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Review

Elimination of Endocrine Disrupting Chemicals using White Rot Fungi and their Lignin Modifying Enzymes: A Review

The ability of white rot fungi (WRF) and their lignin modifying enzymes (LMEs), i.e. laccase and lignin- and manganese-dependent peroxidase, to treat endocrine disrupting chemicals (EDCs) is extensively reviewed in this paper. These chemicals cause adverse health effects by mimicking endogenous hormones in receiving organisms. The alkylphenolic EDCs nonylphenol, bisphenol A and triclosan, the phthalic acid esters dibutylphthalate, diethylphthalate and di-(2-ethylhexyl)phthalate, the natural estrogens estrone, 17β-estradiol, estriol and 17α-ethynylestradiol and the phytoestrogens genistein and β-sitosterol have been shown to be eliminated by several fungi and LMEs. WRF have manifested a highly efficient removal of EDCs in aqueous media and soil matrices using both LME and non LME-systems. The ligninolytic system of WRF could also be used for the elimination of several EDCs and the associated hormone-mimicking activity. The transformation of EDCs by LMEs and WRF is supported by emerging knowledge on the physiology and biochemistry of these organisms and the biocatalytic properties of their enzymes. Due to field reaction conditions, which drastically differ from laboratory conditions, further efforts will have to be directed towards developing robust and reliable biotechnological processes for the treatment of EDC-contaminated environmental matrices.

Keywords: Biodegradation, Biotransformation, Endocrine Disrupting Chemicals (EDC), Fungi

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1 Introduction

There is extensive scientific evidence available showing that low-dose man-made chemicals released into environmental matrices have the potential to modulate or disrupt the endocrine system of living organisms present in receiving ecosystems [1]. This growing environmental problem requires the development of (bio)processes capable of efficiently eliminating these endocrine disrupting chemicals (EDCs) and the associated hormonal disruptions.

Bioprocesses involving microorganisms or enzymes as biocatalysts are cost-competitive technological options with attractive properties such as low energy requirements, easy process control and operability over a wide range of pHs, temperatures and ionic strengths [2]. Among the bioprocesses developed, white rot fungi (WRF) and their lignin modifying enzymes (LMEs) have been successfully used for the treatment of several xenobiotics. They have been applied in bioremediation technologies for the treatment of dyes, PAHs, PCBs, phenols, pesticides and industrial wastes in different environments [2–5].

The aim of this article is to review the different bioprocesses involving WRF and their LMEs for the removal of EDCs in different environmental matrices.

1.1 White Rot Fungi and their Lignin Modifying Enzymes

WRF are a diverse ecophysiological group which includes basidiomycetes and litter-decomposing fungi capable of extensive aerobic lignin depolymerization and mineralization. WRF produce one or more extracellular LMEs whose low substrate specificity enables them to also degrade a wide range of xenobiotics. Their superiority in many cases over bacteria is attributed to the extracellular nature of the LMEs. In addition, they secrete low molecular weight mediators that enlarge the spec-

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trum of compounds they are able to oxidize [5]. Moreover, these eukaryotes can operate over wide ranges of temperatures and pH. LMEs of WRF are expressed under nutrient-deficient conditions, which are prevalent in many soils. The filamentous nature of WRF growth by hyphal extension in soils and other matrices allows them to reach pollutants that may remain inaccessible to bacteria [4].

The main LMEs are lignin peroxidases (LiP, E.C. 1.11.1.14), manganese-dependent peroxidases (MnP, E.C. 1.11.1.13), versatile peroxidases (VP, E.C. 1.11.1.16) and laccases (Lac, E.C. 1.10.3.2). These enzymes are essential for lignin degradation even if the mineralization of this recalcitrant biopolymer requires the combination of its action with other processes involving additional enzymes. Such auxiliary enzymes (by themselves unable to degrade lignin) are glyoxal oxidase and superoxide dismutase for intracellular production of H_2O_2, a co-substrate of LiP and MnP, as well as glucose oxidase, aryl alcohol oxidase and cellobiose dehydrogenase involved in feedback circuits and linking ligninolysis with cellulose and hemicellulose degradation in nature [6]. Such combinations of LMEs with auxiliary oxidoreductive enzymes are also efficient in the degradation of refractory xenobiotics by WRF, often in association with different low molecular weight redox mediators [7].

Varying types and levels of LME are produced by different species of WRF [8, 9], in response to varying culture conditions [10]. Lignin has been found to be partly mineralized in cell-free systems of LMEs, with considerably enhanced rates in the presence of co-oxidants such as fatty acids [11] or thiols [12]. In this way, an older concept of ligninolysis is re-established, namely enzymatic "combustion" [13]. By extension, this enzyme-assisted process is applicable to the degradation of many other recalcitrant molecules, such as EDCs.

The physiology of LME production by WRF for ligninolysis or recalcitrant pollutant degradation has been studied extensively. Several more-or-less general statements can be made despite the many exceptions that are due to the wide variety of fungal strains and of experimental conditions reported: LMEs are produced by WRF during their secondary metabolism since lignin oxidation provides no net energy to the fungus. Synthesis and secretion of these enzymes is often induced by limited nutrient levels (mostly C or N). Production of LiP and MnP is generally optimal at high oxygen partial pressure but is repressed by agitation in submerged WRF liquid culture, while laccase formation is often enhanced by agitation. Frequently, more than one isoform of LMEs are expressed by different strains and culture conditions. The utilization of WRF and their LMEs for the treatment of recalcitrant xenobiotics has been widely reported [2, 14]. Several parameters, such as temperature, pH, additives and the presence of common wastewater constituents or co-contaminants, such as inorganic salts, organic chemicals and heavy metals, could have an impact on the LME-catalyzed removal of EDCs. These parameters influence the enzymatic activity, stability and substrate specificity of the enzyme employed. These features are important in the process design and optimization of fungal and LME treatment of EDC-containing effluents. Tab. 1 reports fungal and LME-mediated processes of elimination of EDCs; the type of treatment, the matrix in which the treatment was performed and the removal rate are specified.

1.2 Endocrine Disrupting Chemicals

Over the last 50 years, a large number of scientific data concerning the hormone-like effects of anthropogenic chemicals in the environment have been accumulated [1, 15]. The release of human hormones into environmental matrices also generates hormonal disruptions in populations present in receiving ecosystems. The effects of these EDCs are believed to be due to their ability to act as a hormone agonist or antagonist and to disrupt the synthesis of endogenous hormones or hormone receptors. These EDCs can disturb the synthesis, secretion, transport, binding, action and elimination of the endogenous hormones which are responsible for maintaining homeostasis, reproduction, development and integrity in living organisms and their progeny. In this review, the authors focus on chemicals that induce endocrine disruptions at typical environmental concentrations, even though no toxicity is exhibited at such low levels. Much attention has been paid to xenobiotics which disrupt the sexual behavior of aquatic organisms at environmentally relevant concentrations [16].

Fig. 1 shows the chemical structure of the EDCs that have been reported as treatable by means of several WRF and their LMEs. These EDCs have structural similarities with endogenous hormones such as estrogens [17]. Tab. 2 presents some of the endocrine disruptions brought about by these chemicals in different living organisms.

1.2.1 Bisphenol A

Bisphenol A (BPA) is used as a raw material for the production of polycarbonates and epoxy resins. Its discharges into the environment can occur from BPA producing factories, from installations that incorporate BPA into plastic [18], from leaching of plastic wastes [19] and landfill sites [20]. Furthermore, BPA is reported as a persistent metabolite from the anaerobic treatment of the flame retardant tetrabromobisphenol A [21].

The concentration of BPA in surface and marine water can reach up to 21,000 μg/L [22–31] and up to 191 ng/L in sediments [32]. BPA is known to be readily biodegraded in river water due to the wide distribution of BPA-degrading bacteria [33]. Concentrations of BPA up to 2.2 μg/L [24, 28, 31, 34–38] in sewage effluents and up to 2.89 μg/g [35] in sewage sludges have been reported. Bacteria able to degrade BPA have been isolated from a sewage treatment plant (STP) [39] and river water [40].

There is a great concern about the impact of the low-dose BPA effect on organisms living in different environmental matrices. Most of the published in vivo studies report significant effects of this EDC on organisms [41]. Tab. 2 presents some of the reports on the endocrine disruptions associated with BPA. This chemical has been reported to interact with estrogen, androgen and thyroid receptors. The hormonal dysfunctions produce transformation of biological activities in exposed organisms [42–46] such as morphological and functional alterations of genitals and mammalian glands [47]. Structure-activity investigations are highlighting the ability of BPA to act as estrogen derive from the presence of a phenolic group on the molecule [17].

Table 1. Elimination of EDCs by WRF and their LMEs.

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	Rate of EDC removal	Rate of removal of estrogenic activity	Comments	Reference
Bjerkandera sp.	WRF	NP	45 mg/L	Aqueous	95 % (at 9.7 mg/L d) after 5 days of incubation.		The rate of NP removal was favored by an agitated culture.	[106]
	WRF	NP	430 mg/kg	Soil	98 % after a 5-week incubation period.		Grew at the surface of soil.	[107]
	WRF	β-sit	0.21 mg/g oven dry wood	Scots pine sapwood matrix	75 % and 100 % after 2 and 4 weeks of incubation.		This strain was able to remove other wood pitch constituents. This strain caused less than 4 % woody mass losses in 4 weeks. Allowed a 16.9-fold detoxification of the wood pitch.	[108, 109]
	WRF	β-sit	0.16 mg/g oven dry wood	Scots pine sapwood matrix	69 % after a 6-week incubation.			[110]
	WRF	β-sit	0.27 mg/g oven dry wood	Scots pine heartwood matrix	18 % after a 6-week incubation.			[110]
Clavariopsis aquatica	Fungi	NP	100–250 μM	Aqueous	50 % and 60 % of a 250 μ solution of t-NP for contact times of 11 and 26 days.			[111, 112]
	Free laccase	NP	203.8–235.1 μM	Aqueous	22.1 % and 14.0 % of 4nNP and t-NP after a contact time of 24 hours.		The laccase/ABTS (1 mM) increases the elimination of t-NP to 97.2 % after 24 hours.	[112]
Coriolopsis polyzona	Free accase	BPA	5 mg/L	Aqueous	40 % and 100 % after contact times of 1 and 4 hours	35 % and 95 % after contact times of 1 and 4 hours.	Determination of optimal conditions of treatment in regard to the controllable parameters, i.e., temperature and pH using a factorial design. The laccase/ABTS system significantly increases the rate of BPA transformation. Identification of high molecular weight chemicals produced by the action of laccase.	[113]
	Free laccase	NP	5 mg/L	Aqueous	80 % and 100 % after contact times of 1 and 4 hours.	80 % and 95 % after contact times of 1 and 4 hours.	Determination of optimal conditions of treatment in regard to the controllable parameters temperature and pH using a factorial design. The laccase/ABTS system significantly increases the rate of NP transformation. Identification of high molecular weight chemicals produced by the action of laccase.	[113]
	Free laccase	TCS	5 mg/L	Aqueous	15 % and 60 % after contact times of 1 and 8 hours.		Identification of high molecular weight chemicals produced by the action of laccase.	[113]

Table 1. Continued.

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	Rate of EDC removal	Rate of removal of estrogenic activity	Comments	Reference
	Insolubilized as CLEA	BPA	5 mg/L	Aqueous	50 % and 90 % after a hydraulic residence time of 40 and 150 minutes		Utilization in a fluidized bed reactor. The kinetic constants K_m and k_{cat} were 0.23 mM and 0.16 μmol/s mg, respectively.	[114]
	Insolubilized as CLEA	NP	5 mg/L	Aqueous	80 % and >95 % after a hydraulic residence time of 40 and 60 minutes.		Utilization in a fluidized bed reactor. The kinetic constants K_m and k_{cat} were 0.45 mM and 0.8 μmol/s mg, respectively.	[114]
	Insolubilized as CLEA	TCS	5 mg/L	Aqueous	80 % and >95 % after a hydraulic residence time of 40 and 60 minutes.		Utilization in a fluidized bed reactor. The kinetic constants K_m and k_{cat} were 0.12 mM and 0.24 μmol/s mg, respectively.	[114]
Coriolus versicolor	Laccase/ reversed micelles	BPA	100 μM	Organic solvent	50 % and 80 % after a contact time of 60 and 140 minutes.		Determination of optimal conditions of treatment in regard to the controllable parameters pH, laccase concentration, hydratation degree and mediator. The utilization of 1-HBT did not improve the conversion rate of BPA.	[115]
	Laccase/ reversed micelles	NP	100 μM	Organic solvent	100 % after a contact time of 1 hour.		Utilization of the optimal conditions determined for BPA conversion. The utilization of the laccase/1-HBT system did not improve the NP-removal.	[115]
	Confined laccase	BPA	2 mM	Aqueous	50–90 % and 100 % for contact times of 24 and 96 hours.		Extent of removal depends of the MWCO of the dialysis membrane.	[116]
	Free laccase	NP	3 μmol/g soil	Soil	65 % and 90 % after a contact time of 0.25 and 1 day.		Impact of the pH on the rate of elimination.	[117]
	Free laccase	BPA	3 μmol/g soil	Soil	80 % and 100 % after a contact time of 5 days.			[117]
	Phytoremediation	BPA	100 μM	Aqueous	90–275 μM of BPA per gram of plant after a 2-month growth period.	Significant diminution of the estrogenic activity.	Hydroponic culture of transgenic tobacco plant secreting laccase.	[118]
Cunninghamella sp.	WRF	NP	11 mg/L	Aqueous	Half-lives: 1 day (4nNP), 2 days (NP).			[119]
Daldinia concentrica	WRF	DBP	28 mg/L	Aqueous	94 % and 100 % after 1 and 6 days of incubation.	50 % and 100 % after 1 and 6 days of incubation.	Identification of metabolites produced. Growth reduced by a factor of 2.5 at a DBP concentration of 500 mg/L.	[120]
Funalia trogii	WRF	β -sit	0.16 mg/g oven dry wood	Scots pine sapwood matrix	50 % after a 6-week incubation.		This strain was able to remove other wood pitch constituents.	[110]
	WRF	β -sit	0.27 mg/g oven dry wood	Scots pine heartwood matrix	41 % after a 6-week incubation.		This strain was able to remove other wood pitch constituents.	[110]

Table 1. Continued.

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	Rate of EDC removal	Rate of removal of estrogenic activity	Comments	Reference
Fusarium sp.	WRF	NP	11 mg/L	Aqueous	Half-lives: 1–2 days (4nNP), >8 days (NP).		Strains isolated from a soil/sludge mixture contaminated by NP.	[119]
Heterobasidium insulare	WRF	BPA	200 mg/L	Aqueous	77 % and 100 % after 3 and 14 days of incubation.	100 % after a 1-day incubation.	High resistance to BPA (up to 100 mg/L).	[121]
Ischnoderma benzoinum	WRF	β -sit	0.24 mg/g oven dry wood	Scots pine sapwood matrix	79.6 % after a 4-week incubation.		This strain was able to remove other wood pitch constituents. Allowed a 6-fold detoxification of the wood pitch.	[109]
Mucor sp.	WRF	NP	11 mg/L	Aqueous	Half-lives: 1.5–2 days (4nNP), 3–5 days (NP).		Strains isolated from a soil/sludge mixture contaminated by NP.	[119]
Myceliopthora sp.	Immobi-lized laccase	E2	4.9 g/L	Organic solvent			Production of E2-dimers. Identification of the dimer structures.	[122]
Peniophora pini	WRF	β -sit	0.23 mg/g oven dry wood	Scots pine sapwood matrix	76.9 % after a 4-week incubation.		This strain was able to remove other wood pitch constituents. Allowed a 4-fold detoxification of the wood pitch.	[109]
Phanerochaete chrysosporium	WRF	NP	11 mg/L	Aqueous	Half-lives: 6 days (4nNP), 3 days (NP).			[119]
	WRF	DBP	28 mg/L	Aqueous	85 % after 6 and 20 days of incubation.		Growth inhibited at a DBP concentration of 500 mg/L.	[120]
	Free MnP	BPA	0.22 mM	Aqueous	90 % and 100 % after contact times of 30 and 60 minutes.	40 % and 90 % after contact times of 4 and 6 hours for a 0.88 mM solution.		[123]
	Free MnP	NP	0.23 mM	Aqueous	90 % and 95 % after contact times of 30 and 60 minutes.	60 % and 80 % after contact times of 1 and 5 hours for a 0.92 mM solution.		[123]
	Free MnP	E2	10^{-5} M	Aqueous	100 % after a contact time of 1 hour.	80 % and 100 % after 1 and 4 hours		[124]
	Free MnP	EE2	10^{-5} M	Aqueous	100 % after a contact time of 1 hour.	90 % and 100 % after 1 and 4 hours		[124]
	LiP/rever-sed micelles	BPA	0.1 mM	Organic solvent	40 % and 50 % after contact times of 30 and 120 minutes.		Determination of optimal conditions: LiP activity in regard to the controllable parameters pH, temperature, organic solvent type, water content, AOT concentration, LiP and H_2O_2 concentrations.	[125]
	LiP/rever-sed micelles	NP	0.1 mM	Organic solvent	70 % and 85 % after contact times of 30 and 120 minutes.			[125]
Phanerochaete sordida	WRF	E1	10^{-5} M	Aqueous	70 % and 100 % after 3 and 6 days of incubation.		2-day lag period.	[126]

Table 1. Continued.

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	Rate of EDC removal	Rate of removal of estrogenic activity	Comments	Reference
	WRF	GEN	10^{-4} M	Aqueous	50 % and 95 % after 3 and 4 days of incubation.		2-day lag period.	[127]
	Free MnP	E1	10^{-5} M	Aqueous	100 % after a contact time of 1 hour.	99 % and 100 % after a contact time of 1 and 2 hours.		[126]
	Free MnP	GEN	10^{-4} M	Aqueous	90 % and 100 % after contact times of 1 and 4 hours.	90 % and 100 % after contact times of 1 and 4 hours.		[127]
Pleurotus ostreatus	WRF	BPA	0.4 mM	Aqueous	80 % and 85 % after 12 and 21 days of cultivation.			[128]
	Free MnP	BPA	0.4 mM	Aqueous	100 % after a 1-hour treatment.		Identification of metabolites.	[128]
	Free laccase	NP	3 µmol/g soil	Soil	35 % and 55 % after a contact time of 1 and 3 days.		Elimination in test tubes.	[117]
Polyporus versicolor	Laccase/ emulsion system	E2		Organic solvent		The 5 metabolites purified did not show estrogenic activity.	Determination of optimal conditions of treatment in regard to the controllable parameters pH, laccase concentration and organic solvent type.	[129]
	Laccase/ emulsion system	E1, E3		Organic solvent			The laccase action produced 3 unidentified chemicals.	[129]
Pycnoporus cinnabarinus	WRF	TCS	0.25 mM	Aqueous			Identification of metabolites produced. Metabolites were less toxic than TCS.	[130]
Pycnoporus coccineus	Free laccase	BPA	3 µmol/g soil	Soil	15 % after a contact time of 5 hours.		Elimination in test tubes.	[131]
	Free laccase	EE2	3 µmol/g soil	Soil	40 % and 80 % after a contact time of 5 and 48 hours.		Elimination in test tubes.	[117, 131]
	Free laccase	NP	3 µmol/g soil	Soil	40 % and 80 % after a contact time of 5 and 48 hours.		Elimination in test tubes.	[117]
	Free laccase	E1	3 µmol/g soil	Soil	20 % and 60 % after a contact time of 1 and 3 days.		Elimination in test tubes.	[117]
Russula delica	Laccase	E1, E2, E3	0.33 g/L	Organic solvent			The oxidation of estrogens was inhibited by KCN.	[132]
	WRF	BPA	200 mg/L	Aqueous	68 % and 100 % after 3 and 14 days of incubation.	40 % and 100 % after 1 and 3 days of incubation.	High resistance to BPA (up to 100 mg/L).	[121]
Stereum hirsutum	WRF	β -sit	0.21 mg/g oven dry wood	Scots pine sapwood matrix	100 % after a 4-week incubation.		This strain was able to remove other wood pitch constituents. This strain caused a 7 % woody mass loss in 4 weeks. Allowed a 13.5-fold detoxification of the wood pitch.	[109]

Table 1. Continued.

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	Rate of EDC removal	Rate of removal of estrogenic activity	Comments	Reference
Stereum sanguinolentum	WRF	β -sit	0.23 mg/g oven dry wood	Scots pine sapwood matrix	53.2 % after a 4-week incubation.		This strain was able to remove other wood pitch constituents. Allowed a 2.5-fold detoxification of the wood pitch.	[109]
Trametes sp.	Combination laccase/ activated sludge	BPA	5–100 mg/L	Aqueous			The treatment efficiency depends on the initial laccase treatment time and the contact time with activated sludge.	[133]
	Laccase / reversed micelles	BPA	200 µM	Organic solvent	40–95 % and 50–100 % after a contact time of 1 and 3 hours depending on the water content (1–4 % v/v).		Determination of the optimal type of organic solvent and water content. Identification of BPA products after laccase oxidation. Treatment of chlorophenols.	[134]
	Immobilized laccase	BPA	0.1–3 mM	Aqueous	134 µg of BPA after a residence time of 30 minutes.		Covalently immobilized on controlled porosity glass. The removal was improved by the electrolytic generation of oxygen. The column performances in regard to BPA removal were maintained over a 10-day period.	[135, 136]
	Free laccase	NP	3 µmol/g soil	Soil	60 % and 80 % for a contact time of 0.5 and 4 hours.	83.3–98.9 % after a contact time of 24 hours in the rotating reactor.	Utilization of test tubes and a rotating reactor. Determination of optimal conditions of treatment in regard to the controllable parameters pH, temperature, rotation speed and enzyme concentration.	[131, 137]
	Free laccase	BPA	3 µmol/g soil	Soil	85 % after a contact time of 5 hours in test tubes. 90 % and 100 % removal after 2 and 8 hours of treatment in the rotating reactor.		The removal was performed in test tubes and in rotating reactor.	[131]
	Free laccase	EE2	3 µmol/g soil	Soil	60 % and 90 % after a contact time of 5 and 48 hours in test tubes. 60 % and 80 % removal after 4 and 8 hours of treatment in the rotating reactor.		The removal was performed in test tubes and in a rotating reactor.	[131]
Trametes hirsuta	Pilot-scale fungal/UF system	NP	2.9 µg/L	Aqueous	> 94% after an overall cycle of 1.5 day.		Sequencing batch mode reactor.	[138]
	Pilot-scale fungal/UF system	DEHP	1.1 µg/L	Aqueous	45 % after an overall cycle of 1.5 day.		Sequencing batch mode reactor.	[138]
Trametes pubescens	Free laccase/ emulsion system	E2	5 g/L	Organic solvent			Production of E2-dimers. Identification of the dimer structures.	[122]

Table 1. Continued.

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	Rate of EDC removal	Rate of removal of estrogenic activity	Comments	Reference
Trametes versicolor	WRF	NP	45 mg/L	Aqueous	90 % (at 2.8 mg/L d) after 15 days of incubation.		The rate of NP removal was favored by a static culture.	[106]
	WRF	NP	11 mg/L	Aqueous	half-lives : 1 day (4nNP), < 1 day (NP)			[119]
	WRF	TCS	0.25 mM	Aqueous	60 % and 90 % after 1 and 4 weeks of incubation.		Identification of metabolites produced. Metabolites less toxic than TCS.	[130]
	WRF	DBP	28 mg/L	Aqueous	83 % and 100 % after 1 and 6 days of incubation.		Growth inhibited at a DBP concentration of 500 mg/L.	[120]
	WRF	NP	430 mg/kg	Soil	98 % after a 5-week incubation period.		Grew diffusely through the soil.	[107]
	WRF	β-sit	0.24 mg/g oven dry wood	Scots pine sapwood matrix			This strain was able to remove other wood pitch constituents. This strain caused less than 4 % woody mass losses in 4 weeks. Allowed a 7.4-fold detoxification of the wood pitch.	[108, 109]
	Free laccase	BPA	0.22 mM	Aqueous	50 % and 70 % after contact times of 30 and 60 minutes.	40 % and 60 % after a contact time of 1 and 6 hours for a 0.88 mM solution.	The action of the laccase/1-HBT system significantly improved the rate of BPA and the removal of estrogenic activity.	[123]
	Free laccase	BPA	120 µM	Aqueous			Determination of optimal conditions of treatment in regard to the controllable parameters temperature, pH and mediator. They also determine the impact of the wastewater constituents on the BPA transformation.	[139]
	Free laccase	NP	0.23 mM	Aqueous	10 % and 60 % after contact times of 30 and 60 minutes.	10 % and 60 % after a contact time of 4 and 9 hours for a 0.92 mM solution.		[123]
	Free laccase	NP (4nNP)	5 mg/L	Aqueous	90 % and 100 % after a contact time of 5 and 90 minutes.		The NP-transformation rate was pH dependent.	[119]
	Free laccase	TCS	20 µM	Aqueous			Determination of optimal conditions of treatment in regard to the controllable parameters temperature, pH and mediator. They also determine the impact of the wastewater constituents on the TCS transformation. K_m and V_{max} were 24 µM and 0.92 µmol/L min, respectively, at pH 5, 25 °C and in the presence of 3000 U/L of laccase activity. V_{max} was improved 65-fold when using ABTS at a concentration of 20 µM as a redox mediator. Finally, the residual toxicity of TCS solution treated by laccase was determined.	[140]

Table 1. Continued.

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	Rate of EDC removal	Rate of removal of estrogenic activity	Comments	Reference
	Free laccase	E1	10^{-5} M	Aqueous	100 % after a contact time of 1 hour.	100 % after a contact time of 1 hour.	The laccase/1-HBT (0.2 mM) system did not increase the transformation rate.	[126]
	Free laccase	E2	10^{-5} M	Aqueous	100 % after a contact time of 1 hour.	80 % and 100 % after 1 and 8 hours.	The laccase/1-HBT (0.2 mM) system did not increase the transformation rate.	[124]
	Free laccase	EE2	10^{-5} M	Aqueous	100 % after a contact time of 1 hour.	10 % and 100 % after 1 and 8 hours.	The laccase/1-HBT (0.2 mM) system did not increase the transformation rate.	[124]
	Free laccase	GEN	10^{-4} M	Aqueous	95 % and 100 % after contact times of 1 and 2 hours.	95 % and 100 % after a contact time of 1 and 2 hours.	The laccase/1-HBT (0.2 mM) system did not increase the transformation rate.	[127]
	Immobilized laccase	BPA	0–5 mM	Aqueous			Immobilized onto a nylon-poly(glycidyl methacrylate) membrane used in a non-isothermal bioreactor. The Michaelis-Menten kinetic parameters K_m and V_{max} vary from 0.10–1.20 mM and 1.8–3.4 μmol/min, respectively, as a function of T_{av} and ΔT.	[141]
Trametes villosa	Free laccase	BPA	2.2 mM	Aqueous	100 % after a contact time of 3 hours.	Both soluble and insoluble fractions of BPA reaction products presented no estrogenic activity.	Recombinant laccase from *T. villosa* produced by *Aspergillus oryzae*. The authors identified metabolites produced by the oxidative action of laccase and they suggested a BPA transformation pathway. The K_m and k_{cat} values for BPA were 14.1 mM and 59.1 μmol/min mg protein, respectively.	[142–144]
Mitosporic strain UHH 1-6-18-4	Fungi	NP	100–250 μM	Aqueous	75 % and 100 % of a 250 μM solution of t-NP for contact times of 11 and 26 days.		Strain isolated from NP-contaminated river.	[111, 112]
	Free laccase	NP	196.7–321.1 μM	Aqueous	46.2 % and 63.5 % of 4nNP and t-NP after a contact time of 24 hours.		The laccase/ABTS (1 mM) increased the removal of t-NP to 97.2 % after 24 hours.	[112]
Strain I-4 isolated from a Japanese soil	Free laccase	BPA	5 mM	Aqueous	95 % and 100 % after contact times of 1 and 3 hours.	After a 24-h treatment, all of the estrogenic activity was removed.	The K_m and k_{cat} values were 10000 μM and 14 s^{-1}, respectively. The laccase/1-HBT (1 mM) did not improve the transformation rate.	[145, 146]
	Free laccase	NP	5 mM	Aqueous	70 % and 100 % after contact times of 1 and 6 hours.	After a 24-h treatment, all of the estrogenic activity was removed	The K_m and k_{cat} values were 5000 μM and 1 s^{-1}, respectively.	[145, 146]

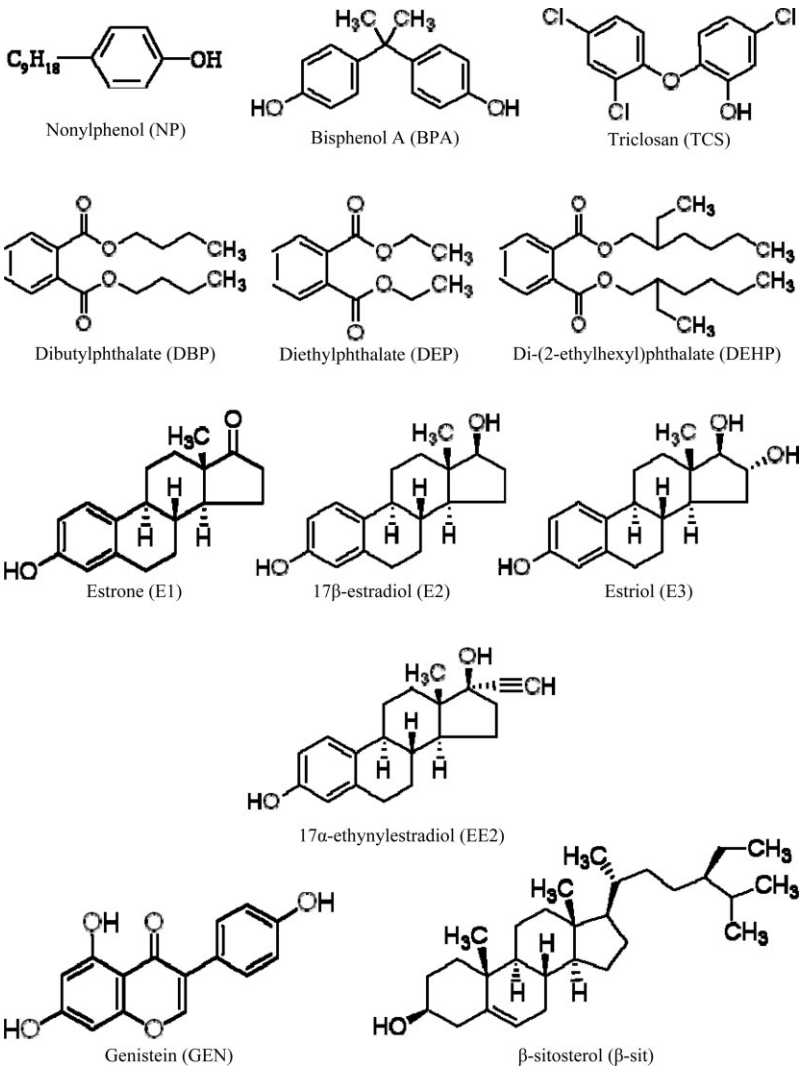

Figure 1. Chemical structure of endocrine disrupting substances eliminated from various environmental matrices by several white rot fungi and their lignin modifying enzymes.

BPA has been shown to be eliminated from aqueous and soil matrices by the action of different WRF and free laccase or MnP and by the action of more industrially relevant treatments such as using immobilized enzymes, transgenic plants, fungal reactors fitted with an ultrafiltration unit or the combination of laccase and activated sludge treatment. These different approaches are presented below.

1.2.2 Nonylphenol

The presence of nonylphenol (NP) isomers in the aquatic environment is related to the biodegradation in STP of nonylphenol ethoxylates [48, 49] which are mainly used as non-ionic surfactants in domestic and industrial applications. The NP produced in this way is thought to be slowly biodegradable under aerobic conditions [50, 51]. This is confirmed by the presence of NP in STP effluents in concentrations ranging from less than 0.2 ng/L to 1000 μg/L [23, 37, 38, 52–62]. Concentrations of NP up to 500 mg/kg in sludges [35, 63] and up to 6.86 μg/L in surface waters [22, 64–67] have been reported. The presence of NP in soil originates from different non-point sources such as atmospheric deposition, application of sewage sludge and the use of plant protection agents [68]. Only a few pure cultures of aerobic bacteria [69–72], one yeast [73] and anaerobic microorganisms [74, 75] have been described to be able to use NP as the sole source of carbon and energy. The sorption to sludges of NP isomers due to their high hydrophobicity (log K_{ow} = 4.48) is the main route of removal of these xenobiotics from wastewaters [62] but it leads to a mere transfer of this type of pollutant to another environmental matrix and not to a destruction or transformation of the substance. The concentration of NP in sediments could be up to 1430 ng/g [32, 76]. Furthermore, the reduction of the available concentration of these chemicals in the aquatic environment is essential due to sorption to particles and sediments and to bioaccumulation in the tissues of aquatic organisms [77]. This accumulation represents a potentially serious environmental and health problem partly due to the well known endocrine disrupting activity associated with this chemical (see Tab. 2). According to Fang et al. [17], the xenoestrogenic activity of NP is linked to the phenolic group in the chemical structure and the high hydrophobicity of the substance.

1.2.3 Triclosan

Triclosan (TCS) is a broad spectrum antimicrobial agent. It has been incorporated into a wide range of Personal care products (PCPs) such as toothpaste, deodorant sticks, soap and handwash. The presence of TCS in the environment essentially comes from STP effluents and sludges. TCS has been detected in STP effluents at concentrations that can reach up to 4.1 μg/L [36, 52, 78–86], in sludges up to 55 mg/kg [78, 81, 83, 85, 87] and in surface water from <0.2 to 431 ng/L [80, 88–90]. As a result of the high hydrophobicity of this chemical (logK_{ow} = 4.8), its dissipation in the aquatic environment occurs by sorption to particles and sediments and it tends to bioaccumulate in aquatic organisms [91]. Attention has been drawn to TCS due to its structural similarity to that of the EDC BPA and of highly toxic contaminants such as dioxins. Little is known about potential endocrine disruption activities linked to TCS. As presented in Tab. 2, TCS is suspected to induce weak estrogenic or androgenic activity or to disrupt gene expression linked with the thyroid hormone. Until now, there exists relatively little research on the removal of TCS using WRF and their LMEs.

Table 2. Some endocrine disruptions associated with EDCs eliminated by LMEs.

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
Bisphenol A (BPA)	10^{-10}–10^{-6} M	Sensitive human breast cancer cells (MCF-7 cells)	Stimulation of glucose-6-phosphate dehydrogenase activity.	[147]
	20–500 mg/kg	Female immature Sprague-Dawley rats	Uterine weight increase. Stimulation of glutathione peroxidase activity.	
		Cell line from fruit fly (*Drosophila melanogaster*)	Antagonistic activity on the ecdysteroid receptor.	[148]
		Premenopausal women	Potential links between BPA exposure and complex endometrial hyperplasia and endometrial cancer.	[149]
	1, 5, 25 and 100 μg/L	Aquatic snail (*Potamopyrgus antipodarum*)	Affects growth of snails. Affects snail embryo production. Interactions with estrogen receptor.	[42]
	500 mg/kg	Immature mouse	Progestogenic and estrogenic properties.	[150]
	0.1, 10 or 100 μg/L	Medaka (*Oryzias latipes*)	Induction of female specific proteins in male fish.	[151]
	10–200 μg/L	Medaka (*Oryzias latipes*)	Induction of choriogenin subunits.	[152]
	10^{-9}–10^{-3} M	Frog (*Xenopus laevis*)	Binding to the liver estrogen receptor.	[153]
	10^{-10}–10^{-5} M	Frog (*Xenopus laevis*)	Induction of the estrogenic biomarker vitellogenin in primary cultured hepatocytes of male.	[154]
	500 μg/d	Male rats	Changes in the volumes of sexually dimorphic brain regions: anteroventral periventricular nucleus of the hypothalamus and the sexually dimorphic nucleus of the preoptic area.	[155]
	10^{-7}–10^{-5} M	Frog tail (*Xenopus laevis*)	Acts as an antagonist of triiodo-thyronine through the suppression of thyroid hormone receptors α and β gene expression.	[156]
	100 μM	Lake trout (*Salvelinus namaycush*)	Inhibition of E2 metabolism.	[157]
Nonylphenol (NP)	0–0.1 mg/L	Daphnia (*Daphnia magna*)	Multi-generational reproductive process disruptions.	[158]
	10^{-10}–10^{-6} M	Sensitive human breast cancer cells (MCF-7 cells)	Stimulation of glucose-6-phosphate dehydrogenase activity.	[147]
	20–500 mg/kg	Female immature Sprague-Dawley rats	Uterine weight increase. Stimulation of glutathione peroxidase activity.	
	250–500 mg/kg	Immature mouse	Progestogenic and estrogenic properties.	[150]
	0.1, 10 or 100 μg/L	Medaka (*Oryzias latipes*)	Induction of female specific proteins in male fish.	[151]
	5–500 μg/L	Medaka (*Oryzias latipes*)	Induction of choriogenin subunits.	[152]
	10^{-9} –10^{-3} M	Frog (*Xenopus laevis*)	Binding to the liver estrogen receptor.	[153]
	10^{-10}–10^{-5} M	Frog (*Xenopus laevis*)	Induction of the estrogenic biomarker vitellogenin in primary cultured hepatocytes of male.	[154]
	300 μg/L	Mangrove Rivulus (*Rivulus marmoratus*)	Down-regulation of androgen receptor and estrogen receptors α and β mRNA was observed in gonadal tissue.	[159]
	0.7–85.6 μg/L	Rainbow trout (*Oncorhynchus mykiss*)	Increased synthesis of vitellogenin. Reduction of plasma follicle stimulating hormone levels and gene expression in the pituitary. Inhibition of gonadal development and steroidogenesis.	[160]

Table 2. Continued.

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
	10^{-12}–10^{-8} M	Clonal rat prolactinoma cell line GH3/B6/F10	Dose-responses for extracellular regulated kinase activation.	[161]
	0.01–100 μg/L	Shrimp (*Neomysis integer*)	Induction of vitellin synthesis.	[162]
Triclosan (TCS)	0.3–30 μg/L	Bullfrog (*Rana catesbeiana*)	Exposure to triclosan disrupts gene expression associated with the presence of thyroid hormones.	[163]
	1–100 μg/L	Medaka (*Oryzias latipes*)	Weak androgenic activity.	[164]
	20–200 μg/L	Yeast two-hybrid assay	No estrogenic activity for TCS alone but estrogenic activity when metabolized by rat S9 liver enzymes.	[165]
		Medaka (*Oryzias latipes*)	Induction of vitellogenin.	
	10^{-8}–10^{-5} M	Human hepatoma cell line (HuH7)	Activation of human pregnane X (PRX) receptor by TCS resulting in an alteration of the metabolism regulated by PRX.	[166]
Dibutylphthalate (DBP)	0–10000 mg/L	Rats	Developmental exposure to DBP affected both male and female sexual development.	[167]
	10^{-6}–10^{-3} M	Atlantic salmon (*Salmo salar*)	Interference with the endocrine functions of steroid-binding protein.	[168]
Diethylphthalate (DEP)	0.1–20 mg/L	Carp (*Cyprinus carpio*)	Variations in metabolic activities such as decrease in activity of acid phosphatase, aspartate aminotransferase, alanine aminotransferase, testiculosomatic and vitellogenin induction.	[169]
Di-(2-ethylhexyl) phthalate (DEHP)	300–750 mg/kg d	Rats	Perinatally exposed males present testicular atrophy, reduced weights of reproductive organs, reduced anogenital distance and an increased number of nipples/areolas.	[170]
Estrone (E1)	10–1000 ng/L	Shrimp (*Neomysis integer*)	Disruption of vitellogenesis.	[162]
17β-estradiol (E2)	10^{-10}–10^{-5} M	Frog (*Xenopus laevis*)	Induction of death and malformations in *X. laevis* embryos.	[171]
Estriol (E3)	0.1–30 μg/L	Zebrafish (*Danio rerio*)	Alteration of sex ratio in the population. Induction of vitellogenin synthesis.	[172]
17*α*-ethynylestradiol (EE2)	1–100 ng/L	Zebrafish (*Danio rerio*)	EE2 showed toxic effects on larvae, embryos and juveniles. Production of vitellogenin into male adult fishes.	[173]
β-sitosterol (β-Sit)	200 μg/g	Goldfish (*Carassius auratus*)	Reduction of steroidogenic acute regulatory protein transcript abundance with decreased plasma components resulting from a non-estrogenic effect.	[174]
	50 mg/kg d	Mink (*Mustela vison*)	Disruption of hormonal balance and development of sexual organs.	[175]
Genistein (GEN)	50 mg/kg d	Mink (*Mustela vison*)	Disruption of hormonal balance and development of sexual organs.	[175]

1.2.4 Phthalic Acid Esters

Phthalic acid esters (PAEs) are used as solvents in the chemical industry and as plasticizers in the formulation of polymers such as PVC [92]. Their presence in the environment is, among other possibilities, a result of leaching because of their weak covalent bonding within polymers. Particular attention has been paid to dibutylphthalate (DBP), diethylphthalate (DEP) and di-(2-ethylhexyl)phthalate (DEHP). The concentrations of DBP, DEP and DEHP in freshwater and STP effluents are reported at several tens of μg/L [34, 93–95], whereas in sediments and activated sludge, biosolids of these same phthalates attain concentrations of tens of mg/kg [93, 96–97]. The concern with PAEs at such levels is their strong poten-

tial interference with reproduction and development of many organisms in the food chain by endocrine or antiandrogenic-mediated processes. Tab. 2 presents some of the endocrine disruption processes linked to DBP, DEP and DEHP.

1.2.5 Natural and Synthetic Hormone Substances

The natural and synthetic steroid estrogens estrone (E1), 17β-estradiol (E2), estriol (E3) and 17α-ethynylestradiol (EE2) are common chemicals detected in sewage effluents [34]. E1, E2 and E3 are endogenous female estrogens and EE2 is a synthetic ovulation inhibitor contained in birth control pills. The occurrence of these EDCs in the aquatic environment is a result of their discharge by municipal STP effluents. Wastewater treatment installations have shown low elimination efficiency for this kind of EDCs [34]. The concentrations of E1, E2, E3 and EE2 in STP effluents are typically 1–147, 0.2–158, 10–30 and 10–78 ng/L [34, 98], respectively.

Furthermore, some substances coming from plant species also exhibit endocrine disruption capabilities. Among such phytoestrogens, genistein (GEN) and β-sitosterol (β-sit) have been treated by WRF and their LMEs. Pulp and paper mill effluents constitute the main anthropogenic source of these EDCs. β-Sit is present in terrestrial vascular plants and, less commonly, in certain species of phytoplankton. This substance, part of wood pitch, has been identified as an agent potentially responsible for the masculinisation of fish [99, 100] in receiving waters downstream from pulp and paper mills [101]. Wood pitch or resin, the main vehicle of β-sit, can cause significant technical and environmental problems in the pulp and paper industries [102]. The concentration of β-sit in municipal and paper mill treatment plant effluents could be up to 10000 ng/L [34, 103, 104]. The isoflavanoid GEN has been detected in municipal sewage at concentrations of 83 ng/L [28], up to 10.5 μg/L in bleach kraft pulp mill effluents [104, 105] and 7 ng/L in surface water [28]. Such environmental concentrations of these naturally-occurring EDCs exert unwanted effects on organisms present in receiving environments (see Tab. 2).

2 Elimination of EDCs in Aqueous Solution using Fungi

BPA was removed using the WRF *Stereum hirsutum*, *Heterobasidium insulare* and *Pleurotus ostreatus* O-48 as whole-cell biocatalysts [121, 128]. *S. hirsutum* and *H. insulare* were incubated in a 200 mg/L of BPA solution [121], while *P. ostreatus* was incubated in 0.4 mM solution [128]. The results obtained suggested that the elimination of BPA was strain dependent. According to Lee et al. [121] the presence of BPA did not induce the secretion of LMEs in the culture media of *S. hirsutum* and *H. insulare*, suggesting that other intra- or extracellular enzyme(s) may be involved in the transformation of BPA. This observation was confirmed by the identification of the metabolites produced during the fermentation. The most abundant products detected were 2-hydroxy-3-phenyl propanoic acid, 1-ethenyl-4-methoxybenzene as well as phenylacetic acid and its hydroxylated derivative at the C2 position. These compounds came from the phenolic moiety of BPA through dehydroxylation, carboxylation, and hydroxylation, respectively, on the side chain. These metabolites have never been detected when using LMEs alone. The estrogenic activity of the BPA-treated effluent was monitored using the E-screen assay which is based on the MCF-7 cell line proliferation [176]. With *S. hirsutum*, a 40 % reduction of the estrogenic activity of a 100 μM solution of BPA was achieved within a 1-day incubation. All of the estrogenic activity was eliminated after 3 days. In comparison, all of the estrogenic activity of the solution was removed after a 1-day cultivation of *H. insulare* [121].

The removal of a technical mixture of NP isomers was achieved using the WRF *Trametes versicolor*, *Phanerochaete chrysosporium*, *Bjerkandera* sp. BOL 13, *Cunninghamella* sp., the mitosporic strain UHH 1-6-18-4 isolated from NP-contaminated river water, the aquatic hyphomycete *Clavariopsis aquatica* and *Fusarium* and *Mucor* strains isolated from a soil/sludge mixture contaminated by NP [106–107, 111–112, 119]. These different fungal strains were able to eliminate NP present in aqueous solution in concentrations ranging from 0.05 to 0.45 mM. The abilities of the fungi to eliminate NP depend on the strain, the culture conditions and the spectrum of NP isomers. For example, among the aquatic strains tested, the autochthonous strain UHH 1-6-18-4 had a higher ability to eliminate NP from aqueous solution than the aquatic strain *C. aquatica* [112].

The biocatalytic strategies used by the different strains were also found to differ. The conversion of NP from aqueous solution by *Bjerkandera* sp. BOL 13 was not associated with an induction of laccase and its secretion in the growth medium unlike in the case of *T. versicolor* [107]. These different strategies were also reflected by the influence of agitation on the elimination of NP from aqueous solution by these fungi. The removal of NP using *T. versicolor* was favored under agitated culture conditions, while the biotransformation carried out by *Bjerkandera* sp. was favored under static conditions [106]. These results support the more-or-less general statement that the production of laccase is favored by agitated culture conditions [14].

Junghanns et al. [112] and Moeder et al. [111] suggested that the metabolic pattern produced during the removal of NP by each of the two cultures, the strain UHH 1-6-18-4 and the aquatic hyphomycete *C. aquatica*, respectively, involved the intracellular hydroxylation of the nonyl moiety, the shortening of the alkyl chain and the formation of carboxylated NP-oligomers. Furthermore, a mass balance on the elimination of ^{14}C-labeled 4-*n*-nonylphenol isomer (4nNP) by *T. versicolor* in liquid culture showed that 23.4 % of 4nNP initially present in the culture medium was incorporated into biomass, 29.2 % was still present in aqueous solution as a metabolites/unreacted 4nNP mixture, 6 % was mineralized to carbon dioxide and the remaining 41.4 % was adsorbed on the recipient's glass walls [119].

The in vivo treatment of TCS has been examined using the WRF *T. versicolor* SBUG-M, DSM 11269 and DSM 11309 and *Pycnoporus cinnabarinus* SBUG-M 1044 [130]. This work focused mainly on the metabolites produced during incubations of a 0.25 mM TCS solution. Two of these metabolites had structures of TCS conjugated with xylose or glucose as shown

by ^{1}H-NMR spectra. These sugar-conjugated products were identified as 2-O-(2,4,4′-trichlorodiphenyl ether)-β-D-xylopyranoside and 2-O-(2,4,4′-trichlorodiphenyl ether)-β-D-glucopyranoside, respectively. Furthermore, the fermentation of *T. versicolor* with TCS was found to result in the formation of 2,4-dichlorophenol [130]. This metabolite could be formed by a phenoxy radical pathway such as the one involved in the production of 4-isopropenylphenol from BPA [142, 177]. Under the same cultivation conditions, *P. cinnabarinus* converted TCS to a glucoside-conjugated and methylated metabolite. This chemical was identified as 2,4,4′-trichloro-2′-methoxydiphenyl ether [130]. The results obtained by Hundt et al. [130] highlighted the involvement of non-lignin-degrading enzymes in the conversion and detoxification of TCS present in aqueous solution using WRF. The glycosylating and xylosylating enzymes involved in the formation of sugar containing metabolites identified by these authors have also been detected during the fungi-catalyzed transformation of phenanthrene [178–180].

DBP was reported to be eliminated by the WRF *P. chrysosporium*, *T. versicolor* and *Daldinia concentrica* [120]. These different strains exhibited differences with respect to a DBP inhibitory effect and DBP consumption. Lee et al. [120] presented the time course of the transformation of a 28 mg/L solution of DBP at a temperature of 30 °C. After a 1-day incubation, *T. versicolor* and *D. concentrica* eliminated 83 and 94 % of the DBP, initially present in solution, respectively. A similar extent of reaction was achieved by *P. chrysosporium* after a 6-day incubation. The *D. concentrica*-mediated removal of DBP led to the formation of *O*-phenylacetic acid, isobenzofuranone, *O*-anisic acid, phenylethyl alcohol, di-butyl-4-methoxy phenol and C8–C18 fatty acids. These metabolites suggest that the elimination of DBP by *D. concentrica* occurred through non-LME biocatalytic actions, given the low LME activity monitored during the whole process of DBP transformation [120]. The metabolites produced did not show any estrogenic activity based on the E-screen and the pS2 mRNA expression assay [120].

The bioconversion of 10^{-5} M solution of E1 and 10^{-4} M solution GEN was studied using shake cultures (150 rpm) of the WRF *Phanerochaete sordida* YK-624 at 30 °C [126, 127]. After a 2-day lag period, 70 % and 100 % of E1 was removed after 3- and 6-day incubations, respectively, and the GEN concentration decreased by 90 % between day 2 and 4 of cultivation. These transformations were correlated with the secretion of the LMEs MnP and laccase, suggesting that E1 bioconversion by *P. sordida* could be due to the action of its LME [126, 127].

The conversion of NP and DEHP contained in the secondary effluent from a biological process treating night soil was evaluated in a 200-L pilot plant bioreactor employing the WRF *T. hirsuta* IFO 4917 grown on polyurethane foam cubes [138]. The reactor was equipped with an ultrafiltration (UF) unit and was operated in a sequencing batch mode. The duration of the overall cycle in the bioreactor was 1.5 days. The UF unit operated in tangential flow mode and was equipped with a 120 cm^2 polysulfone membrane of a 10 kDa MWCO. This UF unit brought about the physical removal of high molecular weight compounds and the concentration of the LME secreted by the WRF into the system thus enhancing its performance as seen by greater than 94 % NP and 45 % DEHP elimination under optimal conditions [138].

3 Elimination of EDCs in Soil by WRF

Soares et al. [107] tested the ability of *T. versicolor* and *Bjerkandera* sp. BOL13 to eliminate NP from a contaminated soil (430 mg/kg matrix) over a 5-week incubation period. Complete colonization of the contaminated soil by *Bjerkandera* sp. BOL13 and by *T. versicolor* was achieved after 5 weeks compared to 2 weeks (*Bjerkandera* sp. BOL13) and 3 weeks (*T. versicolor*) for the control (uncontaminated soil) [107]. At the end of the 5-week incubation period, more than 98 % NP removal had been achieved by both WRF [107].

4 Elimination of β-sit in Wood Matrices using WRF

The removal of wood pitch prior to pulp processing can reduce the potential discharge of such EDCs via WWTP effluents. The fungi *Ischnoderma benzoinum*, *Peniophora pini*, *Stereum sanguinolentum*, *Bjerkandera* sp. BOS55, *Stereum hirsutum* PW93-4, *T. versicolor* LaVec94-6, *Funalia trogii*, *Ophiostoma ainoae* and *Ceratocystis allanstospora* were used to transform β-sit from wood pitch prior to using it in a pulp and paper process [108–110]. After a 4-week static fermentation at 27 °C, a complete conversion was achieved by strains *Bjerkandera* sp. and *S. hirsutum* [109]. The strains *T. versicolor*, *I. benzoinum*, *P. pini* and *S. sanguinolentum* reached levels of 86.5, 79.5, 76.7 and 53.2 % transformation, respectively, while the strain *F. trogii* managed only 50 % conversion after 6 weeks of cultivation [109, 110]. The strains *O. ainoae* and *C. allanstospora* were unable to transform β-sit [110]. Although the *Bjerkandera* sp. strain removed β-sit to a higher extent compared to *T. versicolor*, the time course of β-sit removal indicated that half of the EDC had been converted after 1.5 weeks using *Bjerkandera* sp. and after less than a week by *T. versicolor* [108].

5 Elimination of EDCs in Aqueous Solution using Free LMEs

In order to determine the impact of the LMEs secreted during the WRF fermentation in the presence of EDCs, MnP and laccase have been used in vitro for the treatment of these target chemicals. Furthermore, the utilization of this LME alone could lead to a relevant environmental bioprocess.

The treatment of BPA was indeed achieved in vitro using the MnP secreted by the WRF *P. ostreatus* O-48. BPA was eliminated from a 0.4 mM solution using 10 U/mL of MnP, 2.0 mM $MnSO_4$ and 2.0 mM H_2O_2 at a pH of 4.5 and at room temperature [128]. Remarkably, the removal of this EDC was highlighted by the modification of the UV spectra of the BPA-containing solution after a 1-hour treatment.

Partially purified MnP from *Phanerochaete chrysosporium* ME-446 was used for the removal of BPA from a 0.22 mM solution and NP from a 0.23 mM solution [123]. The co-sub-

strate for the MnP enzyme, H_2O_2, was supplied in situ by the action of glucose oxidase. The aqueous treatment occurred at pH 4.5 and 30 °C using 100 U/L of MnP and 50 μM of $MnSO_4$ and resulted in a complete transformation of both EDCs after one hour.

10^{-4} M solution of GEN [127] and 10^{-5} M solutions of E1, E2 and EE2 were subjected to the biocatalytic action of 10 μkat/L of MnP from *P. chrysosporium* ME-446 and *P. sordida* YK-624 [124, 126]. After a 1-hour treatment, none of the three hormones could be found in the reaction media while a residual 10 % of GEN was detected.

Similarly with MnP, the action of free laccase has been studied for the removal of BPA in vitro. Laccases from several strains of WRF have been tested for the removal of BPA, such as *Trametes versicolor*, *Trametes villosa*, strain I-4 isolated from soil and *Coriolopsis polyzona* [113, 123, 139, 142–146]. These different laccase preparations were used to eliminate BPA from aqueous solutions in concentrations from 0.022 to 2.2 mM, which are higher than those present in STP effluents but could be representative of levels downstream of a process unit discharging BPA. Using laccase activities from 10 to 1500 U/L, fast transformations of BPA were achieved. Depending on the operational conditions, all of the BPA present in the spiked solutions could be removed within a contact time of 4 h. The substrate specificity (affinity), as reflected by K_m, and catalytic efficiency, as reflected by k_{cat}, for laccase from strain I-4 and from *T. villosa* were evaluated and pointed to the superiority of strain I-4 with respect to the substrate BPA [142, 146]. However, the value of this kinetic parameter is significantly higher than the one of typical substrates of laccase such as ABTS, guaiacol or syringaldazine [146]. For comparison, Tab. 3 presents the kinetic constants of laccase from strain I-4 for several substrates.

Laccase in free form has been used for the treatment of NP present in aqueous solutions at concentrations ranging from 0.023 to 0.32 mM. The laccase used originated from the WRF *T. versicolor*, I-4 strain isolated from soil, *C. polyzona* and the aquatic fungal strains UHH 1-6-18-4 and *C. aquatica* [112–113, 119, 123, 145, 146]. In regard to the laccase activity used, the highest conversion of NP was achieved with culture supernatant from the WRF *C. polyzona*. Complete removal was achieved for a contact time of less than 4 hours using a laccase activity of 1 U/L [113]. The various laccases secreted by WRF were able to achieve a complete elimination of NP under the different conditions tested [113, 117, 119, 123, 145, 146]. TCS was also found to be removed using free laccase from the previously described *C. polyzona* and *T. versicolor* (see Tab. 1) [113, 140]. The conversion of TCS by free laccase from *C. polyzona* appeared to be less effective than the removal of BPA and NP [113].

Table 3. Michaelis-Menten kinetic constants of laccase from strain I-4 for the oxidation of different substrates [146].

Substrate	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [μmol/s L]
ABTS	652	26	0.04
Guaiacol	16	36	2.25
Syringaldazine	9	4	0.44
Catechol	1050	46	0.04
Hydroquinone	123	55	0.45
NP	5000	1	0.0002
BPA	10000	14	0.0014

The treatment of 10^{-5} M solution of E1 and 10^{-4} M solution of GEN with unsupplemented laccase and of E2 and EE2 with the laccase/1-HBT system was also investigated by Tamagawa et al. [126, 127] and Suzuki et al. [124]. A 10 μkat/L activity of laccase from the WRF *T. versicolor* IFO-6482 was used in solution at pH 4.5 and 30 °C. After a 1-hour treatment, the estrogens were not detected in solution [124, 126] while 95 % of the phytoestrogen was removed [127].

Finally, an extensive screening of twenty enzyme-containing culture fluids from different WRF has been carried out in regard to their ability to eliminate the EDCs BPA, NP, DEHP, E1, E2 and E3 [138]. The authors did not report the enzyme activities detected and the enzyme concentrations for the preparations used. The screening was performed over a 24-hour contact time. The highest BPA conversion was achieved with culture supernatants from the WRF *T. hirsuta* 1567 and *Pycnoporus coccineus* 866, NP transformation from the WRF *T. hirsuta* 1567, 1674 and IFO 4917, DEHP removal from *Fomes fomentarius* 150 and E1, E2 and E3 with fluids from *T. hirsuta* IFO 4917 and *Phellinus gilvus* 110 [138].

Attention has also been paid to the reaction conditions for the transformation of BPA, NP and TCS using commercially available laccase from *T. versicolor,* and laccase secreted by *T. villosa* and *C. polyzona* [113, 139, 140, 142, 181, 182]. Among the pH and temperature conditions tested, the best performances were obtained at a pH between 5 and 6 and a temperature between 45 and 60 °C. These results represent a combination of stability occurring at a higher pH and catalytic activity resulting from a higher temperature. The results obtained with laccase from *T. versicolor* showed that this LME is quite stable over a pH range from 4 to 8 [139]. The divergence between the pH dependent elimination of BPA and laccase stability suggest that the changes in the efficiency of conversion are mainly due to variations in interactions between the catalytic site of laccase and BPA [139]. In this work it was also demonstrated that free laccase from *T. versicolor* lost its activity faster under reacting conditions with BPA compared to the same laccase incubated only in buffer. This inactivation is linked to the interaction of bisphenolic radicals with the enzyme [139]. These results differ from other data indicating that laccase stability had been improved by the presence of phenolic chemicals that could be oxidized by laccase but which are poor substrates [183]. It could be of interest to determine the potential stabilizing effect of BPA on laccase under non-reactive conditions (e.g. in the absence of oxygen). It is well known that natural substrates of enzymes and their structural analogs could act as stabilizers [184].

Regarding the impact of compounds typically present in wastewaters on the laccase-catalyzed abatement of BPA, the reducing anions nitrite, sulfite, sulfide and thiosulfate tended to decrease BPA conversion by laccase from *T. versicolor* [139]. The sulfite and sulfide anions acted as competitors to laccase for the consumption of dissolved oxygen. The organic dena-

turants methanol (5 and 10 % w/w), acetone (5 and 10 % w/w) and formaldehyde (1 and 2 % w/w) significantly decreased the conversion of BPA. This reduction of the elimination efficiency could be due to the hydrophobic interactions between the organic compounds and amino acid residues, resulting in laccase denaturation. Conversely, phenol, ε-caprolactam and isoprene at concentrations as high as 1000 μM and urea at a concentration of 50 mg/L did not influence BPA conversion [139]. The presence of metal ions, Fe^{3+} and Cu^{2+}, also decreased the conversion of BPA significantly. These metals could interrupt the electron transport in the catalytic site of the laccase and thus inhibit BPA conversion [2]. Calcium, cobalt and zinc chlorides had a negative impact on the laccase-catalyzed transformation of BPA possibly due to the chaotropic effect of these salts [114]. Finally, cyanide, a reagent often used in the plastics industries, also decreased the conversion of BPA by laccase by tending to dissociate copper ion from the catalytic site which results in denaturation of the enzyme [139].

Several approaches could be used to enhance the stability of laccase against these inactivation/inhibition effects and improve the biocatalytic activity of the enzyme. One option is the addition of stabilizing chemicals such as polyethylene glycol (PEG), polyvinyl alcohol, Ficoll or alkyl betaine [181, 185]. All of these substances enhance the conversion of BPA, under non-denaturing conditions; hence a lower laccase activity is needed to achieve the same BPA conversion. This increased conversion is due to two distinct phenomena: enzyme activity reinforcement and stability boost [185]. The presence of PEG did not improve the kinetic parameters of the laccase from *T. versicolor* indicating that the enhanced elimination of BPA in the presence of PEG was linked to enzyme protection and BPA-PEG coupling and did not stem from a modification of the reaction rate [181, 185]. Kim and Nicell [139] were able to boost both the rate and extent of transformation of TCS using PEG as a protecting/activity-enhancing agent. The removal of TCS as a function of treatment conditions such as the presence of redox mediator showed similar trends to the elimination of BPA under similar conditions [140, 181]. However, the quantity of laccase from *T. versicolor* necessary for the conversion of TCS was significantly higher than the level required for the complete conversion of BPA: 3000 U/L for the treatment of a 20 μmol/L solution of TCS compared to 150 U/L for a 120 μmol/L solution of BPA [140, 181]. However, PEG did not improve the stability of laccase against cyanide or fluoride ions [181]. Finally, the relative residual toxicity, determined by a Microtox toxicity assay, following complete transformation of BPA from a 120 μM solution using the laccase supplemented with 5 mg/L PEG was found to be 4.5 % while for TCS the residual toxicity of the products formed by the laccase alone, the laccase-PEG (50 mg/L) system and the laccase/ABTS-mediated system represented 21, 41 and 1200 % of the initial toxicity compared to 100 % for the untreated solution, 0.9 % for the solution treated with unsupplemented laccase and 0.2 % for the PEG alone [140, 181]. These results point out that the residual toxicity may be a function of the nature of the putative metabolites, which, in turn, differ according to the treatment used, i.e., depending on whether or not the system received supplemental stabilizers such as PEG [181]. For TCS, the higher toxicity in the presence of PEG could be due to the enhanced solubility of the polymeric metabolites produced.

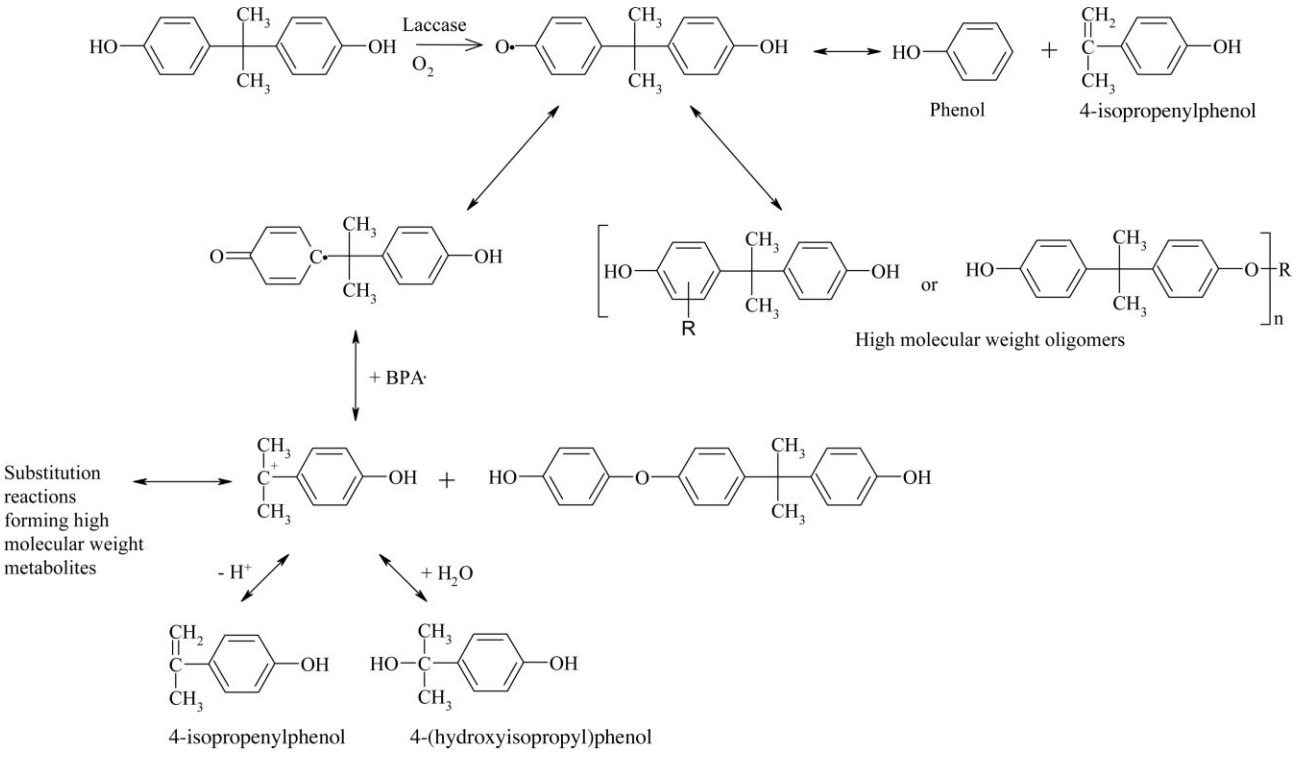

Figure 2. Reaction pathway of laccase-oxidized BPA (modified from Huang and Weber (2005)).

Finally, the laccase/mediator system could have unintentionally enhanced the toxicity of the solution by the presence of the reactive species $ABTS^{\cdot+}$ and $ABTS^{++}$, formed by the laccase action [140].

The use of low-molecular weight oxidizable substances (mediators) in the biocatalytic cycle of laccase expands the activity of this enzyme. This mediated oxidation involves two oxidative steps. In the first one, the laccase oxidizes a primary substrate, the mediator, and this substance acts as an electron transferring compound. The mediator finally transfers the electron from the substance of interest. These mediators are known to increase the substrate range of laccase [186]. The action of the mediators 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), violuric acid (VLA), 1-hydroxy-benzotriazole (1-HBT) and 2,2,6,6-tetramethoxypiperidine 1-oxyl (TEMPO) has been tested in the context of BPA, NP, TCS, E1, E2, EE2 and GEN treatment [112–113, 123–124, 126–127, 139, 140, 145]. The results depended on the laccase source, the EDC and mediator concentrations. For example, using 100 μM mediator solutions, the action of laccase from *T. versicolor* for the removal of BPA was improved by the addition of ABTS and VLA, while the corresponding action of laccase from *P. ostreatus* was improved by 1-HBT [123, 139]. On the other hand, amongst several mediators tested at a concentration of 10 μM, ABTS significantly enhanced the transformation of BPA by *C. polyzona* laccase [113]. Finally, the laccase/1-HBT system did not improve the removal of E1, E2, EE2 and GEN [124, 126–127].

In order to complete this survey of the enzyme-catalyzed removal of EDCs, it is important to address the fate of its estrogenic activity following MnP and laccase treatments. All of the reports assaying the variation of the estrogenic activity of BPA, NP, E1 and GEN in solution following laccase treatment point out that the removal of the parent compound is correlated with a reduction of the solution's estrogenic activity, suggesting that the metabolites produced by the laccase-mediated oxidation process exhibited lower or no estrogenic activity [113, 123, 126, 127, 142]. On the other hand, the removal of the estrogenic activity of E2 and EE2 was not related to the elimination of the parent chemicals by the action of laccase [124]. The complete disappearance of such activity was achieved after a contact time of 8 h. These residual estrogenic activities could be due to the ability of these chemicals to bind to the estrogen receptor even at very low concentrations [187]. The same trends were observed for the MnP treatment of estrogens and phytoestrogen [124, 126, 127]. Tsutsumi et al. [123] also demonstrated that the elimination of the estrogenic activity of a 0.88 mM and a 0.92 mM solution of BPA and NP by MnP are not directly related to the elimination of the EDC and suggested that the chemicals produced still have estrogenic activity. Globally, these outcomes may reflect the loss of the chemicals' structural similarities with estrogens: for instance due to their bulk they could no longer interact with hormone receptors (for example see Figs. 2 and 3).

Finally, due to its high demonstrated efficiency for BPA removal, laccase could be used in a more complex wastewater treatment process. For example, laccase could lead to the removal of BPA from the effluent of a process unit in situ. This conversion could improve the overall efficiency of the biotreatment of the complete effluent stream. Based on this logic, the impact of the action of laccase from *Trametes* sp. in combination with activated sludge treatment on the overall removal of BPA was investigated [133]. Neither the action of laccase alone nor the activated sludge alone provided a significant reduction of the effluents' chemical oxygen demand (COD). However, when the laccase treatment and the activated sludge

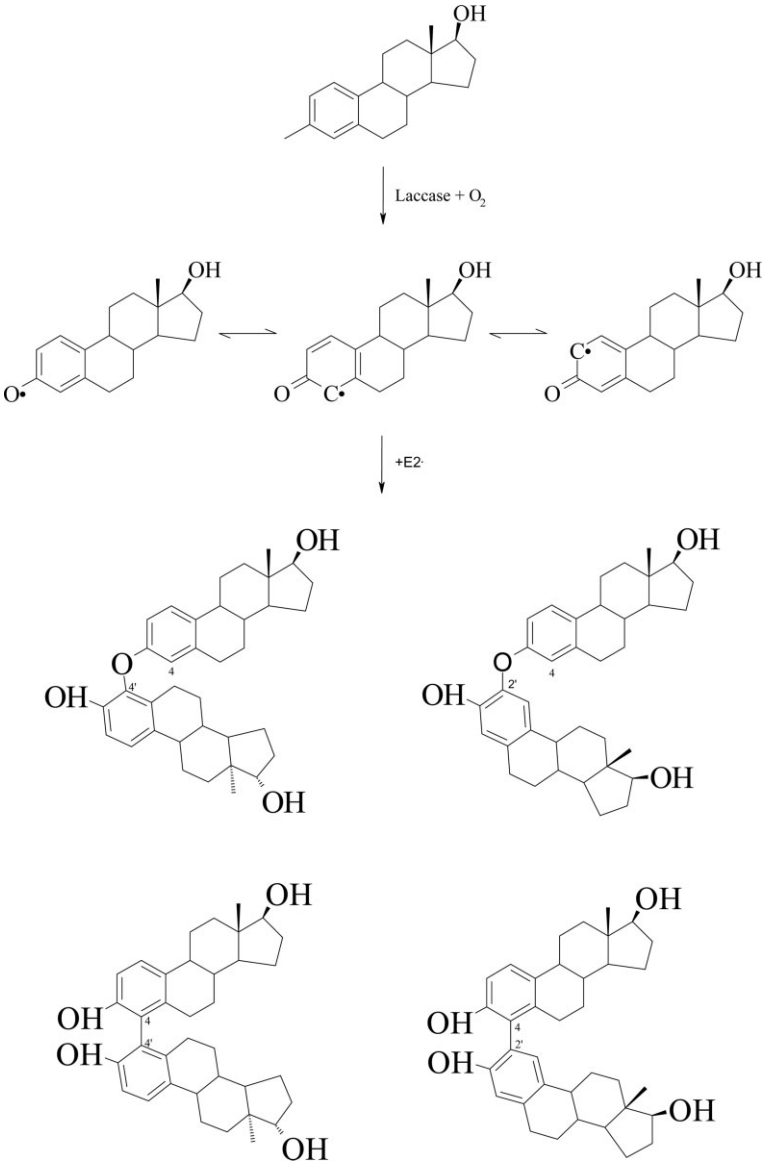

Figure 3. Reaction pathway of E2 oxidized by laccase and structures of dimers produced (modified from Nicotra et al. (2004)).

treatment were combined, a considerable COD removal was achieved [133]. This outcome was a function of the laccase treatment time. For example, after a 3-hour incubation in activated sludge, COD removal extents of 20 %, 25 % and 65 % were achieved for pre-treatment with laccase of 1, 2 or 6 h, respectively [133]. This laccase/activated sludge-combination is promising for the treatment of BPA from real industrial effluents.

6 Elimination of EDCs in Aqueous Solution using Immobilized/Insolubilized LMEs

In order to enhance the industrial benefit of laccases for wastewater treatment, including the possibility to reuse them and to improve their stability, efforts have been made to immobilize the enzyme on solid supports based on chemical and physical mechanisms [188]. Furthermore, stabilization by immobilization could be more valuable especially for continuous bioprocesses.

Conversion of BPA in aqueous solution by heterogeneous catalysis has been demonstrated using laccase from *Trametes* sp. immobilized onto alkylaminated controlled porosity glass in a lab scale packed bed reactor [136]. Flow rates between 0.25 and 1 mL/min in the prototype unit resulted in an elimination proportional to the hydraulic residence time [135]. For a flow rate of 2 mL/min, the conversion was 1.7-fold less than that observed at lower flow rates possibly due to mass transfer limitations. No noticeable loss of activity was exhibited after fifty injections of BPA through the packed column [135] which represents a great advantage of immobilized laccase over the free form of the enzyme. The same Japanese group coupled an electrolysis device to supply additional oxygen to the system, ensuring that this substrate did not limit the BPA conversion by immobilized laccase. The coupling of the electrolysis device with the laccase-immobilized column significantly enhanced the conversion of BPA [136]. This higher removal could be due to counteracting the oxygen limiting conditions or to an additional transformation by the electrochemical oxidation of phenolics [189]. Furthermore, the electrolysis unit provided flexibility to the system because of its ready response to shock loads of BPA [135].

Laccase from *T. versicolor* immobilized onto a nylon-poly-(glycidyl methacrylate) membrane was used within a non-isothermal reactor to treat water polluted with BPA [141]. Non-isothermal conditions profoundly affected the catalytic properties of the membrane as seen by the variation of the Michaelis-Menten kinetic parameters, leading to an overall enhancement of the rate of BPA transformation. It was observed that for each bioreactor average temperature, the laccase presented more affinity, based on the K_m values, under non-isothermal conditions than under corresponding isothermal ones. Furthermore, the affinity for BPA increased, for a given average temperature, when the difference between the warm and cold sides of the membrane increased. Finally, the K_m values (either under isothermal or non-isothermal conditions) decreased with the rise of the average temperature of the bioreactor [141]. These results were explained to be due to an increase of BPA fluxes through the membrane under non-isothermal conditions.

Another way of using enzymes in a continuous mode is the confinement of the enzyme in a physically defined matrix such as a dialysis tube. Laccase from the WRF *C. versicolor* IFO 4937 was confined inside dialysis tubes with varying molecular weight cut-offs (MWCO) [116]. This system enabled the recovery of the enzyme for further applications and the removal of the high molecular weight chemicals produced by the action of this laccase. The results obtained by Hoshino et al. [116] demonstrated the influence of the MWCO of the membrane on the extent of BPA conversion. It is well known that, for a given pressure, the water flux across a dialysis tube is influenced by the MWCO of the membrane [190]. The utilization of higher MWCO membrane resulted in a higher BPA mass transfer rate from bulk to the confined enzyme.

Finally, to overcome the disadvantage of the low enzyme/support weight ratio inherent in enzyme immobilization on a solid support, laccase from *C. polyzona* was insolubilized by forming cross-linked enzyme aggregates (CLEAs). These were used in a fluidized bed reactor for the elimination of the EDCs BPA, NP and TCS from solutions containing 5 mg/L of each at pH 5 and room temperature [114]. At a 150 min hydraulic residence time, a 90 % conversion of BPA was achieved. A 90 % elimination of NP and TCS was obtained with a residence time of 60 minutes. This solid biocatalyst had higher affinity for, respectively NP, BPA and TCS based on K_m values. However, the maximum rate of NP transformation was higher than the one of BPA and TCS as shown by the k_{cat} value. Based on the K_m values, this biocatalyst had higher affinity for BPA and NP than free laccases from strain I-4 and from *T. villosa* [142, 146].

7 Elimination of EDCs in Organic Media using LMEs

Organic solvents are often necessary to dissolve high concentrations of many phenolic EDCs because of their high hydrophobicity. However, LMEs have shown low activity and low stability in organic media [191]. To overcome this limitation, reversed micelles (RM) have been used as a vehicle for LMEs. RMs are composed of enzyme-containing, surfactant-stabilized aqueous microdroplets in a continuous organic phase. This molecular arrangement is thermodynamically stable [192]. These enzymatic vehicles make possible the use of LMEs in organic matrices. The presence of an aqueous layer around the LME and of a surfactant shell protects the LME against inactivation by the surrounding bulk organic phase [193].

An RM emulsion system containing laccase from the WRF *C. versicolor* or *Trametes* sp. was prepared by a Japanese group using bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) solutions in isooctane [115, 134]. The RM formation conditions affected the conversion of BPA in such a system; with isooctane instead of tetrahydrofuran as the organic phase laccase activity on 2,6-dimethoxyphenol as substrate was increased by a factor of fifty. The degree of hydration (W_o) of the RM also influenced the initial rate of BPA transformation, from 280 μmol/min g when W_o was 15 down to 150 μmol/min g when W_o was shifted to 20; the removal of BPA from a 200 μM solution after a contact time of 1 h was complete when the

water content was 4 % v/v whereas just a 50 % removal was reached with a water content of 2 % [115, 134]. The new environment surrounding the laccase modified the biocatalytic properties of the enzyme. For example, the optimal pH for the conversion of BPA in the RM system was 5 compared to 3 for the native laccase in aqueous buffer [115]. The authors explained that shift in optimal pH by the attraction of protons by the negatively charged potential field of the AOT surfactant [115]. This system was also used for the conversion of NP. A complete elimination was achieved when using the optimal conditions [115]. Finally, the RM laccase system supplemented with the mediator 1-HBT was tested for the removal of BPA and NP but no gains were noted, suggesting that the mediator was not essential for this application when using RM-enclosed laccase from *C. versicolor* [115].

The same Japanese group studied the oxidation of 0.1 mM solutions of BPA and NP in organic media by using LiP from *P. chrysosporium* in a RM system [125]. The authors used the optimal conditions in regard to LiP activity, i.e. 100 mM AOT, 0.1 μM H_2O_2, a water content of 50 %, 3 μM LiP, a pH of 5 and a temperature of 40 °C in isooctane for the elimination of these EDCs. Under these conditions, a 50 % and 80 % transformation of BPA and NP was achieved after a treatment time of 2 h. Compared to this, Michizoe et al. [134] obtained a complete removal of BPA using the same concentration of laccase in an RM system.

The treatment of a 0.33 g/L solution of E1, E2 and E3 estrogens by a laccase solution from *Russula delica* was studied using 20.0 % to 26.66 % v/v of alcohol [132]. This enzyme had decreasing affinity for E1, E2 and E3, respectively, which directly correlated with their water solubility (E3>E2>E1). Thus, the laccase oxidation could be mass-transfer limited under the high concentration conditions tested by Graubard and Pincus [132].

The oxidation of E2 in a 1:1 [v:v] water-ethyl acetate emulsion system was tested using laccase from *Polyporus versicolor* [129]. After a 12-hour treatment, five unidentified metabolites were detected by thin layer chromatography, but none of them showed any estrogenic activity as measured using the uterotrophic test [194]. The optimal aqueous phase pH in terms of transformation rate over the range 4–9 was 5.4. The solvents that resulted in the highest reaction rate of E2 were, in descending order, ethyl acetate, diethyl ether, butyl acetate and methyl ethyl ketone [129]. Less polar solvents limited the solubility of E2 while solvents with higher solubility led to a greater inactivation of the laccase. Finally, laccase from *P. versicolor* could also oxidize E1 and E3 forming three unidentified compounds for each EDC in the above emulsion system [129].

8 Elimination of EDCs in Soil using LMEs

The removal of the EDCs BPA, NP, E1 and EE2 (3 μmol/g) adsorbed to model soils (sea sand, 20–35 mesh) by the action of free laccase from the WRF *Pycnoporus coccineus, C. versicolor* and *Trametes* sp. was studied in test tubes and in a rotating reactor [117, 131, 195]. The EDCs were essentially removed by the action of the enzyme instead of by adsorption to the soil particles. The extent of EDC transformations was strain dependent. For example, the laccase secreted *by P. coccineus* removed 15 % of BPA adsorbed to the model soil while the laccase from *Trametes* sp. eliminated 85 %, both after a contact time of 5 hours in test tubes [131]. Furthermore, the laccase from *T. versicolor*, *P. coccineus* and *P. ostreatus* were found to have a decreasing affinity for NP as it seems from the kinetic trends in NP residual concentration [117, 131].

The use of the rotating reactor represents a powerful technology for ex situ soil bioremediation [195]. This system was used for the treatment of BPA, NP and EE2 [131, 137]. For example, after a contact time of less than 5 hours, more than 90 % of the BPA adsorbed to 15 g of soil in a concentration of 3 μmol/g using 36 U of laccase activity from *Trametes* sp. at a pH 5 and a rotation speed of 10 rpm [131]. The rotation speed of this reactor affected its performance as expected from mass transfer considerations (accessibility of laccase to soil-sorbed EDC) [131]. Furthermore, the reaction rate calculated per unit of laccase activity was found to decrease as enzyme concentration increased. The explanation given for that phenomenon alluded to the simultaneous occurrence of enzymatic and non-enzymatic processes of NP polymerization [137].

These authors also measured the residual estrogenic activity (by *Oryzias latipes* vitellogenin assay [196]) of the acetone-soluble mixture eluted from NP-contaminated sand after the laccase treatment in the rotating reactor [137]. The residual estrogenic activity decreased by 83.3 to 98.9 % depending on the dilution used (maximum concentrations of NP of 100 or 300 μg/L) in the fish aquarium [137].

Finally, a phytoremediation process based on the use of transgenic tobacco plants secreting laccase from the WRF *C. versicolor* into the rhizosphere was tested for the ability to remove BPA from spiked soil [118]. The degree of BPA removal by these transgenic plants was 10-fold higher compared to non-laccase producing plants. Furthermore, the estrogenic activity of the BPA solutions treated with laccase producing plants was stated to be significantly lower than that of the same solution treated with control plants but no numerical data were given [118].

9 LME-Catalyzed Transformation of EDCs: The Phenoxy Radical Mechanism

A number of reports have focused on the products formed by the action of LMEs on the EDCs BPA, NP, TCS and E2. The formation of phenoxy radicals by the MnP, laccase or laccase/mediator systems appears to result in coupling reactions [177].

The polymerization products of the EDCs NP, BPA and TCS detected by MS and MS^2 analyses were identified as dimers, trimers and tetramers for BPA [113], dimers, trimers, tetramers and pentamers for NP and dimers, trimers and tetramers for TCS [113]. These results are reinforced by the inferred oligomers' molecular weights which were determined by gel permeation chromatography using standardized molecular markers [112, 123, 143, 177]. The high molecular weight chemicals detected suggest a reaction pathway involving the oxidative coupling of the primary oxidation product (formed by abstracting one electron from the OH-group of the original molecule). Uchida et al. [143] identified the structure of the dimer obtained by the action of laccase from *T. villosa* on BPA

as 5,5′-bis-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]biphenyl-2,2′-diol. This oligomer resulted from the formation of a C–C bond between phenolic moieties of BPA. Recently, a phenoxy radical pathway was proposed, in which the polymerization of BPA also occurs through the formation of a C–O bond [177]. Reaction products representing oligomers condensed with phenol molecules [142] as well as the formation of low molecular weight metabolites such as phenol, 4-isopropenylphenol, 4-isopropylphenol and hexestrol have been reported [115, 128, 134, 142–144, 177, 197]. Furthermore, the intermediate 2,4-dichlorophenol identified during the fermentation of *T. versicolor* in the presence of TCS [130] indicates that the LME-mediated conversion of TCS could occur in a manner analogous to the BPA reaction spectrum following two different mechanisms, i.e, *(i)* a condensation phase resulting in the production of higher molecular weight metabolites and *(ii)* a fragmentation phase at the C–O level. Due to lack of sufficiently detailed information on the structure of the oligomers formed it is quite difficult to propose a precise mechanistic pathway. A general reaction network was proposed by Fukuda et al. [142] and a modeled mechanistic one was proposed by Huang and Weber [177]. Fig. 2 schematically shows the reactions undergone by BPA as a result of laccase-catalyzed oxidation.

Lugaro et al. [129] and Nicotra et al. [122] identified the chemical structures of the dimer produced by the action of laccase on E2. This condensation could occur through C–C or C–O bond formation [122, 129]. The less polar products (C–O dimer) were a mixture of atropoisomers in a 1:3 ratio when the reaction was catalyzed by free laccase from *T. pubescens* and in a 1:2 ratio when catalyzed by immobilized *Myceliopthora* laccase. The other form of dimer, occurring via C–C bonds was also a mixture of stereoisomers in a 1:1 ratio [122]. Fig. 3 presents the reaction pathway of E2 oxidized by laccase and the isomeric structures of the dimers formed.

These final compounds did not show a structural analogy with estrogens (e.g. biomolecular recognition of estrogen receptors), hence, their formation by the action of the LME resulted in the elimination of the xenoestrogenic character of the solutions treated [113, 118, 123, 142].

10 General Discussion

10.1 Fungal Treatment

The removal of EDCs from several idealized environmental matrices has been achieved using different strains of WRF. The elimination efficiency is strain- and cultivation condition-dependent resulting in the use of different enzymatic strategies to promote the transformation of these xenobiotics such as, for example, an overlapping of laccase and cellobiose dehydrogenase [198]. Little is known about the conditions supporting these different biocatalytic strategies. Furthermore, the cumulative action between these LMEs and non-LMEs has not been characterized nor have the reaction pathways involved been established when using the whole microorganisms. A more complete understanding of the mechanisms is necessary to gain insight into the conversion of the different EDCs and the removal of the estrogenic activity. The knowledge of the reaction products resulting at least partially from non-LME actions is important to characterize the long-term fate of these chemicals in the environment. The potential panel of isoenzymes should be characterized in regard to the LMEs secreted into the growth media during EDC elimination. It is well-established that the biocatalytic behavior of LMEs is influenced by the enzyme isoforms secreted by the WRF [199]. An understanding of the fundamental biocatalytic nature of the LME is essential to determine the reaction spectrum and extent with respect to the environmental matrix characteristics. For example, most of the laccases show maximum activity at a pH between 3 and 6, but some isoforms exhibit maximum activity at basic pH [200]. Furthermore, the properties of the different isoforms, such as stability and kinetic features could also be different. Insight into the enzyme isoforms is, moreover, important for the production of LMEs which will be most useful in processes for the in vitro conversion of EDCs.

Most of the investigations using WRF cultivation for the elimination of EDCs deal with pure cultures of these microorganisms. Fujita et al. [138] are the only authors who addressed the treatment of non-sterilized effluents from the bioprocessing of night soil containing EDCs. These researchers reduced by 80 % the unwanted air/water-born microorganisms by heating the UF concentrate of the effluent stream that served as culture medium for the WRF used [138].

In order to develop biotechnological processes based on whole WRF catalysis for the transformation of EDCs from aqueous effluents, it is necessary to obtain reliable kinetic data of the whole system that can subsequently serve for pilot scale process demonstration. Depending on their robustness, the relevant kinetic parameters could be used for the design and scale-up of bioreactors with different operating modes.

The elimination of EDCs in soil could be achieved by bioaugmentation of the fungi naturally occurring in soil (e.g. litter-decomposing fungi with an appropriate enzyme spectrum) or by the addition of exogenously grown strains, which is a more challenging prospect. Several strategies for the bioaugmentation of contaminated environments have been reviewed [201]. Even if WRF are efficient EDC-degrading microorganisms, the operational difficulties associated with the in-situ conditions of cultivation (e.g. the need to add an appropriate lignocellulosic support like sawdust to serve as an anchoring substratum) limits the use of such fungi for the treatment of real contaminated matrices.

10.2 Free and Immobilized/Insolubilized LME Treatment

One way to overcome the operational difficulties associated with the cultivation of whole WRF is to use the enzymes present in the culture supernatants. The LMEs laccase and MnP have been successfully implemented for the removal of phenolic EDCs in aqueous, organic and soil environments. The removal efficiency of a given LME depends significantly on the producer strain used. These biocatalysts have been tested for the abatement of numerous EDCs, over a wide range of operational conditions. Little is known about the removal of EDCs either in the presence of denaturants of LMEs or in real ma-

trices. However, it is well known that some constituents or characteristics of wastewaters such as ions, heavy metals, organic solvents, proteolytic enzymes or temperature are implicated in denaturing or inactivating mechanisms of such LMEs. Furthermore, the coexistence of diverse environmental matrices such as the presence of particulate matter in wastewater effluents or sludge slurries could limit the bioavailability of the EDCs to LME- or WRF-mediated transformation and, by extension, could decrease the elimination efficiency of such biocatalysts. This decreased availability is linked with the low water solubility and high hydrophobicity of a large set of phenolic EDCs. Furthermore, the interactions with these particles could change the biocatalytic properties of the enzymes and contribute to the denaturation process of LMEs [202].

Cost effective production methods for enzymes which can be used in a free or immobilized form will likely involve an improved WRF cultivation technology. It will be necessary to determine the properties of the enzymes secreted and by extension the range of xenobiotics oxidized and the economic feasibility of the proposed treatment technologies. Depending on the strain used and the culture conditions, the panel of LME isoforms differs and, with it, the physicochemical and stability properties of the enzyme preparation which are crucial for the treatment of xenobiotics. Furthermore, the economic dimension of the LME production by WRF fermentation is also an important consideration for environmental applications of such biocatalysts. For example, the source of carbon used could greatly influence their economic attractiveness. Low-cost substrates, such as waste biomass, have been successfully used for the production of enzyme preparations with high laccase activity [203]. The availability of LMEs could be markedly increased by their production in recombinant systems [203, 204]. Furthermore, a given fungal LME abundantly expressed in an appropriate host microorganism can also be manipulated by directed evolution to selectively enhance several aspects of its performance. For instance, the enzymatic activity of a thermophilic laccase, which could be functionally expressed in *Saccharomyces cerevisiae,* was improved up to 170-fold following ten rounds of directed evolution [205]. Of course, the particular host organism chosen, the cultivation conditions and also the downstream processing used to separate/purify the enzymes produced will be expected to influence the level, functionality and cost of the LMEs. As with other enzymes, the future improvement of LMEs will be based more and more on protein engineering strategies such as directed evolution, polypeptide chain extension, manipulation of chimeric proteins and ab initio rational design [206]. These advances make it already possible to provide tailor-made enzymes displaying new activities and thus enabling an enhancement of LME performances for the biotreatment of EDCs.

The application of LMEs in free form (in solution) for the treatment of EDCs is beset by significant operational barriers: reusability, cost and denaturation of the enzyme. Strategies to overcome these limitations such as utilization of additives or immobilization have been successfully developed [181, 185, 188, 207]. These techniques have been proposed in order to maximize the enzyme's biocatalytic effectiveness, to ensure a maximum enzyme recovery and to stabilize the LMEs against inactivation. The immobilization of LMEs on various supports has been recognized as a promising way for the (re)utilization of such biocatalysts. A detailed review of the immobilization procedures of phenoloxidases such as laccase and tyrosinase was compiled a few years ago [188]. Generally, the immobilization of such LMEs on solid supports significantly enhances the enzyme stability against several denaturing conditions and allows the continuous utilization of these biocatalysts. However, as a rule, the immobilization of most proteins on such supports results in low activity/weight ratios. Recently, to overcome this important limitation, procedures for the production of insoluble but catalytically functional enzyme aggregates have been proposed [208]. The general approach involves the precipitation of soluble enzyme with an appropriate aggregation additive and its subsequent cross-linking with a bifunctional agent. The resulting insolubilized enzymes (CLEAs) exhibit high activity/mass ratios. Additives such as PEG or amphiphilic copolymers such as polyalkyleneoxide-*co*-maleic anhydride have been previously found to improve both the stability and the catalytic properties of laccases [181, 185, 207]. Thus PEG has been recently employed as a precipitant for the formation of laccase CLEAs that were successfully applied to the removal of EDCs [114].

Globally, it is becoming progressively clear that the action of WRF or their LMEs on EDCs results in the formation of chemicals without apparent estrogenic activity [113, 118, 120, 121, 123–124, 126–127, 142, 145]. However, nothing is known about the further long term transformation of such metabolites by biocatalytic or physicochemical processes in complex matrices and the endocrine disruption potential of such newly formed chemicals in the case of desorption and leaching.

An additional problem that is in need of novel technical solutions is the usually very low concentration at which many EDCs are occurring in effluent streams. This could represent a challenge for microorganisms or isolated free or insoluble enzymes that, although capable of degrading the target compounds, they do so with an affinity constant considerably higher than the actual concentrations of the EDCs. In such cases, hybrid solutions involving the combination of a membrane module that would increase the concentration of the EDCs to be contacted with the biocatalyst could be implemented. In addition, a step involving a favorable partition of the EDC, for instance, in a biphasic system where one hydrophobic phase will tend to concentrate the target pollutant, itself also a hydrophobic molecule, could offer an advantage: this could contribute to an increase in the local concentration of the EDC in contact with the biocatalyst, provided of course that the hydrophobic phase would be compatible with the biocatalyst (i.e., not a major denaturant). Finally, always in the same quest for increasing the local bioavailable concentration of the target EDCs for the biocatalyst, novel immobilization matrices could be envisioned, that would contain a (co)polymeric hydrophobic domain partitioning favorably the hydrophobic EDCs in the vicinity of the immobilized biocatalyst.

11 Concluding Remarks

The elimination of EDCs by WRF cultures or their LMEs are promising processes for the treatment of different contami-

nated environmental matrices. However, several issues must be considered before the use of these biocatalytic systems for the treatment of real EDCs and, by extension, of xenobiotic contaminated sites, can be packaged into well-established, stringently validated technological solutions. Further studies must be conducted to optimize the fermentation conditions, the reactor designs, the properties of the enzyme(s), the formulation of LMEs and the ultimate cost of such enzymes. Furthermore, there is still a lot to be learned on the transformation pathways involved in the removal of EDCs by WRF and their LMEs. Finally, the long-term environmental fates of the chemicals produced have to be elucidated. Despite these challenges, fungal and enzymatic treatments do represent green environmental technologies that environmental managers and engineers have to promote in order to deal effectively with the insidious and far-reaching threat of hormone-disrupting micropollutants.

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