatment with other technologies for comprehensive wastewater treatment.

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**Figure 1**

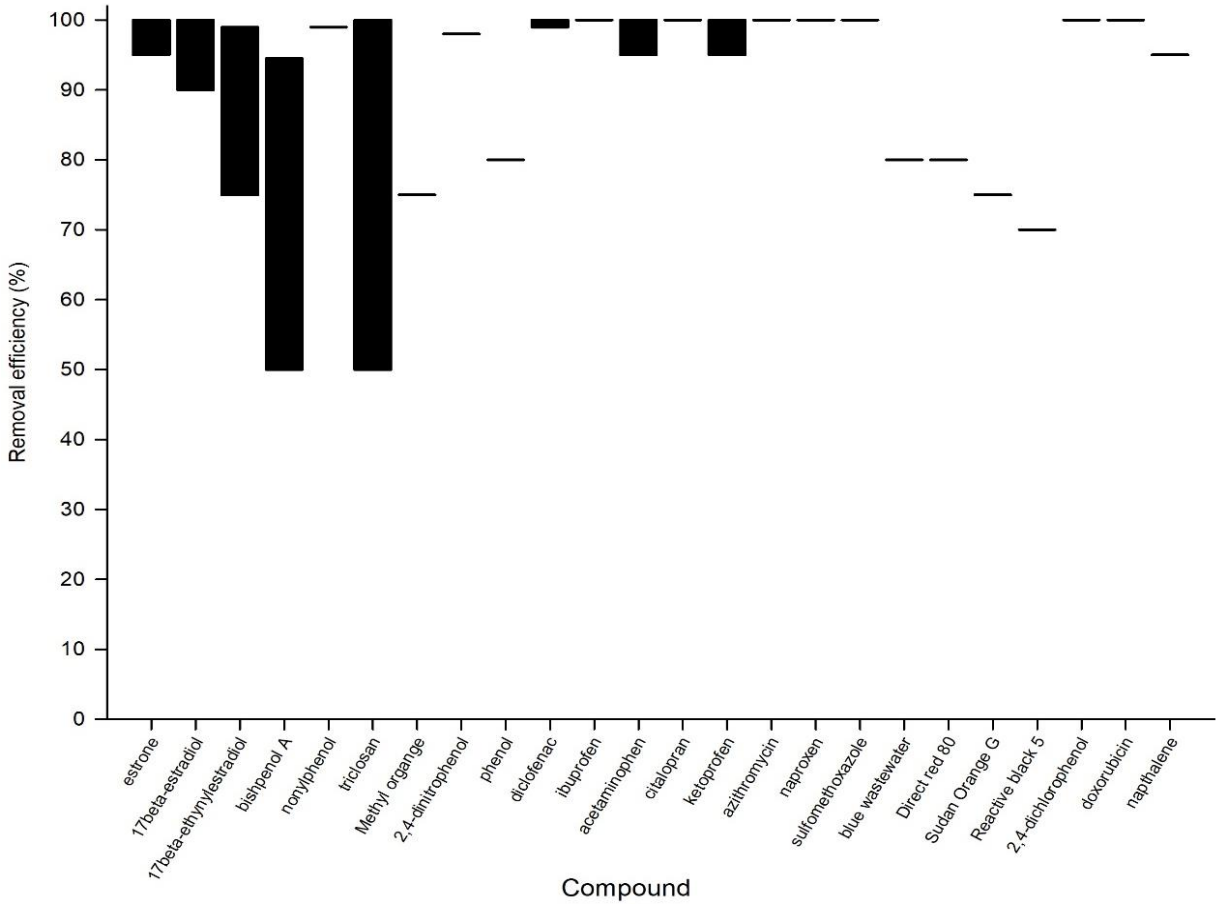


**Figure 1.** Variations in the removal efficiency of micropollutants catalyzed by free oxidoreductases. Based on data presented in Section 3.1.

**Figure 2**

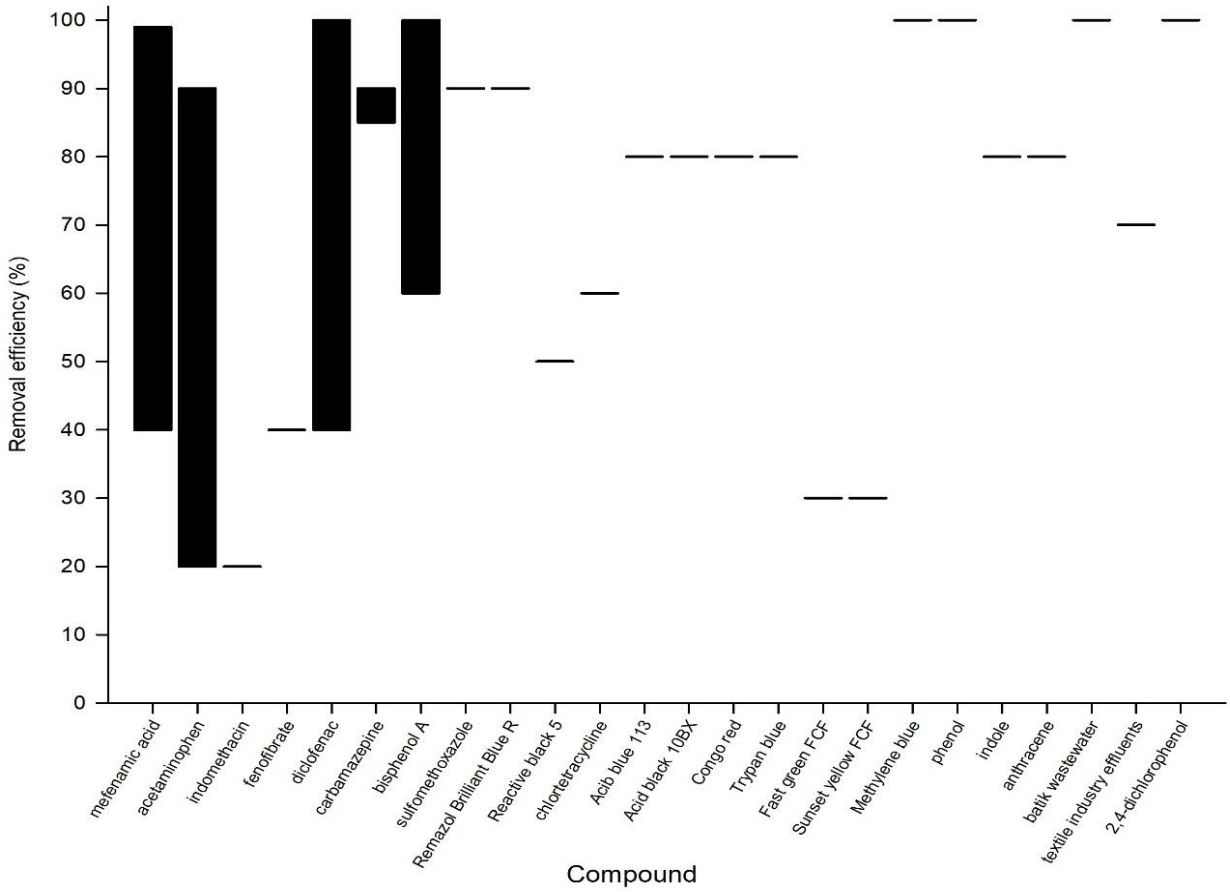


**Figure 2.** Variations in the removal efficiency of micropollutants catalyzed by immobilized oxidoreductases. Based on data presented in Section 3.2.

**Figure 3**

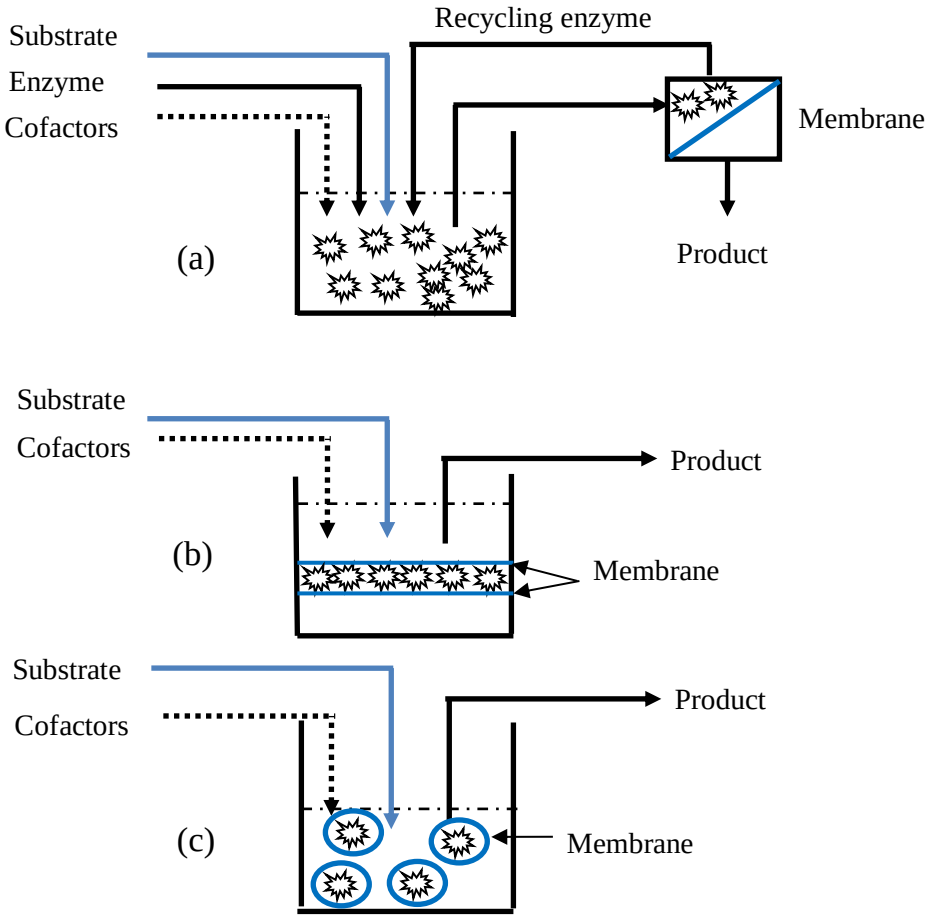


**Figure 3.** Schematic diagram of three different configurations of EMRs. (a) enzyme in suspension in a reactor coupled with membrane units, (b) enzyme immobilized within the membrane matrix itself and, (c) enzyme entrapped in gels or microcapsules.

**Table 1**

**Table 1.** The effect of water constituents of removal efficiency of micropollutants by free or immobilized peroxidases. The removal efficiency values are presented

| Wastewater constituents | | Enzyme | Form | Micropollutant | Removal efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| cations and ions | $Cd^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ca^{2+}$, $Mg^{2+}$ **inhibitory effect** | laccase | free | tetrabromobisphenol A | from 15% to 40% | (Yang et al., 2020) |
| | $Co^{2+}$, $Cu^{2+}$, $Mn^{2+}$ **activation effect** | laccase | immobilized on magnetic silica nanoparticles | phenol, 4-chlorophenol, 4-fluorophenol | 80% 70% 68% | (Yadav et al., 2021) |
| | $Cu^{2+}$, $Ca^{2+}$, $Al^{3+}$, $Fe^{2+}$ **inhibitory effect** | laccase | free | triclosan | 95% 40% | (Sun et al., 2017) |
| | $Cu^{2+}$, $Mg^{2+}$, $K^{+}$, $Ni^{2+}$ **activation effect** | laccase | free | industrial dyes in textile effluents | 50% | (Motamedi et al., 2021) |
| | $Fe^{3+}$, $Cr^{3+}$ activation effect | horseradish peroxidase | free | phenol | over 95% | (Wagner and Nicell, 2002) |
| | $Cu^{2+}$, $Fe^{3+}$ **activation effect** | horseradish peroxidase | immobilized onto magnetic polymethyl methacrylate fibers | phenol | 55% | (Abdulaal et al., 2020) |
| | $Cl^{-}$ **inhibitory effect** | laccase | free | bisphenol A, diclofenac mefenamic acid | from 60% to 65% | (Margot et al., 2013) |
| organic solvents and acids | methanol, acetone **inhibitory effect** | laccase | immobilized onto $TiO_2$ nanoparticles | bisphenol A | 90% | (Hou et al., 2014) |
| | acetonitrile, acetone **inhibitory effect** | laccase | free | bisphenol A | 15% 40% | (Nelson and Anne, 2021) |
| | oxalic acid, citric acid **inhibitory effect** | laccase | free | Congo Red, Methyl Green, Crystal Violet | over 70% | (Kumar et al., 2012) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | ethylenediamine-tetraacetic acid (EDTA)<br>**neutral effect** | laccase | free | Congo Red,<br>Neutral Red | 80%<br>75% | (Si et al., 2013) |
| **natural organic matter** | natural organic matter<br>**inhibitory effect** | laccase | free | 17$\beta$-estradiol | 94% | (Xia et al., 2014) |
| | natural organic matter<br>**inhibitory effect** | laccase | free | triclosan | 60% | (Sun et al., 2016) |
| | natural organic matter<br>**inhibitory effect** | horseradish peroxidase | free | 17$\alpha$-ethinylestradiol | 30% | (Yang et al., 2018) |
| | syringaldehyde, syringic acid, acetosyringone<br>(natural organic matter mediators)<br>**activation effect** | laccase | immobilized onto magnetic particles | sulfadiazine, sulfamethazine, sulfamethoxazole | over 95% | (Shi et al., 2014) |
| | humic acid<br>**inhibitory effect** | laccase | free | 17$\beta$-estradiol | 85% | (Sun et al., 2016) |
| | humic acid<br>**activation effect** | horseradish peroxidase | free | bisphenol A | 82% | (Jiang et al., 2017) |
| **surface active agents** | Triton X-100<br>**inhibitory effect** | laccase | free | indole | 80% | (Azimi et al., 2016) |
| | Triton X-100<br>**activation effect** | laccase | free | bisphenol A | 58% | (Alshabib and Onaizi (2020)) |
| | sodium dodecylbenzene-sulfonate, hexadecyl-trimethyl-ammonium bromide<br>**inhibitory effect** | | | | around 40% | |
| | rhamnolipid<br>**activation effect** | laccase | free | bisphenol A | 65% | (Onaizi et al., 2021 |
| | rhamnolipid<br>**activation effect** | laccase | free | bisphenol A | 60% | Onaizi and Alshabib, 2021 |
| | Merpol<br>**neutral effect** | laccase | free | Reactive Blue 19 | 85% | (Champagne et al., 2012) |

**Table 2**

**Table 2.** Summary of recent studies on EMR for removal of micropollutants.

| Membrane material (MWCO, kDa) | Enzyme (activity) | Compound (concentration, mg/L) | HRT (h) | Reactor volume (mL) | Reference |
|---|---|---|---|---|---|
| UF polyethersulfone membrane (10) | laccase (500 and 1000 U/L) | estriol and 17$\beta$-estradiol (4) | 1–4 | 250 | (Lloret et al. 2012a) |
| UF polyethersulfone membrane (10) | laccase (500 U/L) | estriol and 17$\beta$-estradiol (4) | 2–4 | 250 | (Lloret et al. 2012b) |
| UF polyacrylonitrile membrane (6) | laccase (70-100 µM/min) | diclofenac and bisphenol A (0.5) | 8 | 1500 | (Nguyen et al. 2014) |
| UF polyethersulfone membrane (10) | manganese peroxidase (7000 U/L) | Orange II (100) | 0.3, 0.75, 1 and 1.5 | 250 | (Lopez et al. 2004) |
| UF polyacrylonitrile membrane (20) | laccase (100 U/L) | Acid Violet 17 (76) | 4, 6, 8, 10, and 16 | 250 | (Chhabra et al. 2009) |
| UF polyethersulfone membrane (10) | purified laccase (8750 U/L) | Acid azo (20) | 3 | 70 | (Katuri et al. 2009) |
| UF polyacrylonitrile membrane | laccase (180 µM/min) | 29 emerging trace organic compounds | 24 | 3000 | (Asif et al. 2020) |
| NF membrane | laccase (180 µM/min) | 29 emerging trace organic compounds | 24 | 3000 | (Asif et al. 2020) |
| UF regenerated cellulose membrane (100) | phosphotriesterase (6.4 µM/min•mg) | paraoxon-ethyl (1mM) | >1 | 50 | (Vitola et al., 2021) |
| polyvinylidene fluoride/carboxylated multi-walled carbon nanotubes (-) | laccase (4.46 U/cm$^2$) | diclofenac and carbamazepine (5 ppm) | 48 | 50 | (Masjoudi et al., 2021) |
| NF polyethersulfone membrane (75) | laccase (0.59 U/ cm$^2$) | bisphenol A (34.2) | 7 | 5000 | (Li et al., 2020) |
| NF polysulfone membrane (0.2–0.3) | laccase (0.53 U/mg) | bisphenol A (10) | 36 | 30 | (Zhang et al., 2021) |
| Porous zeolite-like geopolymer membrane (-) | laccase (100 U/mg) | Crystal Violet (5) | 10 | 100 | (Zhang et al., 2020) |