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Removal of micropollutants by fungal laccases in model solution and municipal wastewater: evaluation of estrogenic activity and ecotoxicity

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ABSTRACT

This study describes a multidisciplinary approach that investigates the breakdown potential of a laccase mediated system from *Trametes pubescens* MUT 2400 against several micropollutants including already recognized endocrine disrupting chemicals at their natural residual concentrations (from μg/L up to ng/L).

In model solution, the chemical speciation focused on a mixture of 18 analytes and adopted stir bar sorptive extraction with directed in-situ derivatization followed by gas chromatography and mass spectroscopy analysis. The method's key performance parameters were evaluated in consideration of the chemical peculiarities and complexity of real wastewaters: precision, accuracy, estimated working range extended to a wide residual concentration interval (10 ng/L to100 μg/L) indicated its fitness for purpose.

Laccases were extremely active towards all the target compounds, both in term of removal yields and rate. The maximal percentage of removal was obtained for 4-*t*-butylphenol, 2-hydroxybiphenyl, 4-*n*-octylphenol, salicylic acid and estrone (percentage of removal above 90%). Enzymes concentration played a central role and in most of the cases, the catalyzed reactions were very fast: the initial concentration of 9 compounds was halved within the first 3 h.

The laccase-mediated treatment was then applied to a municipal wastewater collected in a real wastewater treatment plant, containing at least 9 xenobiotics as drugs, pesticides, plasticizers and personal care products. Although the harsh chemical and biological conditions of the effluent influenced enzyme stability, the reaction took place, and above 70% transformation was obtained for most analytes during the 24 h experiment. Bioassays were carried out to estimate the estrogenic activity (the *E-screen* test and the MELN gene-reporter luciferase assay) and the ecotoxicity (*Lepidium sativum*, *Pseudokirchneriella subcapitata* and *Vibrio fischeri*), demonstrating the capability of laccases to mediate an effective detoxification of the wastewater and a decrease of the estrogenic activity.

Abbreviation: EDCs, endocrine disrupting chemicals; PCPs, personal care products; ER, estrogen receptor; SBSE, multi-shot stir bar sorptive extraction; TDU, thermal desorption unit; GC, gas chromatography; MS, mass spectrometry; WWTP, wastewater treatment plant; U/L, enzymatic activity expressed as International Unit per liter; PR, percentage of removal; MEC, minimal effective concentration; $t_{1/2}$, analyte half-life; Ti, Target Ions; RSD%, relative standard deviation percentage; LOQ, limit of quantification; QCx, quality control samples; Rel.Err %, relative error percentage; COD, chemical oxygen demand; GI%, germination index; I%, inhibition percentage of the algal growth or of the bacterial bioluminescence; EEQ, 17-β-estradiol equivalent quantity; RPE %, relative proliferative effect; TRANS%, rate of luciferase gene expression.

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1. Introduction

Endocrine disrupting chemicals (EDCs) are defined as "exogenous substances that cause adverse health effects in an organism, or its progeny, consequent to changes in endocrine functions" and have become a major issue due to their ability to interact with human estrogenic receptors. In this context, European Union has prioritized the reduction of surface-water pollution by municipal and industrial wastewaters, so as to limit the presence of EDCs and other harmful chemicals in the water cycle (Directive 2000/60/EC).

EDCs include biologically active compounds (pesticides, herbicides, and pharmaceuticals), heat stabilizers, plasticizers, personal care products (PCPs), etc. but a complete list is not available yet. One of the main limitations to detect their presence in water bodies is the very low concentration level (from μg/L up to pg/L), prioritizing the development of high-performance analytical techniques (Fatta-Kassinos et al., 2011).

Since several EDCs are designed to be chemically and biologically stable over a wide range of environmental conditions, wastewater treatment plant (WWTP) often proves ineffective causing accumulation phenomena, even into drinking waters (Chong et al., 2012; Mompelat et al., 2009; Snyder et al., 2003). Novel environmentally-sustainable biological processes are an attractive option to the costly and energy-consuming chemical and physical approaches, which often cause undesired by-product formation (Husain and Qayyum, 2012; Ikehata et al., 2006). Many fungi have already been recognized capable to degrade several xenobiotics, including some EDCs as pesticides, plasticizers, pharmaceuticals, etc. by means of their extracellular enzymes (Corvini et al., 2006; Kabiersch et al., 2011; Karas et al., 2011).

Laccases are glycosilated multicopper oxidases, able to catalyze the electron transfer from a substrate to a molecule of oxygen, which is thereby reduced to water. Their activity mainly targets phenolic moieties, but very often thanks to natural or synthetic mediators oxidation of non-phenolic compounds is also possible through an indirect electron transfer (Strong and Claus, 2011). On the whole, laccase-mediated system triggers oxidative reaction cascades, showing low substrate specificity (Strong and Claus, 2011). Owing to their biochemical and catalytic properties, laccase are considered as good green biocatalysts, potentially exploitable for in-field uses: they indeed mediate versatile reactions, being thermostable with a long shelf-life even at room temperature (Youshuang et al., 2011; Liu et al., 2013). Treatments based on these enzymes have been successfully applied towards many xenobiotics including EDCs (Cabana et al., 2007a; Manda et al., 2014). However mostly model single-component solutions have been tested (Catapane et al., 2013; Hommes et al., 2012; Murugesan et al., 2010).

Furthermore because of the heterogeneous and often unknown composition and the mixture interactions of municipal wastewaters, their chemical characterization is not sufficient to examine the intrinsic toxicity. In detail, synergic behavior has been associated to many compounds: for example, the effects of 17-β-estradiol, bisphenol A and DDT were additive (Rajapakse et al., 2001). In this context, bioassays represent effective monitoring tools to estimate the estrogenic activity and the ecotoxicity (Leusch et al., 2010). Moreover, considering the intrinsic sensitivity of each model assay, a battery of tests is generally recommended, using organisms belonging to different trophic levels and different end points as lethality, growth ability, respiration rate, etc. (Leusch et al., 2010; Soupilas et al., 2008; Tigini et al., 2011). Several *in vitro* bioassays have recently been suggested as a screening tool for suspected estrogenic chemicals. The most common ecotoxicological tests involve terrestrial plants (*Cucumis sativus*, *Lepidium sativum*, *Sorghum bicolour*, *Triticum aestivum*, etc.), aquatic plants (*Lemna minor*), algae (*Pseudokirchneriella subcapitata*, *Selenastrum capricornutum*, etc.), crustacea (*Daphnia magna*, *Artemia franciscana*) and bacteria (*Vibrio fischeri*) (Lundstrom et al., 2010; Tigini et al., 2011) whereas the endocrine interference is generally evaluated by means of human cell lines or yeasts (Jobling, 1998). Assays include estrogen receptor (ER) binding, ER-dependent transcription system, and proliferation of estrogen dependent cell lines such as MCF-7 cells; they can determine the total estrogenic EDCs activity of environmental samples (Berckmans et al., 2007; Nelson et al., 2007; Schilirò et al., 2009).

Because of the large number of micropollutants potentially present in WWTP samples, the present multidisciplinary study investigated the effectiveness of laccases of *T. pubescens* MUT 2400 against a complex mixture of EDCs, pharmaceuticals, pesticides and PCPs. A multi-residue screening analytical approach, based on multi-shot stir bar sorptive extraction (SBSE) with directed in-situ derivatization followed by gas chromatography and mass spectrometry (GC–MS) was identified as the most suitable method to contemporarily quantify 18 target compounds on model solution and a real wastewater sample in order to evaluate the full potential of the enzymatic treatment. In this intriguing context, the present research covered some scientific areas in order to critically discuss the potentials of a biological oxidation method for the treatment of civil wastewaters. The risk assessment was carried out combining advanced multi-residue analytical technique and biological tests to globally evaluate the effectiveness of fungal laccases.

This study was then focused to describe the capability of fungal laccases: i) to remove micropollutants including EDCs, PCPs, etc. in model and real solutions through the definition of the maximal analyte removal and half-life, and the minimal effective concentration of laccases; ii) to reduce the ecotoxicological hazard and the estrogenic activity of a real municipal wastewater.

2. Materials and methods

Certified EDCs, pharmaceuticals and PCPs with known or suspected estrogenic activity were used: 2,4-dichlorophenol, 4-t-butylphenol, diethyl phthalate, 2-hydroxybiphenyl, 4-n-octylphenol, salicylic acid, alachlor, 4-n-nonylphenol, oxybenzone naproxen, diclofenac, triclosan, ketoprofen, bisphenol A, bis(2-ethylhexyl) phthalate, estrone, 17-α-ethynyl estradiol, 17-β-estradiol. Standard stock solutions of each compound were prepared in acetone at a concentration of 1 g/L, and stored at −18 °C. All the chemicals (purity 97–99%) were purchased from Sigma–Aldrich (Milan, Italy).

2.1. Municipal wastewater

Sample was collected from a real municipal WWTP in northwestern Italy (Turin), treating more than 260 million m^3 of sewage from an area of about 450 km^2. The mean treated wastewater flow is approximately 615,000 m^3/day mainly released by 1.5 million inhabitants and 1800 industries, amounting to a total equivalent population of 3 million.

Twenty-four hour composite sample (4 L) was collected after primary sedimentation and stored in brown glass bottles at 4 °C. The effluent parameters are listed in Table 1.

2.2. Enzymatic treatment

The strain selected for the study and its enzymatic pathway is the result of screening more than 300 different Basidiomycetes with interesting key-features for this specific application: high chemical stability in complex environmental samples, wide range of operative pH, high production yield in optimized cultural