A contemporary review of enzymatic applications in the remediation of emerging estrogenic compounds

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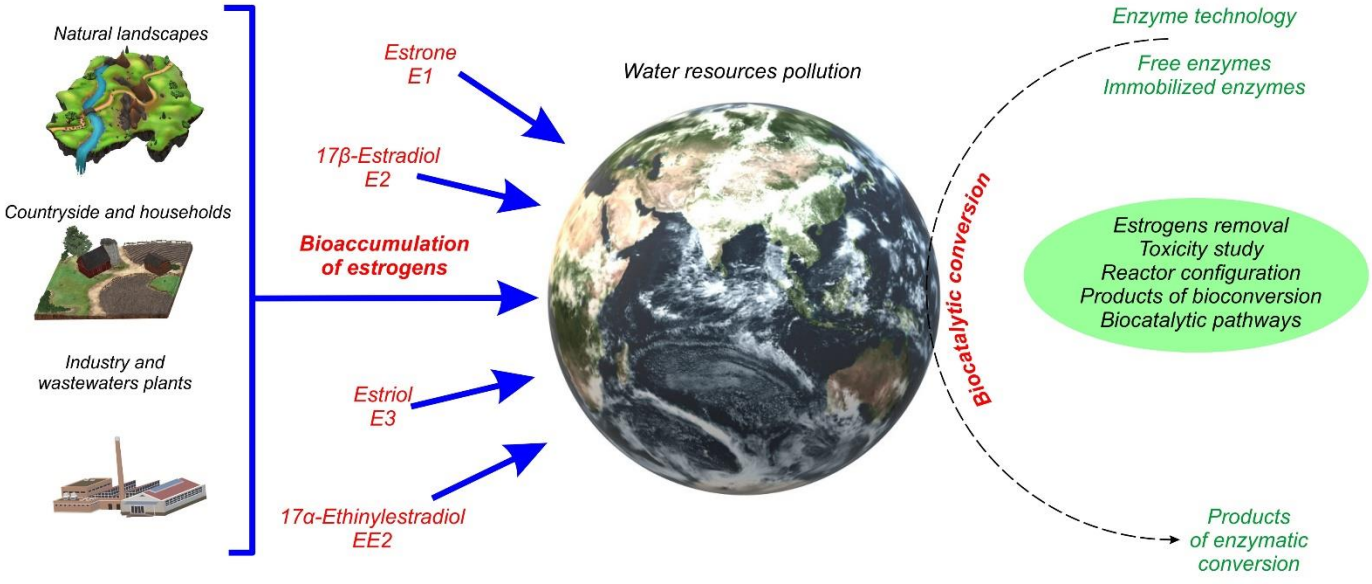
Abstract

The occurrence of emerging contaminants, such as estrogens, in secondary and tertiary treated effluents and in sewage-impacted water bodies is one of the major obstacles to the implementation of water reuse. This review critically evaluates the performance of emerging process of enzymatic degradation of estrogens, and its efficiency. The data collected from peer-review literature show that enzymes have been extensively applied (in both free and immobilized form) in estrogen removal. Amongst others, the use of laccase as a catalyst provides over 90% removal of estrogens. Immobilized enzymes can overcome some limitations of the free biocatalysts, including reusability. Research evidence points to the formation of by-products, such as dimers and trimers. Nevertheless, estrogenic activity assessment indicates a reduction in toxicity after enzyme treatment. The cost and stability of enzymes, as well as their performance in a real wastewater matrix, are the major obstacles to the implementation of enzymatic processes in wastewater treatment. Continued endeavors are required to enhance the successful application of enzymes in the wastewater treatment industry.

Keywords: Estrogens; Enzymatic conversion; Enzymatic membrane reactor; Catalytic by-products; Toxicity

Graphical abstract



Keywords: Estrogens; Enzymatic conversion; Catalytic by-products; Toxicity

Highlights

- Processes of enzyme-supported conversion of estrogens are reviewed and discussed
- Laccase is the most commonly applied enzyme and achieves over 90% estrogen removal
- Immobilization is suggested as an effective tool for enhancement of estrogen removal
- Dimers and trimers have been identified as main bioconversion products of estrogens
- Existing research gaps are highlighted and future recommendations are provided

Abbreviations list:

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

BSTR – batch stirred tank reactor

CLEAs – cross-linked enzyme aggregates

DyP – dye decolorizing peroxidases

E1 – estrone

E2 – 17β-estradiol

E3 – estriol

EA – estrogenic activity

ECs – emerging contaminants

EE2 – 17α-ethinylestradiol

EMR – enzymatic membrane reactors

HA – humic acid

HBT – 1-hydroxy-benzotriazole

HRP – horseradish peroxidase

LiP – lignin peroxidase

LYES – yeast estrogen screen-assay assisted by enzymatic digestion with lyticase

MELN – luciferase-transfected human breast cancer cell line gene reporter

MnP – manganese peroxidase

NOM – natural organic matter

PBR – packed bed reactor

SA – syringaldehyde

UPOs – peroxygenases

VA – veratryl alcohol

VP – versatile peroxidase

YES – yeast estrogen screen

1. Introduction

Water reclamation refers to the treatment of used water, or wastewater, to make it suitable for either potable (e.g. drinking) or non-potable (e.g. irrigation, agricultural applications and toilet flushing) uses. Water reclamation provides an alternative source of water that affords an additional level of certainty and security of water supplies in the face of a changing climate. In recent years, there has been an upward trajectory in both technological development and full-scale implementation of water reclamation. Although water reclamation has been operated recently (e.g. NEWater in Singapore), there are several barriers to the acceptance of water reclamation, including capital and operational costs, the presence of emerging contaminants (ECs), as well as public attitudes. Research efforts to reduce the cost, treat and remove ECs, and improve public awareness are ongoing.

One group of ECs of particular concern is the estrogenic hormones, which include estrone, estriol, 17β-estradiol, and 17α-ethinylestradiol (Bilal et al., 2020a). The occurrence of these compounds in the environment is beginning to receive considerable attention from the scientific community and from public health and ecological conservation authorities. The concerns relate mainly to endocrine disruption and negative impact on the reproductive and sexual functions of wildlife, fish and humans (Hamid and Eskicioglu, 2012; Sutherland and Ralph, 2019; Tran et al., 2018). Estrogens have been detected in both influent and effluent of wastewater treatment plants and in the receiving environment at concentrations from a few to several hundred ng/L (Havens et al., 2020; Tran et al., 2018). The concentration of estrogens may exceed the predicted no-effect concentration threshold of some aquatic organisms. The European Community document COM(2011)876 suggested environmental quality standards for 17α-ethinylestradiol and 17β-estradiol at 0.035 and 0.4 ng/L respectively (Johnson et al., 2013). Currently, there are no statutory requirements for wastewater and water reclamation plants to monitor the concentration of estrogens in the water, and in most instances, it is not routinely

monitored. However, with the increased use of water reclamation and improved understanding of the impacts of estrogens in reclaimed water, technologies for the treatment or removal of estrogens will need to be developed.

Enzymatic degradation of estrogens may be considered a promising method compared with conventional physical and chemical oxidation process (Sami et al., 2020; Bilal et al., 2019a). Enzymes such as laccase, horseradish peroxidase, manganese peroxidase, and lignin peroxidase can oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism. For example, the oxidoreductase laccase is a multi-copper protein, which is able to oxidize the phenolic moiety of organic compounds by reducing molecular oxygen to water (Claus, 2004; Nguyen et al., 2016). On the other hand, horseradish peroxidase (HRP, EC 1.11.1.7) as well manganese, and lignin peroxidases utilize peroxide as an electron acceptor. These enzymes originate from fungi (e.g. white-rot fungi, brown-rot fungi) and plants (e.g. roots of radish). White-rot fungi produce the extracellular lignin-modifying enzymes laccase (EC 1.10.3.2), lignin peroxidase (LiP, EC 1.11.1.14), and manganese peroxidase (MnP, EC 1.11.1.13) to oxidize lignin—the structural polymer found in woody plants. In addition, other oxidoreductases/peroxidase such as versatile peroxidases (EC 1.11.1.16), dye decolorizing peroxidases (DyP, EC 1.11.1.19) and peroxygenases (UPOs, EC 1.11.2.1) might be found in fungi. These enzymes have the potential to be useful biocatalysts due to their broad substrate coverage (Asif et al., 2020; Harms et al., 2011; Marco-Urrea et al., 2010; Nguyen et al., 2016). In addition, enzymatic processes do not produce a large amount of chemical sludge, which is a significant attribute of chemical treatment processes (Claus, 2004).

The use of enzymes for the removal of estrogenic compounds has been on an upward trajectory, with efforts made to enhance these techniques in real-world applications. For example, the capacity of laccase to remove estrogens has been demonstrated in laboratory-scale studies (Lloret et al., 2012a; Nguyen et al., 2015). However, enzymatic denaturation and

enzyme washout with the treated effluent are critical problems encountered in continuous systems such as wastewater treatment plants. Enzymatic immobilization and the use of enzymatic membrane bioreactors (with a filtration membrane that can retain enzyme in the reactor) have emerged as potential solutions. The enzymatic membrane reactor offers several advantages, such as its ability to retain enzymes, operate with free enzymes, and allow the periodic replenishment of enzymes (Modin et al., 2019).

This paper aims to provide a systematic perspective on applications of enzymes in the treatment of estrogenic compounds. The applications of enzymes in free suspension (i.e. in both batch and enzymatic membrane bioreactors) and in immobilized form are reviewed. The efficiency of removal of estrogens by enzymatic treatment is assessed in terms of i) the disappearance of the original compounds, ii) the formation of by-products, and iii) reduction of estrogenic activity.

2. Removal of estrogenic compounds by free enzymes

The biological approach for removal of pollutants from water and wastewater concerns use of microorganisms or extracted enzymes. Selected enzymes are capable of effective oxidation of a broad range of organic compounds including estrogenic compounds, from water and wastewater (Martínez et al., 2017; Mate and Alcalde, 2017). The removal of estrogenic compounds by enzymes has attracted particular interest. This is due to i) their potential impact on the reproductive system of aquatic organisms, ii) their low level of removal in the conventional wastewater treatment process, and iii) the presence of a phenolic moiety in their chemical structures (Auriol et al., 2008; Ba and Kumar, 2017; Bilal et al., 2019b, 2019c; Mukherjee et al., 2013). Enzymes include free laccases, horseradish peroxidase, lignin peroxidase and manganese peroxidase, and their immobilized counterparts that have been used to remove estrogens including estrone (E1), 17β-estradiol (E2), estriol (E3) and 17α-

ethinylestradiol (EE2). Tests have been carried out mainly on real and model wastewater solutions, under different operational conditions (pH, temperature, enzyme dose, level of estrogens, reaction time) (Table 1).

Table 1

2.1 Estrogenic compounds removal by laccase

Laccases have been the most widely investigated enzymes for use in the bioconversion of estrogens (Table 1). This is mainly due to their substrate specificity (laccase has high affinity to phenolic compounds) and availability (they are produced by a number of fungi and yeast) (Auriol et al., 2008). For instance, Xia et al. (2014) investigated the removal of 17β-estradiol in catalyzed oxidative coupling processes supported by laccase from *Trametes versicolor*. The efficiency of removal of 17β-estradiol was up to 100% at pH between 4 and 6. However, in the presence of natural organic matter (NOM), a slight decrease in the removal of 17β-estradiol was observed. Auriol et al. (2008) also observed the total removal of 17β-estradiol, estriol, and 17α-ethinylestradiol, and of their estrogenic activity, from municipal wastewater after 8 h of a process at a temperature of 25 °C and at pH 7 using laccase from *Trametes versicolor*.

Free laccase has been applied for the removal of estrogenic compounds in continuous systems such as enzymatic membrane bioreactors. The free enzyme is retained by the membrane, which has a pore size smaller than the size of the enzyme. This configuration prevents the enzyme loss in the treated water. Lloret et al. (2012a) achieved a continuous removal of estrogens (>90%) when applied laccase from *Myceliophthora thermophila* in a stirred tank reactor coupled with an ultrafiltration membrane. The utilized ultrafiltration membrane could retain laccase for continuous operation. There is some variation in the reported removal rates. These variations are mainly due to differences in laccase origin, enzyme dosage

and process duration, as well as the different pH and temperature optima and different redox potential of laccases from different species.

2.2 Estrogenic compounds removal by horseradish peroxidase

HRP is a typical enzyme of plant origin (found in the roots of horseradish), which has been used as an efficient catalyst for the conversion of estrogenic compounds. HRP is a heme-containing enzyme which catalyzes the oxidation of aromatic phenols and non-aromatic amines in the presence of hydrogen peroxide which is reduced to water during the reaction (Bilal et al., 2018a). Auriol et al. (2007a) used HRP to treat a mixture of estrone, 17β-estradiol, estriol and 17α-ethinylestradiol in synthetic wastewater. An initial HRP activity of 0.02 U/mL was sufficient to completely remove 17α-ethinylestradiol, but greater HRP doses, up to 0.06 U/mL, were required for efficient removal of estrone, 17β-estradiol, and estriol. Since HRP requires H_2O_2 to perform the catalytic function, the ratio of H_2O_2 to substrate (e.g. estrogens) is a factor that influences the overall removal rate. They observed that the optimal molar peroxide-to-substrate ratio, to achieve the highest removal rate of all studied estrogens, was 0.45.

There are reports on the use of HRP for the removal of estrogens from real wastewater solutions. The removal efficiency was between 95 and 100% for estrone, 17β-estradiol and estriol and 17α-ethinylestradiol estrogens with the process conditions of pH 7 and temperature 25 °C, and enzyme activity of 8 U/mL (Auriol et al., 2006). Organic compounds, surfactants and metal ions could inhibit the catalytic action of HRP, requiring a higher dose. Thus, enzymatic estrogen removal is often carried out as a finishing step in wastewater treatment, when other constituents have been removed.

Different types of HRPs have been used for the removal of estrogens. Rathner et al. (2017) used two different HRP isoenzyme variants—of herbal origin and expressed by recombinant yeasts—for the removal of 17α-ethinylestradiol from differently polluted wastewater bodies. It

was found that both isoenzymes are capable of efficient treatment of 17α-ethinylestradiol. The plant-origin enzyme could completely remove estrogens under conditions of pH 4.5, ambient temperature, and 24 h. The presence of organic compounds and availability of H_2O_2 significantly decreased the removal rate of 17α-ethinylestradiol. Huang et al. (2013) observed that natural organic matter reduced by 20% the rate of removal of 17β-estradiol. Nevertheless, the described solution should be considered highly efficient, because over 80% of the natural estrogen was removed from the simulated wastewater solution. The foregoing examples indicate HRP as an efficient biocatalyst for estrogen treatment. Nevertheless, the requirements for a continuous supply of H_2O_2 and pre-treatment of NOM before HRP application are the main drawbacks of the application of this enzyme.

2.3 Estrogenic compounds removal by lignin and manganese peroxidase

Beside laccase and HRP, also manganese peroxidase and lignin peroxidase might be used for conversion of estrogens, however, available literature reports are very limited. Manganese peroxidase oxidizes a wide spectrum of different mono- and dimeric phenols, and even dyes. Meanwhile, lignin peroxidase catalyzes the oxidation of a wide range of aromatic phenolic and non-phenolic compounds and even xenobiotics (Bilal et al., 2019c). However, LiP and MnP require H_2O_2 as a co-substrate and other cofactors in the reaction that limits their applications in comparison to laccase, which uses free oxygen as an electron acceptor. Further, the drawbacks of peroxidases include also their poor stability and low operationability. Nevertheless, LiP and MnP have been investigated in the removal of estrogens. Tamagawa et al. (2006) reported a complete removal of estrone by MnP from the white-rot fungus *Phanerochaete sordida* YK-624.C after 1 h reaction at pH 4.5, and room temperature. Unlike MnP, LiP was not effective to remove performance estrogens. Wang et al. (2012) observed 25%, 38%, and 45% removal of estrone, 17β-estradiol and 17α-ethinylestradiol, respectively

by LiP. The lower removal rates of estrone and 17*β*-estradiol as compared to 17*α*-ethinylestradiol are related to the origin of the estrogens and their chemical structure. Whereas the low biodegradation of 17*α*-ethinylestradiol may be explained by the lower substrate affinity of LiP towards 17*α*-ethinylestradiol.

2.4 Application of free enzymes in a continuous reactor

Various reactor setups have been developed for the continuous application of enzymes for estrogenic compound removal in laboratory-scale studies. For example, Tanaka et al. (2001) used laccase from *Trametes* sp. in a rotating reactor for the removal of 17*α*-ethinylestradiol. Total removal of 17*α*-ethinylestradiol was achieved in a continuous rotating reactor (pH 5, 24 h, mixing at 10 rpm). Thus, enzymatic treatment supported by continuous stirring facilitates the rapid remediation of ECs by improving enzyme–substrate contact. In another study, laccase from *Myceliophthora thermophila* was placed in an enzymatic stirred tank reactor for continuous treatment of a mixture of estrone, 17*β*-estradiol, and 17*α*-ethinylestradiol (Fig. 1). After 100 h of the process, over 90% of each of the pollutants was converted to less toxic products. Furthermore, the presented continuous flow bioreactor demonstrated enhanced effectivity, even at lower residence times, compared with other bioreactors. This is mainly due to rapid mass transfer and large surface-to-volume ratio, as well as preventing enzyme elution and its inactivation (Lloret et al., 2013a). These studies represent important steps towards the implementation of an enzymatic reactor for the continuous removal of estrogens.

Figure 1

3. Removal of estrogenic compounds by immobilized enzymes

3.1 Enzyme immobilization techniques

Numerous techniques have been developed to improve the stability, efficiency and reusability of enzymes. Enzyme immobilization has been established as the most promising and practically important to improve enzyme stability, efficiency, and reusability (Ba and Kumar, 2017). It is also considered as the most effective technique for practical application of immobilized biocatalysts. Enzyme immobilization techniques are principally based on the attachment of biomolecules, by way of adsorption and/or covalent interactions, to insoluble support materials. However, methods of enzyme immobilization by entrapment and encapsulation into the matrix structure have been also developed (Bilal et al., 2018b, Zdarta et al., 2018a, 2018b). One of the greatest advantages of the enzyme immobilization is stabilization of the biocatalysts structure that prevents enzyme against inactivation at harsh reaction conditions (Jesionowski et al., 2014). Immobilization may also reduce enzyme inactivation due to denaturation and inhibition, leading to the longer retention of high catalytic activity. Further, separation of the immobilized enzyme from the reaction mixture and retention in continuous processes are also improved (Arca-Ramos et al., 2016). Finally, the use of immobilized enzymes expands the range of possible bioreactor configurations for highly efficient biocatalytic processes (Mateo et al., 2007). The described advantages of immobilization enhance the utility and reusability of immobilized enzymes in the removal of estrogens.

Enzymes such as laccase, tyrosinase, HRP, soybean peroxidase and MnP have been immobilized using numerous types of support materials (Ba et al., 2013; Bilal and Iqbal, 2019a; Bilal et al., 2018c, 2019d; Rodrigues et al., 2013; Zdarta et al., 2018c, 2020a, 2020b). A suitable support material should be characterized by a well-developed porous structure, high operational stability and mechanical resistance. It should also provide protection of the biomolecules against inactivation under process conditions (Bilal and Iqbal, 2019b; Sheldon and van Pelt,

2013). Furthermore, the presence of numerous functional groups exhibiting affinity to the enzyme facilitates the formation of stable enzyme–support interactions. A variety of materials have previously been reported as effective supports for the immobilization of enzymes, the most frequently used being inorganic oxides, synthetic polymers and biopolymers (Ansorge-Schumacher and Thum, 2013; Arana-Pena et al., 2020; Liese and Hilterhaus, 2013; Liu et al., 2018; Shakerian et al., 2020). Based on these materials, stable and catalytically active biocatalytic systems have been formed that may find application in the removal of estrogenic compounds (Table 2).

Table 2

3.2 Use of immobilized enzymes in estrogen elimination

3.2.1 Estrogen removal using enzymatic reactors with immobilized laccases

A promising approach was presented by Lloret et al. (2011), who used laccase from *Myceliophthora thermophila* immobilized by an encapsulation as catalytic beads in a batch stirred tank reactor (BSTR) and a packed bed reactor (PBR) for the removal of estrone, 17β-estradiol and 17α-ethinylestradiol. Over 85% of each of the estrogens was removed after 8 h of the process carried out in the BSTR, that was around 20% higher than in PBR. The lower removal efficiency in continuous operation is related to the poorer accessibility of the enzyme's active sites upon encapsulation, as well as the excessively high flow rate of the mixture, which resulted in an insufficient enzyme-estrogen contact time. The immobilized laccase exhibited exceptional reusability: after five cycles of batch operation (each lasting 8 h) over 90% of the initial enzyme activity was preserved. To reduce diffusional limitations in the transport of substrates, commercially available laccase and laccase from *Myceliophthora thermophila* were covalently immobilized using Eupergit C250L commercial oxirane acrylic beads (Lloret et al., 2012b, 2012c). Immobilized laccase was used for the removal of a mixture of estrogens in a

packed bed reactor achieving 60% of estrone removal and around 80% of 17β-estradiol and 17α-ethinylestradiol degradation. To improve process efficiency a fluidized packed bed reactor was tested. In this case estrone, 17β-estradiol and 17α-ethinylestradiol were degraded with efficiencies of 90%, 100% and 80% respectively. Furthermore, after 16 days of continuous use, over 60% of each of the estrogens was removed by immobilized laccase. This clearly indicates that a fluidized bed reactor with immobilized laccase may be considered as a versatile tool for the continuous removal of estrogens. Becker et al. (2017) immobilized laccase from *Trametes versicolor* by adsorption onto a ceramic membrane modified by gelatin, and laccase from *Myceliophthora thermophila* by covalent binding using wet IB-EC-1 beads modified by glutaraldehyde for the removal of estriol from artificial mixtures and real wastewater. It was shown that using the membrane bioreactor (with laccase from *Trametes versicolor*), over 95% of estriol was removed, compared with around 80% in the case of the packed bed bioreactor (with laccase from *Myceliophthora thermophila*). The reason for the lower removal rate in the packed bed bioreactor are the diffusional limitations and possible enzyme inhibition. Although the membrane-immobilized laccase produced a higher removal rate, IB-EC-1-immobilized laccase exhibited a higher capacity for the elimination of estrogenic and androgenic activity in real wastewater. Over 99% removal of estrogenic and androgenic activity was obtained even at very low biocatalyst dosage. Nguyen et al. (2015) used an enzymatic membrane reactor with immobilized laccase for the removal of estrone, and recorded over 90% estriol removal. Collective results from the literature suggest that immobilized laccase can be effective (>90%) in the removal of estrogenic compounds (Parra-Arroyo et al., 2020; Bilal et al., 2020b; Zakaria et al., 2020; Datta et al., 2020; Zhoua et al., 2021).

Immobilized oxidoreductases may be used in a variety of reactor configurations, which in general can be divided into two types according to their operational mode: batch and continuous. Batch reactors are frequently used due to their simplicity, easy process control and

346 flexibility (Srikanlayanukul et al., 2016). Batch reactor types have been used for the removal
347 of estrogens from water solutions and wastewaters, enabling the efficient removal of these
348 compounds over a wide range of process conditions, even in long-term processes with relatively
349 high efficiencies. The inability to achieve total removal of pollutants results from several
350 drawbacks of batch systems, which include limited contact time between the immobilized
351 enzyme and the substrate, diffusional limitations, and enzyme inhibition (Boudrant et al., 2020;
352 Oh and Lim, 2019). Furthermore, the separation of biocatalysts from the post-reaction mixture
353 may be complicated, and the reusability of oxidoreductases in batch reactors has been shown
354 to be limited (Ai et al., 2017; Lacerda et al., 2019).

355 To overcome these limitations, continuous reactors may be used. The great advantage of
356 such reactors is the constant contact of the enzyme with the substrate, due to the continuous
357 delivery of fresh substrate solution (Du et al., 2013). Further, inhibition of the enzyme by the
358 products is limited, and separation of the biocatalyst from the reaction mixture is usually
359 avoided (Bolivar et al., 2011). For the removal of estriol, 17β-estradiol and 17α-ethinylestradiol
360 from model water solutions, usually laccases immobilized by covalent binding have been used,
361 as stable attachment of the biomolecule is crucial for the high efficiency of a continuous
362 bioreactor. Higher removal rates were achieved as compared to batch systems: in most cases
363 over 90% of the estrogens were biodegraded. Nevertheless, in our opinion, among other
364 continuous systems, particular attention should be paid to enzymatic membrane reactors
365 (EMRs). This solution, through a combination of simultaneous biocatalytic conversion and
366 selective mass transport through the membrane, enables an uncontaminated stream of products
367 of improved purity to be obtained (Rasera et al., 2009). In addition, enzymatic membrane
368 bioreactors allow the achievement of high biodegradation rates due to reduced diffusional
369 limitations, under mild process conditions (neutral pH and ambient temperature), and without
370 toxic reagents (Arca-Ramos et al., 2015). Moreover, it should be noted that the choice of

371 immobilization methodology is in most cases governed by the type of the bioreactor and its
372 configuration. Lloret et al. (2013b) immobilized laccase from *Trametes versicolor* on the inner
373 wall of microtubes by covalent binding and formed laccase-immobilized microreactors (Fig.
374 2). The developed system offered significantly improved stability under various conditions (pH,
375 temperature and chemical inactivation agents), as well as enhanced storage stability and
376 feasibility. The designed microreactor exhibited very high efficiency and performance under a
377 continuous flow regime; the rates of biodegradation of estrone, 17β-estradiol and 17α-
378 ethinylestradiol exceeded 99%.
379 **Figure 2**
380
381 **3.2.2 Use of immobilized peroxidase for estrogen elimination**
382 Although the performance of peroxidase (LiP, MnP and HRP) in estrogen removal is lower
383 than that of laccase, some studies have attempted to immobilize these enzymes to enhance their
384 performance. Ai et al. (2017) immobilized HRP by covalent binding onto amine-modified
385 magnetite-silica composite support and cross-linked HRP by glutaraldehyde to prevent its
386 elution. Immobilization has no negative effect on enzyme activity, while the thermal and
387 chemical stability were significantly improved upon immobilization. After 3 h of the process at
388 pH 7 and 30 °C, around 80% of the 17β-estradiol had been converted by the biocatalytic system.
389 In addition, the presence of natural organic matter does not affect the removal of 17β-estradiol.
390 As the lower removal efficiency might be a limitation of this method, to improve the removal
391 rate we suggest the use of mediators to boost the oxidizing potential of the immobilized HRP.
392 Finally, immobilized HRP exhibited good reusability: after seven repeated catalytic cycles, the
393 efficiency of removal of 17β-estradiol was above 45%. Taboada-Puig et al. (2011) insolubilized
394 versatile peroxidase (VP) from *Bjerkandera adusta* using glutaraldehyde to form cross-linked
395 enzyme aggregates. (CLEAs), however due to changes in the enzyme structure, less than 70%

of the initial activity was preserved. To improve the catalytic properties of CLEAs, peroxidase was co-immobilized with glucose oxidase from *Aspergillus niger*, resulting in increased activity retention, up to around 90%. As-prepared CLEAs were used for the removal of 17β-estradiol and 17α-ethinylestradiol and removed over 90% of the pollutant. The high removal rate is related to the continuous supply of H_2O_2 to the co-immobilized VP, and the improved stability of both enzymes against inactivation. Surprisingly, only a slight rise in the kinetic parameters of the versatile peroxidase was recorded upon immobilization, indicating the high substrate affinity of the immobilized enzyme and showing that diffusional limitations might be neglected. From the above examples it is evident that, immobilized peroxidases can also be used efficiently for the treatment of estrogens. In contrast to free HRP, LiP and MnP, the immobilized enzymes were reported to be effective over five and more consecutive reaction cycles. Finally, the efficiency of immobilized peroxidases was found to be as high as that of immobilized laccases. However, the proper selection of immobilization conditions, as well as the provision of an adequate H_2O_2 supply, is required to achieve high estrogen biodegradation rates. In addition, due to the use of immobilized peroxidase, a significant reduction in the estrogenic activity of treated compounds can be achieved.

3.2.3 Use of immobilized enzymes for estrogen elimination: the effect of the support material

The support materials used, due to the presence of different functional groups, various surface charges, morphological characteristics and/or particle size, can affect the enzyme structure, cause/limit diffusional limitations or provide a stabilizing and protective effect on the enzyme biomolecules. In consequence, support materials have an impact on a number of parameters, such as immobilization efficiency, the stability of the enzyme and its activity, also affecting the removal of pollutants. Cardinal-Watkins and Nicell (2011) used controlled

porosity silica beads for multipoint covalent attachment of laccase from *Trametes versicolor*. The immobilized enzyme was used in a continuous flow packed bed reactor for the removal of 17*β*-estradiol. It was demonstrated that over 75% removal rate was achieved after 3 h at pH 5 and 21 °C, using a flow-through reactor, whereas long-term storage stability was much greater at pH 7. Over a broad range of temperatures, the reaction is governed by Arrhenius' law, indicating that due to stabilization of the tertiary enzyme structure upon immobilization and the protective effect of the silica support, temperature has a greater impact on substrate transport than on enzyme inactivation. The main limitations of this method are the construction of the reactor, its poor flow characteristic, and the presence of dead zones that are inaccessible for efficient biocatalytic conversion. Therefore, to improve process efficiency, the design of the reactor should be improved to enhance contact between the substrate and the immobilized enzyme. In turn biopolymeric *Luffa cylindrica* fibers were used as a support for adsorption immobilization of laccase from *Pleurotus ostreatus*. Biopolymeric sponges, besides their high availability and low cost, are characterized by open three-dimensional structure that reduces diffusional limitations. The highest rate of removal of 17*α*-ethinylestradiol by the immobilized biocatalysts was recorded after 8 h of the process at pH 5, and amounted to 75%. It was found, however, that the reaction media played a crucial role in enzymatic activity. The biopolymer-bound laccase retained around 50% of its initial activity after 30 days of storage, and less than 30% of the 17*α*-ethinylestradiol was removed after ten repeated biocatalytic cycles (Lacerda et al. 2019). The relatively low storage stability and recyclability might be explained by the use of adsorption immobilization and the formation of weak enzyme–support interactions, which leads to the elution of biomolecules from the support. A possible way to overcome this limitation might be functionalization of the sponge surface to introduce chemical groups allowing the formation of more stable, covalent interactions.

Numerous inorganic and organic supports have previously been used for the immobilization of oxidoreductases (Table 2). Nevertheless, the development and synthesis of a novel group of composite/hybrid materials, with properties tailor-made for the enzyme and biocatalytic process, make these materials interesting and suitable alternatives as supports for enzyme immobilization. Garcia et al. (2019) used chitosan as the stabilizing factor to improve the mechanical resistance and biocompatibility of calcium and copper alginate beads with entrapped laccase from *Pycnoporus sanguineus*. It was shown that an alginate/laccase ratio of 5:0.5 (v/v) and a chitosan/copper ion ratio of 3:7 (v/v) used results in the highest activity retention and immobilization yield. The pH and temperature optima did not change upon immobilization; however, removal of 17α-ethinylestradiol by the immobilized laccase was more efficient over a broader range of pH and temperature. After 24 h of the process at pH 5 and a temperature of 28 °C, almost 80% of the estrogen was converted into less toxic dimers of 17α-ethinylestradiol. Storage stability (60% after 56 days) and reusability (20% after 4 cycles) were also determined in the study. These data indicate that, although the produced immobilized laccase might be considered as an alternative for the removal of 17α-ethinylestradiol, further study related to improvement of the recyclability of the biocatalysts is required.

4. Catalytic products of estrogen conversion

The removal of estrogens in usually assessed in terms of the disappearance of parent compounds. However, recently increases interest in the question of whether such removal entails the complete elimination of estrogen compounds, or their conversion to intermediates (by-products). This is because there is evidence of the environmental impact of estrogen-derived compounds. Therefore, studies have been undertaken to identify metabolites of enzymatic conversion of estrogens, to determine the degradation pathways of such compounds, and to suggest possible mechanisms of catalytic conversion (Nicotra et al., 2004). In addition,

catalytic pathways clearly show the type of the reactions occurring, as well as required co-substrates and their role in the conversion process. Evaluation of possible degradation routes allows the identification of bottlenecks in the process, and the indication of possible enzyme inhibitors and their effect on enzyme activity (Rathner et al., 2017). Further, examination of the final reaction products can help to enhance the purity of the final stream of products by the proper selection of a separation method. Moreover, determination of the reaction pathway facilitates selection of the reactor type and operational mode. In consequence, process operability and estrogen removal efficiency might be improved.

In general, oxidoreductases, including laccases and peroxidases, oxidize the phenolic moiety section in the substrate molecule and generate large amounts of free phenoxyl radicals (Beck et al., 2018; Maciel et al., 2010). In most cases, the formed phenoxyl radicals tend to combine and form dimeric forms of the initial compound. In the next step these dimers may be oxidized again by the oxidoreductase, resulting in the formation of trimers, tetramers and even oligomers (Huang et al., 2004; Xia et al., 2014). The rapid formation of phenoxyl radicals explains the disappearance of the substrate monomers at the initial stages of the reaction. This assumption has been put forward in previous investigations concerning the oxidoreductase-catalyzed oxidation of estrogens, and explains the difficulties in characterizing the reaction products (Lloret et al., 2013a; Mao et al., 2010a). However, also alternative degradation pathways and novel products of enzymatic conversion of estrogens have been presented indicating complex and not fully characterized mechanism of these biocatalytic conversions. There have recently been published literature reports, though very limited in number, that present the enzymatic treatment of estrogens and their mixtures by free and/or immobilized oxidoreductases (Table 3).

Table 3

4.1 Pathways and products of enzymatic conversion of 17*β*-estradiol

The mechanism of enzymatic conversion of 17*β*-estradiol involves mainly a radical oxidation mechanism and the formation of dimers and trimers (Table 3). Nicotra et al. (2004) observed that free laccase from *Mycelyopthora* could oxidize the substrate to oxygen radicals and subsequently couple the reactive intermediates to form C–C or C–O dimers. (Fig. 3) that could be further oxidized to generate oligomers and even polymers. Results of mass spectrometry, showing a peak at m/z 541, indicated that the brown precipitate formed during the reaction consisted mainly of dimeric forms of the initial compound. Similar observations were made by Xia et al. (2014), who used free laccase from *Trametes versicolor* for the removal of 17*β*-estradiol in the presence of natural organic matter (removal efficiency 90%). This was due to resonation of the single electron on the phenoxide oxygen to benzene, and the formation of potential reactive sites on the estrogen molecule. However, the mass spectra also contained a peak at m/z 271, representing the residue of the initial 17*β*-estradiol resulting from incomplete removal.

Figure 3

Intra et al. (2005) used free laccase from *Myceliophtora thermophyla* to perform coupling oxidation of 17*β*-estradiol. Although various 17*β*-estradiol dimers were found to be the main products of the laccase-catalyzed conversion, the formation of trimers and even oligomers is also expected. Surprisingly, the ratio of C–O and C–C is strongly affected by the type of solvent used. This might be explained by interactions between the organic solvent and enzyme amino acids that lead to slight changes in the enzyme structure, as well as the mobility of phenoxyl radicals in various organic media. This conclusion is in agreement with a study by Sun et al. (2020a), who used free laccase from *Trametes hirsuta* for conversion of 17*β*-estradiol from model water solutions. The results of high-resolution mass spectrometry in combination with ^{13}C-isotope labeling demonstrated that the main products of 17*β*-estradiol metabolism were

dimers, alongside trimers and tetramers. Sun et al. (2016) used laccase from *Plerotus ostreatus* for removal of 17*β*-estradiol in the presence of humic acid (HA), whose presence significantly affects the laccase-assisted conversion of 17*β*-estradiol. Results of high-resolution mass spectrometry showed peaks at m/z 541, 811 and 1084, attributed respectively to dimers, trimers and tetramers of 17*β*-estradiol. The addition of humic acid altered the degradation pathway of enzyme-catalyzed conversion due to inhibition by HA the self-coupling tendency of phenoxyl radicals and the formation of oligomeric compounds. Instead, promotion of cross-coupling between 17*β*-estradiol and HA was observed resulting in the formation of products of various molecular masses. However, it should be noted that not all of the compounds formed were identified. Therefore, in our opinion, further study in this area is still required to examine the effect of NOM on the enzyme-assisted conversion of estrogens. Mao and others published a series of studies (Mao et al., 2009, 2010a, 2010b) concerning the use of free lignin peroxidase from the white-rot fungus *Phanerochaete chrysosporium* in the presence of NOM, with removal efficiency over 95%. The addition of NOM did not affect the enzyme coupling mechanism of the conversion and final products: according to the HPLC and MS results, in the presence and absence of NOM, 17*β*-estradiol dimeric and trimeric forms were determined as the main products. Further, it was suggested that due to charge density distribution in the 17*β*-estradiol molecule, phenoxyl radicals are likely to couple covalently between the unsubstituted C3 and C7 carbon atoms in the phenolic ring. However, the formation of bonds between the O1 atom and C3 or C5 carbon atoms was not excluded.

The catalytic mechanism for HRP is more complicated than for the aforementioned enzymes. It consists of a multi-step reaction, and involves two active sites present in the HRP molecule. Huang et al. (2013) used free horseradish peroxidase for the removal of 17*β*-estradiol in the presence of NOM. The addition of NOM significantly altered the degradation pathways of HRP-mediated removal, however removal rate reached 85%. The presence of NOM reduced

545 the accessibility of the HRP active sites to bind 17*β*-estradiol molecules, as NOM molecules
546 compete with estrogen molecules for access to the enzyme's active sites. Further, NOM
547 contains numerous phenolic hydroxyl and phenolic amino groups capable of reacting with
548 horseradish peroxidase and able to form reactive radicals. As a consequence, coupling of 17*β*-
549 estradiol radicals is suppressed and the non-selective coupling of 17*β*-estradiol radicals with
550 NOM radicals is enhanced, leading to the formation of numerous cross-coupled products with
551 various molecular masses. Li et al. (2017) used free HRP for the removal of 17*β*-estradiol from
552 model water solutions containing humic acid. The process was performed under simulated
553 sunlight to obtain photoproduced H_2O_2 and to enhance the supply of this cofactor to the enzyme.
554 Based on the results of liquid chromatography with mass spectrometry, it was shown that the
555 addition of humic acid significantly affects the degradation pathways of 17*β*-estradiol. The
556 main reaction process was polymerization, resulting in the formation of C–O and C–C 17*β*-
557 estradiol dimers at m/z 541 as the main products. However, due to the presence of HA,
558 oxidation and/or hydroxylation processes also occurred, resulting in the formation of products
559 at the lower molecular weight (Fig. 4).

560 **Figure 4**

561 In a study by Pezzella et al. (2004), nitration was used to enhance the performance of an HRP-
562 catalyzed process. The authors observed a different reaction mechanism due to the involvement
563 of NO_2^- ions in the reaction. Initially, free radicals are generated by the peroxidase, and in the
564 next step NO_2^- ions are coupled with the formed radicals to produce final products, which
565 according to the HPLC-MS and NMR results were identified as 2-nitroestradiol, 4-
566 nitroestradiol and 2,4-dinitroestradiol. Therefore, in our opinion, the described enzyme-
567 catalyzed removal of 17*β*-estradiol supported a by nitration process may be an interesting
568 alternative for the removal of persistent estrogens, with the possibility of obtaining low-toxic
569 final products.

570

571 **4.2 Pathways and products of 17α-ethinylestradiol conversion**

572 The catalytic pathways of 17α-ethinylestradiol conversion have been found to follow a
573 similar mechanism as the conversion of 17β-estradiol, with dimers and trimers being the main
574 bioconversion products (Table 3). Tanaka et al. (2009) used free laccase from *Trametes* sp. Ha1
575 for the removal of 17α-ethinylestradiol, and examined the effect of an O_2 supply and its
576 consumption on the degradation pathways of estrogen. It was proved that after 60 min of the
577 process the main product of the reaction was 17α-ethinylestradiol dimer (peak at m/z 591).
578 However, in later stages of the process, instead of coupling of 17α-ethinylestradiol free radicals,
579 there was a decrease in the concentration of estrogen dimers. This is due to direct oxidation of
580 the dimers by laccase molecules, as well as their indirect oxidation with the aid of the monomer
581 radical as a mediator agent. This leads to the formation of oligomeric 17α-ethinylestradiol
582 derivatives. Golveia et al. (2018) used free *Pycnoporus sanguineus* laccase for the removal of
583 17α-ethinylestradiol and reached over 99% removal efficiency. Results obtained by mass
584 spectrometry combined with electrospray ionization and time-of-flight detectors showed a
585 dimer as the main reaction product of 17α-ethinylestradiol conversion. However, the mass
586 spectrum obtained after 6 h contained only a signal at m/z 311 (characteristic for a new
587 degradation product). This product was formed as a result of attack of the •OH radical on 17α-
588 ethinylestradiol, further dehydration, and a further •OH radical attack to form final 17α-
589 ethinylestradiol semiquinone (Fig. 5).

590 **Figure 5**

591 Rathner et al. (2017) used free horseradish peroxidase for the complete degradation of 17α-
592 ethinylestradiol in artificial wastewater samples. The addition of H_2O_2 and humic acids
593 significantly affected the efficiency of 17α-ethinylestradiol conversion. The molar spectra
594 obtained by HPLC-MS analysis contained two peaks at m/z 97 and 195, identified respectively

as unsaturated glutaraldehyde derivative and its dimer. The formation of low-molecular-weight final products was an unexpected phenomenon, and indicates a significantly different degradation mechanism as compared to the previous examples. Nevertheless, in our opinion, more detailed and advanced characterization of the degradation products using sophisticated analytical equipment should be performed to make a clear determination of the catalytic pathways. Garcia et al. (2019) immobilized laccase from *Pycnoporus sanguineus* on calcium and copper alginate–chitosan beads for use in the removal of 17α-ethinylestradiol. Results from electrospray ionization coupled with a time-of-flight mass spectrometer showed two peaks at m/z 295 and 591, characteristic for unreacted 17α-ethinylestradiol and its dimers, respectively.

4.3 Products of conversion of estrogen mixtures

In a study by Lloret et al. (2013a), a mixture of estrone, 17β-estradiol, and 17α-ethinylestradiol was treated using free laccase from *Myceliophthora thermophila*. Based on HPLC and GC-MS results, dimers and trimers of estrone, 17β-estradiol, and 17α-ethinylestradiol were the main products of the laccase-assisted conversion. However, in the case of estrone and 17β-estradiol, other species at smaller molecular weight were detected. This indicates that radical coupling was not the only transformation reaction mechanism. Becker et al. (2018) observed a precipitate as the final conversion product of laccase treatment of mixture of 17β-estradiol and 17α-ethinylestradiol consisting mainly dimers, trimers and oligomers of both estrogens. Lloret et al. (2012a) used free laccase from *Myceliophthora thermophila* for continuous removal of estrone and 17β-estradiol. Based on the GC-MS chromatograms of the samples after 8 h of incubation, it was found that metabolites at m/z 340 and m/z 415 and 430 were produced by bioconversion of estrone and 17β-estradiol respectively. However, more detailed explanation of the observed phenomena is lacking, due to difficulties in identification of the metabolites. Therefore, we strongly believe that it is important to perform more

620 exhaustive studies and to apply other techniques for the identification of bioconversion products
621 and degradation pathways.
622
623 **5. Estrogenic activity and toxicity of catalytic by-products**
624 Estrogens and their estrogenic activity may have an adverse effect both on natural
625 ecosystems and on human health. Their presence in water has a negative impact on the
626 reproduction of aquatic organisms, and might lead to serious disorders in aquatic ecosystems
627 and the extinction of some organisms. It has been reported that 17β-estradiol, estrone and 17α-
628 ethinylestradiol may affect fish life, and exhibit chronic aquatic toxicity at concentrations
629 exceeding, respectively 5 ng/L, 2 ng/L and 0.1 ng/L (Anderson et al., 2012; Laurenson et al.,
630 2014; Adeel et al., 2017). Estrogens have been detected in soil and water streams, WWTP
631 effluents, industrial effluents and others, from which they might easily pass to surface water,
632 groundwater and drinking water (Ben et al., 2018; Ekpeghere et al., 2018). 17β-estradiol and
633 estrone were found in 53 out of 62 drinking and wastewater treatment plants in China. The
634 maximum detected concentrations were 1.7 ng/L and 0.1 ng/L for 17β-estradiol and estrone,
635 respectively, indicating concentration levels that pose a risk to human health (Fan et al., 2013).
636 Further, maximum concentrations of estrone and 17β-estradiol of 68.1 and 2.5 ng/L,
637 respectively were reported in deep groundwater close to drinking water, being significantly
638 higher than the lowest observable effect level (Kjaer et al., 2007).
639 Though the removal/conversion of estrogens is important, even more crucial is the effective
640 elimination of their toxicity. This is quite challenging, due to low bioavailability, low
641 concentrations of these compounds, their complicated structure, as well as resistance to most
642 of the treatment technologies used (Taboda-Puig et al., 2011; Tanaka et al., 2009, Becker et al.,
643 2017; Stalter et al., 2011, Huber et al., 2004). This provokes researchers to develop more
644 advanced and effective treatment techniques to remove estrogenic activity. In this context, the

use of free and immobilized enzymes appears to be an efficient route, as significantly lower estrogenic activity of the reaction products has been reported.

5.1 Methods of determining estrogenic activity

There are different ways of examining the toxicity and estrogenic activity. Animals or their cells are usually used; however, many data come from *in vitro* tests carried out using various bacterial strains, microorganisms and fungi, mainly yeast (Bristan et al., 2012; Suzuki et al., 2003). The most frequently applied methods employ yeast as model organisms sensitive to the activity of estrogens. These techniques are classified as yeast estrogen screen (YES) bioassays, and generally use modified yeast cells, including human estrogen receptor, capable of binding estrogenic compounds (Fenta et al., 2006; Spengler et al., 2001). Yeast, mainly from the species *Saccharomyces cerevisiae*, has been used mainly due to its rapid growth, short sexual cycle and susceptibility to modification, as well as the broad range of plasmids and promoters (Purvis et al., 1991). Various approaches using yeast as a model microorganism have recently been reported, such as the application of a yeast two-hybrid estrogenic assay system (Suzuki et al., 2003; Tamagawa et al., 2006; Wang et al., 2012), use of the yeast strain BY4741 (Auriol et al., 2008), and a yeast estrogen screen assay assisted by enzymatic digestion with lyticase (Lloret et al., 2011, 2012c; Taboada-Puig et al., 2011). Also other methods of estrogenic activity determination have been presented, based on human cells (luciferase-transfected human breast cancer cell line gene reporter (MELN) assay) (Shreve et al., 2016), as well on aquatic microorganisms such as *Artemia salina* or small fish of the species *Danio rerio* or *Oryzias latipes* (Tanaka et al., 2009; Torres-Duarte et al., 2012). All of the above-mentioned techniques are comparable and enable the effective determination of estrogenic activity. Table 4 summarizes literature reports on the toxicity and estrogenic activity of estrogens before and after enzymatic treatment.

Table 4

5.2 Toxicity and estrogenic activity of mixtures after enzymatic treatment

Tanaka et al. (2009) examined the estrogenic activities of the 17*β*-estradiol and 17*α*-ethinylestradiol after complete enzymatic removal by free laccase using male medaka (*Oryzias latipes*) fish and the Medaka Chromato assay kit, which offers simplicity in operation and low cost. Total elimination of the estrogenic activity of the estrogen mixture was recorded. This is related to the formation of dimers and oligomers of the parent compounds, which have significantly lower EA than the initial estrogens. In another study, laccase from *Trametes versicolor* was used to reduce the estrogenic activity of a mixture of estrone, 17*β*-estradiol and 17*α*-ethinylestradiol in wastewater treatment plant effluent (Shreve et al., 2016). Using the YES assay, it was found that the decrease in estrogenic activity follows the same trend as the rate of removal of estrogens. After 12 h of WWTP treatment the initial EA was reduced by over 98% indicating laccase is capable of reducing the estrogenic activity of mixtures of estrogens. Nevertheless, further studies are required to examine the effect of the wastewater matrix on enzyme properties. Similarly, over 98% removal rates of estrone, 17*β*-estradiol and 17*α*-ethinylestradiol, from model solution and municipal wastewater by fungal *Trametes pubescens* laccase were reported (Spina et al., 2015). Based on the MELN assay results, a total removal of the initial estrogenic activity of the estrogen mixture was observed. Also free lignin peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624 is capable of significant reduction in the estrogenic activity of estrone, 17*β*-estradiol and 17*α*-ethinylestradiol (Wang et al., 2012). Based on the results of *in vitro* screening tests for chemicals with hormonal activities, using yeast, it was found that the initial estrogenic activities of estrone, 17*β*-estradiol and 17*α*-ethinylestradiol were reduced by 100%, 72% and 82% respectively after 24 h of treatment. The incomplete elimination of the EA of 17*β*-estradiol and 17*α*-ethinylestradiol is probably related

to the lower affinity of the enzyme towards these compounds. Recombinant yeast strain BY4741 was used to determine the estrogenic activity of estrone, 17β-estradiol, estriol and 17α-ethinylestradiol under environmental conditions supported by horseradish peroxidase and laccase from *Trametes versicolor* before and after enzymatic treatment (Auriol et al., 2008). Due to the oxidative nature of the catalytic reaction and the formation of oligomers of parent compounds, total elimination of acute estrogenic toxicity was achieved with both HRP and laccase after 1 h of the process. These data confirm the feasibility of eliminating estrogenic activity using enzymatic treatments. The treatment of estrone solution by manganese peroxidase and laccase from *Phanerochaete sordida* YK-624 resulted in the total removal rate of pollutant and in total removal of estrogenic activity after 2 h. It was speculated that such significant and rapid decrease of EA was related to the formation of oligomeric reaction products through enzymatic oxidation followed by radical coupling, as estrone has a para-substituted phenol structure, which facilitates oxidation and oligomer formation (Tamagawa et al., 2006). White-rot fungus manganese peroxidase and laccase were used for the elimination of estrogenic activity of 17β-estradiol and 17α-ethinylestradiol (Suzuki et al., 2003). Using the yeast two-hybrid assay system, it was found that the estrogenic activities of 17β-estradiol and 17α-ethinylestradiol were reduced by more than 80% following 1 h of treatment. Extending the treatment time to 8 h resulted in elimination of the remaining estrogenic activity of both estrogens. These results strongly suggest that free ligninolytic enzymes are effective in eliminating the estrogenic activity of estrogens in model and real wastewater solutions.

Taboada-Puig et al. (2011) used versatile peroxidase co-immobilized with glucose oxidase in the form of CLEAs for elimination of estrogenic activity of 17β-estradiol (removal rate 90%) and 17α-ethinylestradiol (removal rate 93%). According to the LYES (yeast estrogen screen-assay assisted by enzymatic digestion with lyticase) assay protocol it was demonstrated that the initial EA of 17α-ethinylestradiol was reduced by around 60%, and that of 17β-estradiol by over

70%. This difference is probably due to the slightly different chemical structures of 17*α*-ethinylestradiol and 17*β*-estradiol. As a consequence, the immobilized enzyme may exhibit different affinity towards the two compounds, but no definitive statements about changed catalytic properties can be made without the identification of metabolites. Lloret et al. (2011) used laccase from *Myceliophthora thermophila* immobilized by encapsulation in a siliceous sol-gel matrix for the removal of estrone, 17*β*-estradiol and 17*α*-ethinylestradiol. Low conversion of parent compounds (around 60%) results in lower reduction of estrogenic activity. Based on the results of the LYES assay protocol, the initial EA was reduced by around 65%. In another study, laccase immobilized onto Eupergit C 250 L was used for the removal of a mixture of estriol, 17*β*-estradiol and 17*α*-ethinylestradiol allowing over 85% conversion of tested compounds and over 90% decrease in estrogenic activity after 16 days of continuous operation of a fluidized bed reactor (Lloret et al., 2012c). Torres-Duarte et al. (2012) immobilized laccase from *Coriolopsis gallica* onto the prepolymer Hypol 2002 for the bioconversion of 17*β*-estradiol. To examine the estrogenic activity of the 17*β*-estradiol and its enzymatic transformation products, *in vitro* tests based on the affinity of human estrogen receptor alpha (hER*α*) for the ligand binding domain of zebrafish (*Danio rerio*) were used. During the exposure experiments of the samples after treatment, no fish mortality was detected; thus it can be concluded that the estrogenic activity of the 17*β*-estradiol was eliminated.

6. Future prospects and challenges

Recently, valuable reports have been published concerning the application of free and immobilized oxidoreductases. Though relatively high removal efficiencies were obtained, the methods presented have drawbacks and limitations. Free enzymes suffer from low stability and restricted reusability. Therefore, immobilization has been presented as a facile solution to improve enzymes' reuse potential. However, there is still a need to develop and optimize—for

instance, in terms of enzyme dosage and the reduction of enzyme leaching—novel and universal immobilization approaches that support the stability and activity of biocatalysts. Furthermore, in our opinion, future research should be focused on the evaluation of biocatalytic systems based on co-immobilized enzymes, in order to limit enzyme inhibition and/or enhance O_2/H_2O_2 supply. Ongoing research should be also focused on the development of novel, tailor-made composite and/or hybrid materials for use as supports for oxidoreductases, to improve the catalytic properties of the enzyme and reduce enzyme leaching. Future studies should consider the synthesis and application of cheap materials characterized by high enzyme capacity, easy preparation and significant mechanical stability, in order to reduce the costs of immobilization and simplify its protocol.

In our opinion also operationability of the process strongly affect its efficiency. In this term, of particular interest are bioreactors and enzymatic membranes reactors, as they facilitate conversion of estrogens and purification of the post reaction mixture. Recently, ceramic membranes have mainly been applied as supports for oxidoreductase immobilization in EMRs, due to their significant mechanical resistance and operational stability. Nevertheless, we strongly believe that the development of novel composite membranes with pore size tailored for effective enzyme binding and efficient separation of reaction components will increase even further the applicability of EMRs for the removal of estrogens. Although we believe that continuous enzymatic membrane reactors are the most promising solution for the efficient removal of estrogens, other bioreactors with immobilized enzymes might also be considered as suitable platforms for the enhancement of oxidoreductase application in wastewater treatment. Nevertheless, proper selection of the enzyme support, the immobilization approach and the reactor's operational mode is crucial for the effective treatment of estrogenic compounds.

Nevertheless, the greatest challenge in future research will be the development of novel and advanced techniques for simultaneous bioconversion of estrogens and their removal from the

reaction mixture. Of particular interest, in our judgement, will be processes of simultaneous conversion of estrogens and their adsorption by support or sorbent materials, as well as dual processes including simultaneous catalytic conversion and separation using membrane technologies. In this context attention should be paid to the development of suitable support/sorbent materials and appropriate selection of the membrane, which acts as a support for the immobilized enzyme. Beside efficient oxidoreductase immobilization, other parameters might also significantly affect enzymatic conversion of estrogens.

In our opinion, one of the most essential issues to be considered is the development of novel methods and improvement of existing methods for separation or precipitation of the products of catalytic conversion. This is of key interest for several reasons, including: (i) obtaining as pure a solution as possible after treatment, (ii) separation of biocatalysts from the reaction mixture, and (iii) improvement the overall process efficiency. We also believe that detailed characterization of the mechanisms of catalytic conversion and optimization of the process according to the degradation pathway may significantly improve process efficiency and will allow the development of suitable techniques for separation of the final products.

A crucial aspect of estrogen removal is also the elimination of estrogenic activity. Examination of the estrogenic activity of estrogens is of particular interest, as in our opinion only a significant EA reduction can justify claims of the ultimate effectiveness and usefulness of each technique. The degradation pathways of catalytic conversion and the type of final reaction products may influence EA reduction. Though various protocols for the examination of estrogenic activity have been developed, future study focused on the evaluation of assay protocols for the examination of EA in terms of sensitivity, simplicity and low cost, and their practical assessment is still required. In our opinion, this is of the greatest importance, as the data obtained might lead to the identification of a novel degradation route resulting in less toxic final products. In spite of the efforts of many researchers, the identification of all of the

metabolites formed and determination of the catalytic pathways still require much study. Therefore, we strongly believe that future work will focus on the use of advanced analytical equipment for the evaluation of novel biocatalytic pathways and identification of the final products.

Finally, in the near future, various problems should be solved in order to enable the large-scale application of oxidoreductases in industrial wastewater treatment and in environmental protection. To achieve this goal it is crucial to evaluate methods that produce cheap, stable and reusable biocatalytic systems in a simple manner. Furthermore, the development and proper configuration and setup of reactors are still important topics of research. These issues should be addressed to facilitate the transfer of laboratory-scale technology to larger scales, and to ensure the stability of the biocatalysts used and the feasibility of estrogen removal.

7. Conclusions and general remarks

This paper reviews current studies on the enzymatic degradation of estrogens and the efficiency of this technique in reducing estrogenic activity and toxicity. Enzymes including laccase, as well as horseradish, manganese and lignin peroxidases, have been applied in the treatment of estrogenic compounds. Amongst all enzymes, laccase is the most studied. This is because laccase can utilize free dissolved oxygen as an electron acceptor, unlike other enzymes which require the addition of hydrogen peroxide. Studies also show that laccase produces a high efficiency (>90%) of removal of estrogens. Enzyme denaturation and enzyme washout are two major challenges which hamper the study of enzymatic processes, particularly on a laboratory scale. The wastewater matrix (pH, organic matter concentration) also has an impact on enzymatic catalysis. The immobilization of enzymes is a very promising technique to overcome the problems of free enzyme application. However, research is still at an early stage, with results available from laboratory-scale studies only. The reported removal of estrogens by

free or immobilized laccase is not always complete, and there is evidence of residual by-products (e.g. dimers and trimers) remaining after treatment. Nevertheless, the estrogenic activity of treated estrogenic solutions is significantly reduced.

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Declaration of interests

The authors declare that they have no conflict of interest.

831 **Tables and Figures**

832 **Table 1.** Bioconversion of estrogens by native oxidoreductases under various operational conditions.

Target compound	Enzyme and source	Process conditions	Mediator	Removal system setup	Removal efficiency	References
estriol	laccase from *Pleurotus eryngii var tuoliensis* C.J. Mou	pH 7, 37 °C, 48 h	-	batch system	98%	(Ueda et al., 2012)
17*β*-estradiol	laccase from *Trametes versicolor*	pH between 4 and 6, room temperature, 24 h	-	batch system	100%	(Xia et al., 2014)
17*α*-ethinylestradiol	laccase from *Pycnoporus sanguineus*	pH 5, 25 °C, 8 h	cupuaçu (*Theobroma grandiflorum*)	batch system	99%	(Golveia et al., 2018)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Trametes pubescens* MUT 2400	pH 6.9, room temperature, 24 h	-	batch system	around 100% for all compounds	(Spina et al., 2015)
17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Trametes versicolor* IFO-6482	pH 4.5, 30 °C, 1 h	1-hydroxy-benzotriazole (HBT)	batch system	around 100% for all compounds	(Suzuki et al., 2003)
17*α*-ethinylestradiol	laccase from *Trametes* sp.	pH 5, 30 °C, 48 h	-	rotating reactor for enzymatic treatment	around 100% for all compounds	(Tanaka et al., 2001)
17*β*-estradiol	laccase from *Plerotus ostreatus*	pH 5.8, 25 °C, 240 min	-	batch system	99%	(Sun et al., 2016)
17*β*-estradiol	laccase from *Trametes hirsuta*	pH 5, 25 °C, 120 min	-	batch system	99.3%	(Sun et al., 2020a)
estrone 17*β*-estradiol	laccase from *Myceliophthora thermophila*	pH 7, 26 °C, 10 h	-	continuous enzymatic membrane reactor	95.6% >98%	(Lloret et al., 2012a)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Myceliophthora thermophila*	pH 7, 26 °C, 100 h	-	continuous flow enzymatic membrane reactor	88% 99% 94%	(Lloret et al., 2013a)

17α-ethinylestradiol	laccase and manganese peroxidase from *Pleurotus* sp. P1	30 °C, 6 days	-	batch system	100%	(Santos et al., 2012)
17α-ethinylestradiol	fungi laccase	pH 5, 25 °C, 24 h	-	batch system	95%	(Sun et al., 2020b)
estrone 17β-estradiol estriol 17α-ethinylestradiol	laccase from *Trametes versicolor*	pH 7, 25 °C, 1 h	1-hydroxy-benzotriazole	batch system	100% for all tested compounds	(Auriol et al., 2007b)
estrone 17β-estradiol 17α-ethinylestradiol	laccase from *Trametes* sp. Ha1	pH 5, 30 °C, 1 h	-	batch system	>90%	(Tanaka et al., 2009)
estrone 17β-estradiol 17α-ethinylestradiol	laccase from *Trametes versicolor*	pH 4.5, 25 °C, 3.5 h	-	batch system	83.5% 100% 71.3%	(Shreve et al., 2016)
estrone	laccase from white-rot fungus *Phanerochaete sordida* manganese peroxidase from white-rot fungus *Phanerochaete sordida*	pH 4.5, 30 °C, 1 h	-	batch system	100% 100%	(Tamagawa et al., 2006)
estrone 17β-estradiol estriol 17α-ethinylestradiol	laccase from *Trametes versicolor* horseradish peroxidase	pH 7, 25 °C, 8 h for laccase pH 8, 25 °C, 1 h for horseradish peroxidase	-	batch system	100% for all compounds after laccase treatment 100% for all compounds after HRP treatment	(Auriol et al., 2008)
estrone 17β-estradiol estriol 17α-ethinylestradiol	horseradish peroxidase	pH 7, 25 °C, 1 h	-	batch system	around 100% for all compounds	(Auriol et al., 2007a)
estrone 17β-estradiol estriol	horseradish peroxidase	pH 7, 25°C, 1 h	-	batch system	92% 92% 93%	(Auriol et al., 2006)

17α-ethinylestradiol					100%	
17α-ethinylestradiol	horseradish peroxidase	pH 4.5, room temperature, 24 h	-	batch system	100%	(Rathner et al., 2017)
17β-estradiol	horseradish peroxidase	pH 7, room temperature, 60 min	-	batch system	84.4%	(Huang et al., 2013)
17β-estradiol	horseradish peroxidase	pH 7, 25 °C, 180 min		batch system	31.7%	(Li et al., 2017)
17α-ethinylestradiol	horseradish peroxidase	pH 7, 25 °C, 30 min	natural organic matter	batch system	75%	(Yang et al., 2018)
estrone 17β-estradiol 17α-ethinylestradiol	lignin peroxidase from *Phanerochaete sordida*	pH 3, 30 °C, 4 h	-	batch system	23.9% 38.2% 45.0%	(Wang et al., 2012)
17β-estradiol	lignin peroxidase from *Phanerochaete chrysosporium*	pH 4.6, room temperature, 90 min	veratryl alcohol (VA)	batch system	100%	(Mao et al., 2009)
17α-ethinylestradiol	laccase, manganese peroxidase and lignin peroxidase from *Trametes versicolor*	pH 4.5, 28 °C, 3 days	-	batch system	100%	(Cajthaml et al., 2009)

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835 **Table 2.** Application of immobilized oxidoreductases in bioconversion of estrogens under various operational conditions. n.a. – not available

Target compound	Enzyme and source	Support material	Immobilization approach	Removal system	Process conditions	Mediator	Reusability, storage stability	Removal efficiency	References
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Trametes versicolor*	poly(tetrafluoroethylene) microtubes modified by poly-l-lysine and glutaraldehyde	covalent binding/cross-linking	continuous flow enzymatic membrane reactor	flow rate 0.5 µL/min, pH 5, 30 °C, 12 h	-	n.a.	>99% >99% >99%	(Lloret et al., 2013b)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Myceliophthora thermophila*	Eupergit C 250L	covalent binding	packed bed reactor	pH 7, 24 °C, 8 h	-	65% after 10 cycles, 98% after 4 months	60% 80% 80%	(Lloret et al., 2012b)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Myceliophthora thermophila*	sol-gel matrix based on methyltrimethoxysilane and tetramethoxysilane	encapsulation	batch stirred tank reactor	pH 7, 23 °C, 8 h	-	90% after 5 cycles, 95% after 3 months	>98% >98% >98%	(Lloret et al., 2011)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Myceliophthora thermophila*	sol-gel matrix based on methyltrimethoxysilane and tetramethoxysilane	encapsulation	packed bed reactor	pH 7, 23 °C, 8 h	-	90% after 5 cycles, 95% after 3 months	55% 75% 60%	(Lloret et al., 2011)
17*α*-ethinylestradiol	laccase from *Pleurotus ostreatus*	*Luffa cylindrica* fibers	adsorption	batch system	pH 5, room temperature, 24 h	-	30% after 10 cycles, 50% after 30 days	76%	(Lacerda et al., 2019)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	commercially available laccase	Eupergit C 250L	covalent binding	fluidized bed reactor	pH 7, 26 °C, 16 days	-	50% after 16 days	92% 100% 92%	(Lloret et al., 2012c)
estrone 17*β*-estradiol estriol 17*α*-ethinylestradiol	laccase from *Trametes versicolor*	ceramic membrane modified by gelatin	adsorption	enzymatic membrane reactor	pH 5, 22 °C, 24 h	-	n.a.	over 95% for all tested compounds	(Becker et al., 2017)

estrone 17*β*-estradiol estriol 17*α*-ethinylestradiol	laccase from *Myceliophthora thermophila*	wet IB-EC-1 beads modified by glutaraldehyde	covalent binding	packed bed reactor	pH 5, 22 °C, 24 h	-	n.a.	around 80% for all tested compounds	(Becker et al., 2017)
17*β*-estradiol	laccase from Myceliophthora thermophila	fumed silica microparticles and magnetized fumed silica microparticles	covalent binding	enzymatic membrane reactor	pH 7, room temperature, 24 h	-	70% after 10 cycles, 80% after 60 days	76%	(Gamallo et al., 2018)
estrone 17*β*-estradiol estriol 17*α*-ethinylestradiol	laccase from *Aspergillus oryzae*	ultrafiltration hollow fiber membrane	adsorption	enzymatic membrane reactor	pH 6.8, 28 °C, 72 h	syringaldehyde, HB	n.a.	>95% >95% 93% 90%	(Nguyen et al., 2015)
17*β*-estradiol	laccase from *Trametes versicolor*	silica beads	covalent binding	continuous flow packed bed reactor	pH 5, 21 °C, 180 min	-	25% after 12 weeks	75%	(Cardinal-Watkins & Nicell, 2011)
17*α*-ethinylestradiol	laccase from *Trametes versicolor*	polyamide 6/chitosan	covalent binding	batch system	pH 5, 25 °C, 6 h		40% after 3 cycles, 80% after 12 days	100%	(Maryšková et al., 2016)
17*α*-ethinylestradiol	laccase from *Coriolopsis gallica*	mesoporous silica spheres modified by glutaraldehyde	adsorption and crosslinking	continuous stirred-tank membrane reactor	pH 5, room temperature, 24 h	-	n.a.	100%	(Nair et al., 2013)
17*β*-estradiol	laccase from *Mycelyopthora* sp.	glass beads	adsorption	batch system	pH 6.5, 45 °C, 7 days	-	n.a.	100%	(Nicotra et al., 2004)
17*α*-ethinylestradiol	laccase from *Pycnoporus sanguineus*	Ca and Cu alginate–chitosan composite	entrapment	batch system	pH 5, 28 °C, 24 h	-	20% after 4 cycles, 60% after 56 days	up to 90%	(Garcia et al., 2019)
17*β*-estradiol 17*α*-ethinylestradiol	versatile peroxidase from *Bjerkandera*	-	cross-linking enzyme aggregates	batch system	pH 5, 30 °C, 60 min	-	n.a.	93% 90%	(Taboada-Puig et al., 2011)

	adusta co-immobilized by cross-linking with glucose oxidase from *Aspergillus niger*								
17*β*-estradiol E2	horseradish peroxidase	Fe_3O_4@SiO_2 microspheres modified by amine groups and glutaraldehyde	covalent binding/cross-linking	batch system	pH 7, 30 °C, 3 h	-	45% after 7 cycles	80%	(Ai et al., 2017)
17*α*-ethinylestradiol	horseradish peroxidase	Fe_3O_4 nanoparticles	adsorption	batch system	pH 7, 30 °C	-	48% after 7 cycles	100%	(Xiao et al., 2020)
estrone	horseradish peroxidase	poly(vinyl alcohol)/ poly(acrylic acid)/SiO_2/Fe_3O_4 composite	adsorption	batch system	pH 7, 25 °C, 40 min	-	n.a.	84.5%	(Xu et al., 2016)

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838 **Table 3.** Main products of catalytic conversion of estrogens by free and immobilized oxidoreductases. n.a. – not available

Target compound	Enzyme	Form of enzyme/bioreactor configuration	Removal efficiency (%)	Mediator	Molecular ion (m/z) and/or suggested products	References
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Myceliophthora thermophila*	free/enzymatic membrane reactor	88% 99% 94%	-	539 – dimer E1 525 – dimer E2, 795 – trimer E2 573 – dimer EE2, 867 – trimer EE2	(Lloret et al., 2013a)
estrone 17*β*-estradiol	laccase from *Myceliophthora thermophila*	free/fed-batch reactor	95.6% >98%	-	340 – metabolite E1 415, 430 – metabolites E2	(Lloret et al., 2012a)
17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Trametes versicolor*	free/batch reactor	n.a.	-	541 – dimer E2, 811 – trimer E2, 1083 – tetramer E2, 1353 – pentamer E2, 1623 – hexamer E2, 1893 – heptamer E2 590 – dimer EE2, 884 – trimer EE2, 1178 – tetramer EE2, 1473 – pentamer EE2, 1767 – hexamer EE2	(Beck et al., 2018)
17*β*-estradiol	laccase from *Trametes versicolor*	free/batch reactor	90%	-	541 – dimer E2	(Xia et al., 2014)
17*β*-estradiol	laccase from *Trametes pubescens*	free/batch reactor	n.a.	-	524 and 541 – dimer E2	(Intra et al., 2005)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Trametes* sp. Ha1	free/batch reactor	>90% >90% >90%	-	dimers E1 dimers E2 590 – dimer EE2 oligomers EE2	(Tanaka et al., 2009)
17*β*-estradiol	laccase from *Mycelyopthora* strain	free/batch reactor	n.a.	-	541 – dimer E2	(Nicotra et al., 2004)
17*β*-estradiol	laccase from *Plerotus ostreatus*	free/batch reactor	99%	-	269 – estrone E1, 539 – cross-coupling products E1-E2, 541 – dimer E2, 811 – trimer E2, 1084 – tetramer E2	(Sun et al., 2016)
17*β*-estradiol	laccase from *Trametes hirsuta*	free/batch reactor	99.3%	-	268 – estrone E1, 541 – dimer E2, 811 – trimer E2, 1081, 1084, 1087, 1090 and 1081 – tetramer E2, 539 – cross-coupling products E1-E2	(Sun et al., 2020)

17*α*-ethinylestradiol	laccase from *Pycnoporus sanguineus*	immobilized onto alginate-chitosan support/batch reactor	89.81%	-	590 – dimer EE2	(Garcia et al., 2019)
17*α*-ethinylestradiol	laccase from *Pycnoporus sanguineus*	free/batch reactor	99.75%	cupuaçu (*Theobroma grandiflorum*)	590 – dimer EE2	(Golveia et al., 2018)
17*β*-estradiol	horseradish peroxidase	free/batch reactor	84.4%	-	541 – dimer E2 811 – trimer E2	(Huang et al., 2013)
17*α*-ethinylestradiol	horseradish peroxidase	free/batch reactor	100%	-	97 - unsaturated derivative of glutaraldehyde 195 - dimeric form of unsaturated derivative of glutaraldehyde	(Rathner et al., 2017)
17*β*-estradiol	horseradish peroxidase	free/batch reactor	n.a.	-	361 – 2,4-dinitroestradiol, 316 – 2-nitroestradiol, 316 – 4-nitroestradiol	(Pezzella et al., 2004)
17*β*-estradiol	horseradish peroxidase	free/batch reactor	n.a.	-	541 – dimer E2	(Li et al., 2017)
17*β*-estradiol	chloroperoxidase from *Caldariomyces fumago*	immobilized onto Eupergit C/batch reactor	n.a.	-	mono- and di-chlorinated 17*β*-estradiol	(Salcedo et al., 2015)
17*β*-estradiol	tyrosinase	free/batch reactor	n.a.	-	301 – 2-hydroxy-6-oxo-estra-1,3,5(10)-trien-3,17*β* -diol 283 – 2-hydroxy-estra-1,3,5,6,8-pentaene-3,17 *β* -diol 285 – 6,7-dehydro-2-hydroxyestradiol 285 – 9,11-dehydro-4-hydroxyestradiol 573 – 2-hydroxy-1-[[(17 *β*)-2,17-dihydroxy-19-norpregna-1,3,5(10)-trien-3-yl]oxy]estra-1,3,5(10)trien-3,17 *β* -diol 569 – 2-hydroxy-1-[[(17 *β*)-2,17-dihydroxy-19-norpregna-1,3,5(10)-trien-3-yl]oxy]estra-1,3,5,6,8 pentaen-3,17 *β*-diol	(Pezzella et al., 2005)
17*β*-estradiol	lignin peroxidase from *Phanerochaete chrysosporium*	free/batch reactor	100%	veratryl alcohol (VA)	270 – estrone E1 541 – dimer E2 811 – trimer E2	(Mao et al., 2009)
17*β*-estradiol	lignin peroxidase from *Phanerochaete chrysosporium*	free/batch reactor	n.a.	veratryl alcohol (VA)	270 – estrone, 541 – dimer E2, 811 – trimer E2	(Mao et al., 2010a)

17*β*-estradiol	lignin peroxidase from *Phanerochaete chrysosporium*	free/batch reactor	n.a.	-	541 – dimer E2, 811 – trimer E2	(Mao et al., 2010b)

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841 **Table 4.** Toxicity and estrogenic activity of mixtures of estrogens after enzymatic treatment by free and immobilized oxidoreductases. n.a. – not

842 available

Target compound	Enzyme and its form (free/immobilized)	Removal efficiency (%)	Methods of toxicity evaluation	Reduction of estrogenic activity	References
estrone 17*β*-estradiol 17*α*-ethinylestradiol	free laccase from *Trametes* sp. Ha1	>90%	medaka vitellogenin assay system	100% (for mixture of estrogens)	(Tanaka et al., 2009)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	free laccase from *Trametes versicolor*	83.5 100% 71.3%	yeast estrogen screen (YES) assay	>98%	(Shreve et al., 2016)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	free laccase from *Trametes pubescens*	100% 100% 100%	MELN gene-reporter luciferase assay	100%	(Spina et al., 2015)
17*β*-estradiol	laccase from *Coriolopsis gallica* immobilized onto prepolymer Hypol 2002	n.a.	zebrafish (*Danio rerio*)	100%	(Torres-Duarte et al., 2012)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	laccase from *Myceliophthora thermophila* immobilized by encapsulation in sol-gel matrix based on methyltrimethoxysilane and tetramethoxysilane	55% 75% 60%	yeast estrogen screen-assay assisted by enzymatic digestion with lyticase (LYES)	63% (for mixture of estrogens)	(Lloret et al., 2011)
estrone 17*β*-estradiol 17*α*-ethinylestradiol	commercially available laccase immobilized on Eupergit C 250 L support	92% 100% 92%	yeast estrogen screen-assay assisted by enzymatic	90% (for mixture of estrogens)	(Lloret et al., 2012c)

			digestion with lyticase (LYES)		
estrone 17β-estradiol 17α-ethinylestradiol	free lignin peroxidase from *Phanerochaete sordida*	23.9% 38.2% 45.0%	yeast two-hybrid estrogenic assay system	100% 72.6% 82.6%	(Wang et al., 2012)
17β-estradiol 17α-ethinylestradiol	free manganese peroxidase from *Phanerochaete chrysosporium* free laccase from *Trametes versicolor*	n.a.	yeast two-hybrid estrogenic system assay	100% 100%	(Suzuki et al., 2003)
estrone	free laccase from white-rot fungus *Phanerochaete sordida* free manganese peroxidase from white-rot fungus *Phanerochaete sordida*	100% 100%	yeast two-hybrid estrogenic assay system	100% 100%	(Tamagawa et al., 2006)
estrone 17β-estradiol	free laccase from *Trametes versicolor*	100% removal of all compounds	yeast strain BY4741	100% (for mixture of estrogens)	(Auriol et al., 2008)
estriol 17α-ethinylestradiol	free horseradish peroxidase *Trametes versicolor*	100% removal of all compounds		100% (for mixture of estrogens)	
17α-ethinylestradiol	free laccase, manganese peroxidase and lignin peroxidase from	100%	yeast estrogen screen (YES) *Saccharomyces cerevisiae*	94%	(Cajthaml et al., 2009)

	Trametes versicolor				
17α-ethinylestradiol 17β-estradiol	versatile peroxidase from *Bjerkandera adusta* co-immobilized by cross-linking with glucose oxidase from *Aspergillus niger*	93.1% 90.1%	yeast estrogen screen-assay assisted by enzymatic digestion with lyticase (LYES)	60.4% 72.5%	(Taboda-Puig et al., 2011)

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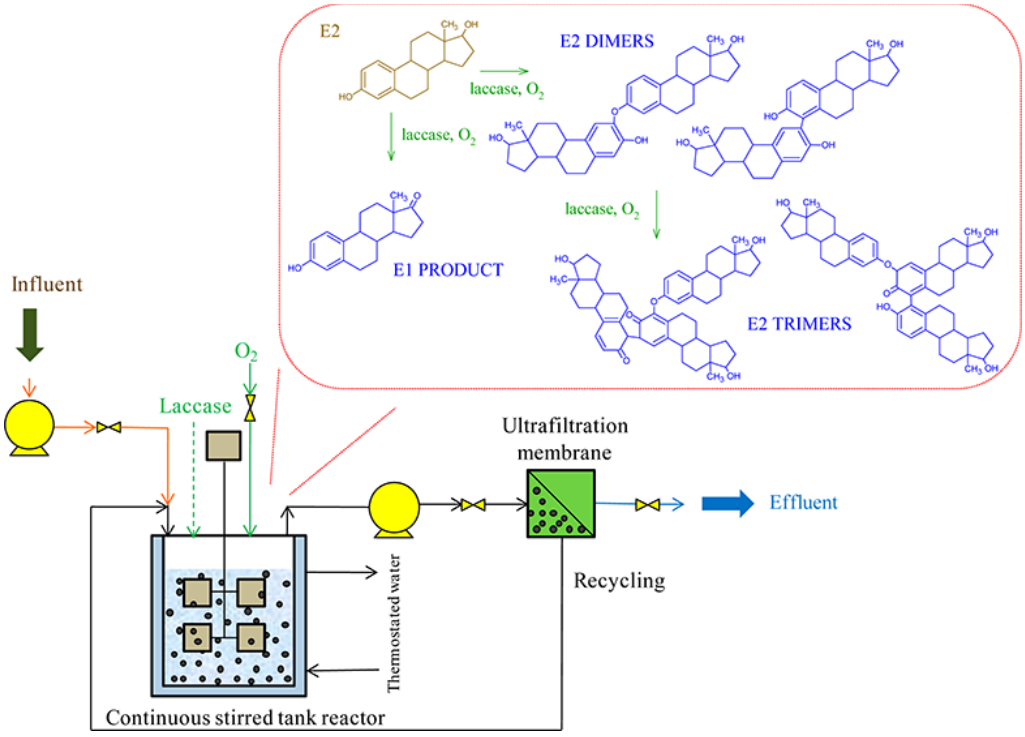
844 **Figure 1**



845

846 **Fig. 1.** Experimental setup with continuous enzymatic stirred tank reactor containing laccase
847 for estrone and 17*β*-estradiol treatment, with by-products (Source: Lloret et al. (2013a), with
848 permission from American Chemical Society. Copyright (2013) American Chemical Society.).

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Figure 2

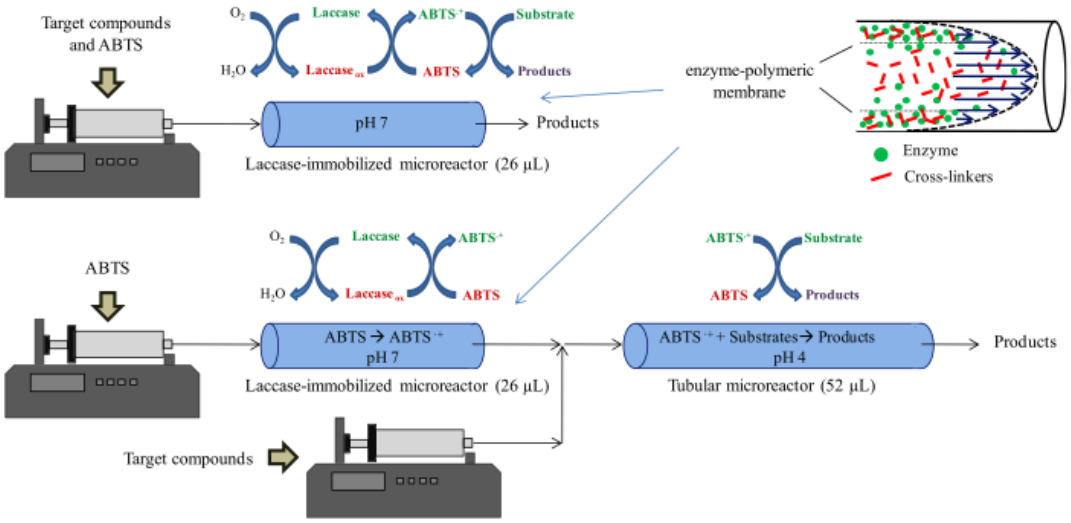


Fig. 2. Experimental setup consisting of laccase from *Trametes versicolor* immobilized by covalent binding on the inner wall of microtubes and formed laccase-immobilized microreactors for removal of estrogens (Source: Lloret et al. (2013b), with permission from Elsevier. Copyright (2013) Elsevier. License number 4965800793519.).

Figure 3

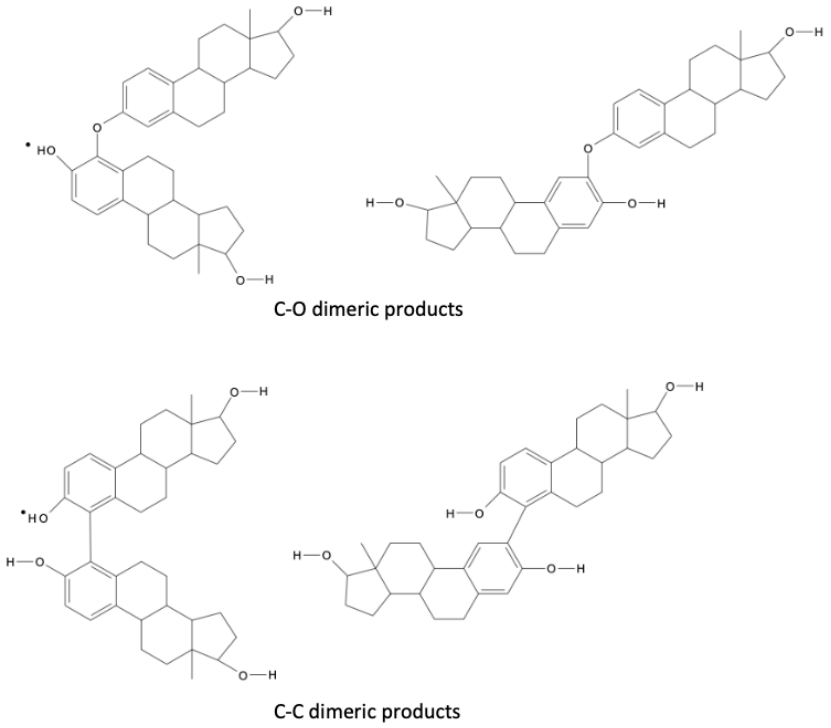
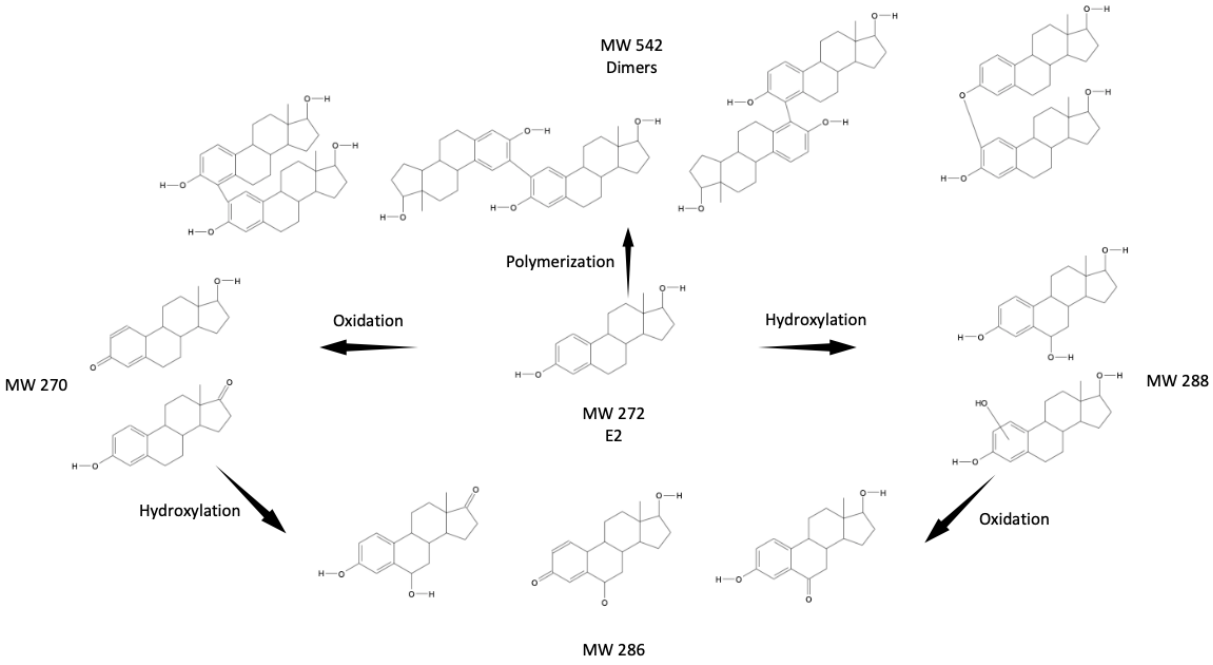


Fig. 3. Main reaction products of 17*β*-estradiol oxidation by laccase from *Myceliophthora thermophila* represents C-O dimeric products and C-C dimeric products. Based on Lloret et al. (2013a).

865 **Figure 4**



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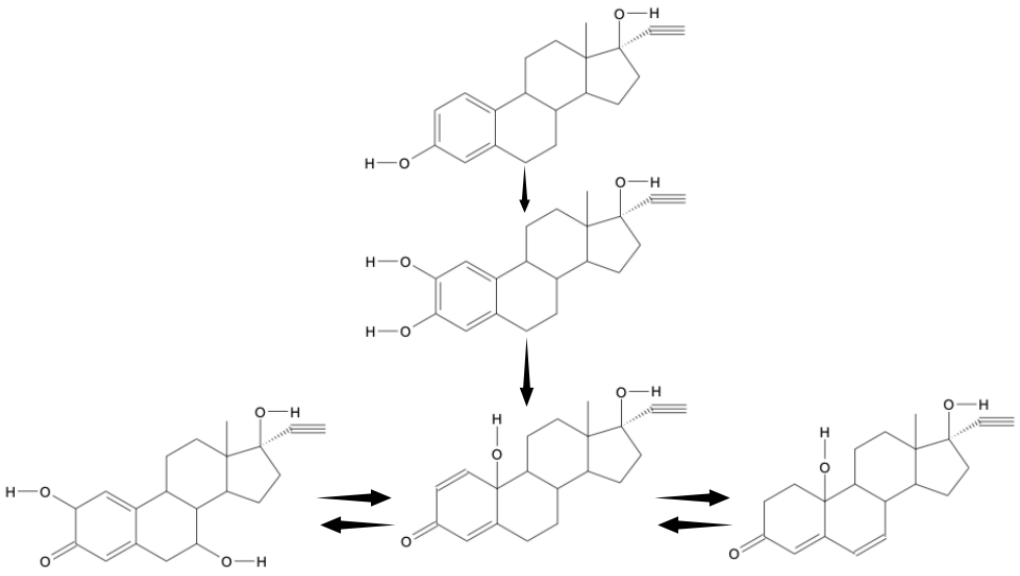
868 **Fig. 4.** Proposed pathways of 17*β*-estradiol and products formed after degradation of 17*β*-

869 estradiol by HRP system. Based on Li et a. (2017).

870 **Figure 5**



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872 **Fig. 5.** Proposed scheme of 17*α*-ethinylestradiol degradation by laccase from *P. sanguineus*

873 supported by Cupuacu (*Theobroma grandiflorum*) residue. Based on Golveia et al. (2018).

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