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Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus *Coriolopsis polyzona*

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Abstract

The biocatalytic elimination of the endocrine disrupting chemicals (EDC) nonylphenol (NP) and bisphenol A (BPA) and the personal care product ingredient triclosan (TCS) by the enzyme preparation from the white rot fungus *Coriolopsis polyzona* was investigated. Analysis of variance methodology showed that the pH and the temperature are statistically significant factors in the removal of NP, BPA and TCS. The elimination of NP and TCS was best at a temperature of 50 °C and the disappearance of BPA at 40 °C, whereas the most suitable pH for all three micropollutants was 5. After a 4-h treatment of the three target compounds at concentrations of 5 mg l^{-1} all of the NP and BPA were eliminated. In the case of TCS, 65% was removed after either a 4 or an 8-h treatment. The utilisation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in the laccase/mediator system significantly increased the efficiency of the enzymatic treatment. The elimination of NP and BPA was directly associated with the disappearance of the estrogenic activity. Mass spectrometry analysis showed that the enzymatic treatment produced high molecular weight metabolites through a radical polymerization mechanism of NP, BPA and TCS. These oligomers were produced through the formation of C–C or C–O bonds. The polymerization of NP produced dimers, trimers, tetramers and pentamers which had molecular weights of 438, 656, 874 and 1092 amu respectively. The polymerization of BPA produced dimers, trimers and tetramers which had molecular weights of 454, 680 and 906 amu. Finally, the polymerization of TCS produced dimers, trimers and tetramers which had molecular weights of 574, 859 and 1146 amu.

Keywords: Laccase; Estrogenic activity; Polymerization; Mediators

1. Introduction

There are increasing concerns about potential adverse health and ecological effects resulting from the production, use, and disposal of numerous chemicals that otherwise offer improvements in human life and economic activities. Household chemicals, pharmaceuticals, and other consumables as well as biogenic hormones are released in the environment after passing through wastewater treatment processes, which are not designed to remove them. Current environmental protection research is focused both on chemicals used in the elaboration of personal care products (PCP) and on substances which are known or

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suspected potential endocrine disrupting chemicals (EDC). Nonylphenol (4-nonylphenol) (NP), bisphenol A (2,2-bis(4-hydroxyphenol)propane) (BPA), and triclosan (5-chloro-2(2,4-dichlorophenoxy) phenol) (TCS) are among the most frequently detected compounds in waters downstream of intense urbanization (Kolpin et al., 2002; Boyd et al., 2004). Furthermore, recent research has demonstrated that these chemicals can or are suspected to mimic or interfere with the action of animal endogenous hormones by acting as estrogen agonists, binding to the estrogen receptor or eliminating a normal biological response (Soto et al., 1991; Jobling et al., 2003; Ishibashi et al., 2004).

The presence of NP in the aquatic environment is linked to the biodegradation in sewage treatment plants (STP) of nonylphenol ethoxylates (NPE) (Schröder, 2001) which are mainly used as non-ionic surfactants in domestic and industrial applications. The NP produced are considered to be slowly biodegradable under aerobic conditions (Staples et al., 1999; Ying et al., 2002). This fact is demonstrated by the presence of NP in the effluents of STP in concentrations up to 398 $\mu g\,l^{-1}$ (Sole et al., 2000). Only a few pure cultures of aerobic bacteria (Tanghe et al., 1999b; Fujii et al., 2001; Soares et al., 2003; Ushiba et al., 2003; Gabriel et al., 2005), one yeast (Corti et al., 1995), two aquatic fungi (Junghanns et al., 2005) and an anaerobic consortium (Chang et al., 2004) are able to use NP as the sole source of carbon and energy have been described. The sorption of NP to sludges due to these compounds' high hydrophobicity ($\log K_{ow} = 4.48$) is supposed to be the main mechanism of removal of these xenobiotics (Ahel et al., 1994). This mechanism leads to a transfer of this pollutant to another environmental matrix and not to its destruction. Furthermore, the elimination of NP in the aquatic environment is essentially due to sorption to particles and sediments and to bioaccumulation in the tissues of aquatic organisms (Uguz et al., 2003), a serious environmental and health problem in itself.

BPA is used as raw material for the production of polycarbonates and epoxy resins. Its discharge in the environment can occur from factories producing BPA or incorporating it into plastics (Staples et al., 1998), from leaching of plastic wastes (Sajiki and Yonekubo, 2003) and landfill sites (Asakura et al., 2004). Concentrations of BPA can reach up to 21 $\mu g\,l^{-1}$ in surface and marine water (Belfroid et al., 2002) and up to 702 $ng\,l^{-1}$ in sewage effluents. Bacteria able to degrade BPA have been isolated from a STP (Lobos et al., 1992) and river water (Kang et al., 2004).

TCS is a broad spectrum antimicrobial agent. It has been incorporated into a wide range of PCP such as toothpaste, deodorant sticks, soap and handwash. The presence of TCS in the environment essentially comes from STP effluents and sludges. The concentration of TCS in STP effluents can reach up to 37.8 $\mu g\,l^{-1}$ (Hua et al., 2005) and in surface water up to 431 $ng\,l^{-1}$ (Morrall et al., 2004). As a result of the high hydrophobicity of this chemical ($\log K_{ow} = 4.8$), its dissipation in the aquatic environment occurs by sorption to particles and sediments and it tends to bioaccumulate in aquatic organisms (Adolfsson-Erici et al., 2002). Attention has been drawn to TCS due to the similarity of its chemical structure with those of the xenoestrogen BPA and of highly toxic contaminants such as dioxins. Little is known about potential endocrine disruption activities associated with TCS. Studies on medaka (*Oryzias latipes*) suggest that TCS could be a weak androgenic substance (Foran et al., 2000) and that its metabolites can act as estrogen receptor antagonists (Ishibashi et al., 2004).

Recently there has been a great interest in white rot fungi (WRF) and their ligninolytic enzymes for the biodegradation of a wide range of xenobiotics (Torres et al., 2003). WRF produce oxidative enzymes such as laccase, lignin and manganese peroxidase, which are relatively non-specific biocatalysts (Wesenberg et al., 2003). A few studies have used fungi and ligninolytic enzymes to eliminate EDC and PCP. Two aquatic fungi (Junghanns et al., 2005) and four strains of WRF (Soares et al., 2005) were studied as means to remove NP. In addition, one strain of *Pleurotus ostreatus* was used for BPA elimination (Hirano et al., 2000) and two strains, *Trametes versicolor* and *Pycnoporus cinnabarinus*, were used to degrade TCS (Hundt et al., 2000). Finally, crude and purified laccase (polyphenoloxidase, EC 1.10.3.2) were used to degrade NP and BPA (Tsutsumi et al., 2001; Saito et al., 2004; Kim and Nicell, 2006). None of these studies has addressed the potential mechanism of elimination nor has there been a precise determination of the products formed from these substances upon enzymatic treatment.

The aim of this study was to examine the removal of NP, BPA and TCS by the enzyme preparation from the WRF *Coriolopsis polyzona*. Specific aspects addressed included the effect of the pH and temperature on their removal, the elimination of their estrogenic activity and potential improvement of the transformation in the presence of redox mediators. Finally, the transformation mechanism of NP, BPA and TCS was assessed by determining the metabolites produced.

2. Experimental section

2.1. Organism and cultivation conditions

The WRF strain *C. polyzona* (MUCL 38443) was provided by the Belgian Coordinated Collection of Microorganisms (BCCM^{TM}/MUCL). The inoculum was prepared by growing the fungus on a rotary shaker at 150 rpm and 27 °C in 250-ml flasks containing 100 ml of medium: 10 $g\,l^{-1}$ glucose, 2 $g\,l^{-1}$ NH_4NO_3, 0.8 $g\,l^{-1}$ KH_2PO_4, 0.4 $g\,l^{-1}$ Na_2HPO_4, 0.5 $g\,l^{-1}$ $MgSO_4 \cdot 7H_2O$, 2 $g\,l^{-1}$ yeast extract. After 5 d of cultivation mycelial pellets were harvested and homogenized with a Waring laboratory blender. For enzyme preparation, submerged fermentation of poplar (*Populus* sp.) leaves (50 $g\,l^{-1}$, oven dried at 50 °C and milled) was carried out on a rotary shaker at 150 rpm and

27 °C in 250-ml flasks containing 100 ml of the above standard medium without glucose. The following trace elements were added to the medium: 1 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 5 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 60 mg l^{-1} $CaCl_2 \cdot 2H_2O$ and 5 mg l^{-1} $CuSO_4 \cdot 7H_2O$. The medium was adjusted to pH 6.0 with 2 M NaOH prior to sterilization. After 10 d of fungus cultivation the biomass was filtered, the solids were separated by centrifugation (2500 g for 15 min) at 4 °C, and the supernatant was used as the source of crude enzyme.

2.2. Enzyme assay

Laccase activity was determined by monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to its cation radical ($ABTS^{\cdot+}$) at 420 nm ($\varepsilon_{max} = 3.6 \times 10^4$ M^{-1} cm^{-1}). The assay mixture contained 0.5 mM ABTS. The pH was adjusted to 3 using citric acid/di-sodium hydrogen phosphate buffer and the temperature was set at 30 °C. One unit (U) of activity was defined as the amount of enzyme forming 1 μmol of $ABTS^{\cdot+}$ per min.

2.3. Enzymatic treatment

The reaction mixture consisted of 5 mg l^{-1} of each of NP (technical mixture, 0.023 mM), BPA (0.022 mM) or TCS (0.018 mM), 5 U l^{-1} catalase from *Aspergillus niger*, crude enzyme preparation from *C. polyzona*, citric acid/di-sodium hydrogen phosphate buffer and 1% v/v methanol. Concentrations of enzyme preparation containing 1, 10 and 100 U l^{-1} laccase were used to treat respectively NP, BPA and TCS except where other concentrations are cited. The enzymatic treatment occurred at 20 °C, 40 °C or 50 °C and at pH 3, 4 or 5. For the laccase mediator-supplemented system, 10 μM of 1-hydroxybenzotriazole (1-HBT) or ABTS was added to the mixture. Prior to treatment, air was bubbled in the buffer solution overnight to saturate it with oxygen. The enzymatic treatment was stopped by boiling the solution for 5 min. The solution was kept frozen at −20 °C up to the time of analysis. The enzymatic treatment was performed three times and samples from each treatment were analyzed twice.

2.4. Extraction

NP was extracted using a C_{18} solid phase extraction cartridge. The cartridge was conditioned with 4 ml of methanol and 4 ml of distilled water prior to extraction. It was filled with 5 ml of the treated solution and eluted at a flow rate of approximately 2 ml min^{-1} under vacuum. The NP was eluted from the cartridge using 3 ml of ethyl acetate and 6 ml of methylene chloride. The organic phase was then evaporated under a gentle stream of nitrogen. The NP recovery was 89% (±3%).

BPA and TCS were extracted using ethyl acetate in a 1:1 volumetric ratio (ethyl acetate:treated solution). The solutions were acidified to pH 2 with HCl, shaken for 20 min and then frozen overnight. The organic phase was separated and evaporated under a gentle stream of nitrogen. Each sample was dissolved in 100 μl of methanol prior to quantitative chemical analysis. BPA and TCS recoveries were respectively 95% (±2%) and 91% (±4%).

2.5. HPLC analysis

The quantitative analysis of NP, BPA and TCS was performed on a HPLC system consisting of a 600 controller, a 717 plus autosampler and a 996 photodiode array detector (Waters, USA). An Adsorbosphere XL Silica 90A 5U 250 × 4.6 mm column (Alltech, USA) was used for the chromatographic separation. A 1 ml min^{-1} gradient elution by means of (A) ethyl acetate with 1% bi-distilled water and (B) tetrahydrofuran with 5% bi-distilled water was applied. The gradient program was set as follows: Initially the eluent was constituted of 100% A and then the concentration was decreased linearly to 87.5% A within 5 min. The composition was kept constant for 15 min. NP, BPA and TCS were detected at a wavelength of 277 nm. The limit of detection was 60 μg l^{-1} for NP and 35 μg l^{-1} for BPA and TCS.

2.6. Estrogenic activity

The estrogenic activity of the treated solution was determined using the recombinant Yeast Estrogenic Screen (YES) kindly provided by Prof. J.P. Sumpter (Brunel University, UK). This test allows to identify the chemical compounds that can bind to the human estrogen receptor (hER). The assay makes use of a *Saccharomyces cerevisiae* strain genetically modified to express the hER gene. When the hER is bound to an estrogen-like compound, the receptor is co-expressed with the reporter gene lacZ, which codes for the enzyme β-galactosidase. This enzyme is secreted into the growth medium and catalyzes the transformation of the chromogenic substance chlorophenol red-β-D-galactopyranoside, which is subsequently measured colorimetrically in the medium. Handling of the yeast culture, preparation of the growth medium and test procedure are described in detail elsewhere (Routledge and Sumpter, 1996). In this study, we used a double strength assay medium (Tanghe et al., 1999a). The estrogenic activity of the solution was correlated to its absorbance by the expression

$$A_{\text{corrected}} = (A_{540\,\text{sample}} - A_{540\,\text{blank}}) - (A_{620\,\text{sample}} - A_{620\,\text{blank}}). \quad (1)$$

The corrected absorbance of the treated solution was compared to that of a solution not subjected to enzymatic treatment.

2.7. Identification of high molecular weight metabolites

To identify high molecular weight metabolites, a 5 ml reaction mixture consisting of 100 mg l^{-1} of NP

(0.46 mM), BPA (0.44 mM) or TCS (0.36 mM), 5 U l^{-1} catalase from *A. niger*, citric acid/di-sodium hydrogen phosphate buffer and 1% v/v methanol was used. The temperature was set at 30 °C and the pH at 5. The concentration of laccase was 50 U l^{-1}. The enzymatic reaction was allowed to take place for 24 h and it was stopped by acidifying the solution at a pH of approximately 1. The reaction mixture was centrifuged at 1252 g for 90 min. The precipitate was dried under a gentle stream of nitrogen and suspended in chloroform. This solution was subjected to mass spectra (MS) analysis.

2.8. MS analysis

MS were acquired using a TSQ Quantum triple stage quadrupole instrument (Thermo Finnigan, USA) equipped with an electrospray atmospheric pressure ionization (ESI) source in the negative ion monitoring mode, hereafter indicated as ESI(-). The ESI(-) conditions were set as follows: Spray voltage, 2 kV; capillary voltage, −35 V; capillary temperature, 270 °C; sheath gas, 17 U and collision gas pressure, 0.2 Pa.

3. Results

3.1. Effect of temperature and pH on the enzymatic elimination of NP, BPA and TCS

Preliminary results had shown the disappearance of NP, BPA and TCS at room temperature and at pH 5. In order to investigate the effect of pH and temperature on the removal of NP, BPA and TCS, a factorial experimental design was used. This design can determine statistically the impact of each condition on the reduction of the concentration of each substance in solution. The temperatures studied were 20, 40 and 50 °C and the pHs were 3, 4 and 5. The corresponding analysis of variance (ANOVA) is presented in Table 1. This statistical analysis is based on the model presented in Eq. (2):

$$y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \varepsilon_{ijk}. \quad (2)$$

The coefficient of determination (R^2) value provides a measure of how much variability in the observed response values can be attributed to the experimental factors and their interactions. The model R^2 of 0.995 for the elimination of NP, 0.996 for that of BPA and 0.994 for that of TCS suggested that the fitted linear-plus-interactions models could explain 99.5%, 99.6% and 99.4% respectively of the total variation. This implies satisfactory representations of the processes by the models. The *F*-values of 426.0 for NP elimination, 622.5 for BPA elimination and 361.3 for TCS elimination and a *p* value <0.001 for all eliminations indicate that the present models are statistically significant and can predict well the experimental results.

3.2. Elimination of NP, BPA and TCS

The removal by the enzyme preparation from *C. polyzona* of the three phenolic compounds NP, BPA and TCS was pursued using pH 5 and 50 °C. Fig. 1 shows the time course of the removal of NP, BPA and TCS. A 55% elimination of NP was achieved after a 0.5-h and 80% after a 1-h treatment. After a 4-h treatment, more than 95% of the NP had been eliminated from the solution. The crude enzyme preparation from *C. polyzona* helped remove 25% of BPA after 30 min, 35% after 1 h and 100% after 4 h.

Table 1
Analysis of variance (ANOVA) for the selected linear-plus-interactions model for elimination of NP, BPA and TCS with laccase from *C. polyzona*

Substance	Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Prob > *F*	Coefficient of determination (R^2)
NP[a]	Model	8055.5	8	1006.9	426.0	<0.0001	0.995
	Temperature	3148.4	2	1574.2	665.9	<0.0001	–
	pH	4683.9	2	2342.0	990.7	<0.0001	–
	Interaction temperature/pH	223.2	4	55.8	23.6	<0.0001	–
	Residual error	42.6	18	2.4	–	–	–
	Total	8098.1	26	–	–	–	–
BPA[a]	Model	32660.5	8	4082.6	622.5	<0.0001	0.996
	Temperature	11557.5	2	5778.7	881.2	<0.0001	–
	pH	17741.2	2	8870.6	1352.6	<0.0001	–
	Interaction temperature/pH	3361.8	4	840.4	128.2	<0.0001	–
	Residual error	118.1	18	6.6	–	–	–
	Total	32778.5	26	–	–	–	–
TCS[b]	Model	7038.2	8	879.8	361.3	<0.0001	0.994
	Temperature	1646.2	2	823.1	338.0	<0.0001	–
	pH	5154.1	2	2577.1	1058.2	<0.0001	–
	Interaction temperature/pH	238.0	4	59.5	24.4	<0.0001	–
	Residual error	43.8	18	2.4	–	–	–
	Total	7082.1	26	–	–	–	–

[a] Analysis for a 4-h treatment.
[b] Analysis for a 8-h treatment.

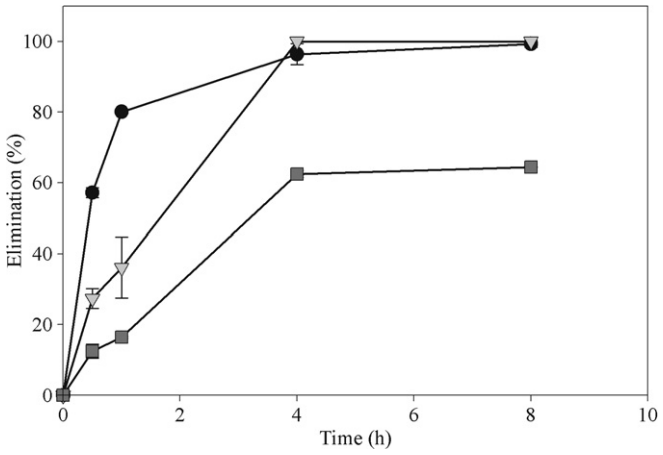

Fig. 1. Elimination of (●) NP with 1 U l^{-1}, (▼) BPA with 10 U l^{-1} and (■) TCS with 100 U l^{-1} of laccase activity from *C. polyzona* at the previously established pH of 5 and temperature of 50 °C.

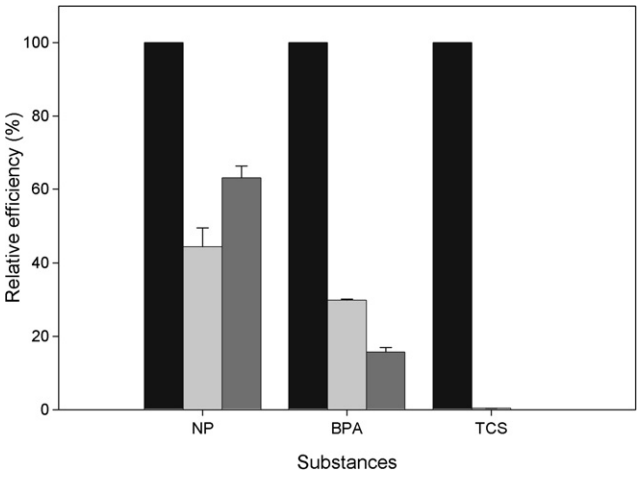

Fig. 2. Effect of 10 μM of (■) ABTS, (▒) 1-HBT or (▒) absence of mediator on the removal of NP, BPA and TCS after a 1-h treatment at pH 4 and a temperature of 40 °C with the enzyme preparation containing 10 U l^{-1} of laccase from *C. polyzona*.

After half an hour, less than 15% of the TCS was removed from the solution. A 1-h treatment resulted in less than 20% removal, whereas after either a 4-h or an 8-h treatment, 65% of the TCS had been removed from the solution.

3.3. Use of the enzyme preparation/mediator system

The use of low-molecular weight oxidizable substances 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 (Bourbonnais and Paice, 1990). In this study, we compared the ability of ABTS and 1-HBT to improve the elimination of NP, BPA and TCS at less favorable conditions. For this, a 1-h treatment at 40 °C and pH 4 involved the enzyme preparation containing 10 U l^{-1} of laccase and 10 μM of mediator. The relative efficiency of the mediators used is compared in Fig. 2. The use of ABTS significantly increased the removal of all chemicals tested ($\alpha = 0.05$). Using ABTS, an absolute elimination of 66% for NP and of 50% for BPA and TCS was reached under the conditions tested. The presence of the mediator alone did not eliminate NP, BPA or TCS (results not shown).

3.4. Elimination of the estrogenic activity of NP and BPA upon laccase treatment

Having already studied the enzyme-catalyzed removal of NP, BPA and TCS, it was subsequently important to address the elimination of the estrogenic activity of these compounds. For this, the YES assay was used. Fig. 3 shows that BPA and NP could bind to the hER at concentrations higher than 25×10^{-1} and 50×10^{-1} mg l^{-1} respectively, i.e., BPA had a higher tendency to bind to the hER

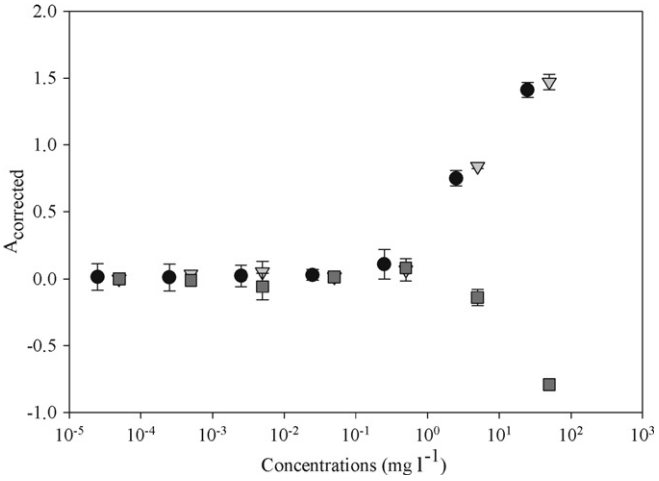

Fig. 3. Responses of the Yeast Estrogenic Screen assay to (▼) NP, (●) BPA and (■) TCS.

than NP. The drop in corrected absorbance for TCS observed at concentrations higher than 5 mg l^{-1} is due to lysis of the yeast. At concentrations lower than 5 mg l^{-1}, TCS did not show any ability to bind to the hER.

Fig. 4 shows the time course of the elimination of the estrogenic activity of the NP and BPA solutions. For the NP, a reduction of approximately 80% in estrogenic activity was obtained after 1 h and 95% after 4 h of enzymatic treatment. For the BPA, the estrogenic activity of the solution decreased by 35% after 1 h and by 90% after 4 h of treatment. After 8 h, the enzymatic treatment had removed all of the estrogenic activity associated with NP and BPA.

3.5. Identification of high molecular weight metabolites produced by the enzyme preparation treatment

Fig. 5a shows the ESI(-) MS spectra of chloroform soluble metabolites of NP. On the full ESI(-) MS spectrum,

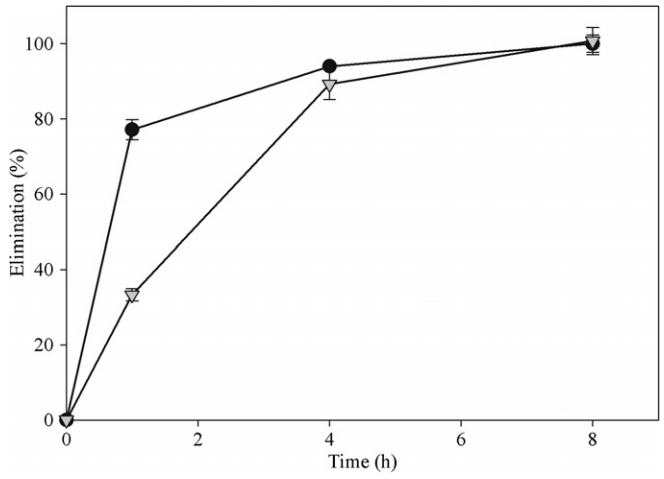

Fig. 4. Elimination of the estrogenic activity of (●) NP and (▽) BPA with the enzyme preparation containing respectively 1 U l^{-1} and 10 U l^{-1} of laccase from *C. polyzona* at a pH of 5 and a temperature of 50 °C.

m/*z* values of 437, 655, 873 and 1091 represent respectively $[2M-H]^-$, $[3M-H]^-$, $[4M-H]^-$ and $[5M-H]^-$ of NP. The molecular weights of these compounds are respectively 438, 656, 874 and 1092 amu. The MS^2 spectrum of the dimer shows ions at *m*/*z* of 337, 351 and 365 which are due to the fragmentation of the nonyl chain from the dimer produced.

Fig. 5b shows the ESI(-) MS spectra of chloroform-soluble metabolites of BPA. On the full ESI(-) MS spectrum, *m*/*z* values of 453, 679 and 905 represent respectively $[2M-H]^-$, $[3M-H]^-$ and $[4M-H]^-$ of BPA. The molecular weights of the BPA dimer, trimer and tetramer detected are respectively 454, 680 and 906 amu. The MS^2 spectrum of the dimer of BPA shows only one fragment at *m*/*z* 227 corresponding to the dimer truncated by one of the monomers forming this molecule.

Fig. 5c shows the ESI(-) MS spectra of chloroform-soluble metabolites of TCS. On the full ESI(-) MS spectrum, *m*/*z* values of 287, 573, 859 and 1145 represent respectively $[M-H]^-$, $[2M-H]^-$, $[3M-H]^-$ and $[4M-H]^-$ of TCS. The molecular weights of these compounds are respectively 288, 574, 860 and 1146 amu. The MS^2 spectrum of the dimer shows an intense fragment at *m*/*z* 161 corresponding to the loss of $C_6H_3Cl_2O$. The fragment at *m*/*z* 411 corresponds to the loss of $C_6H_3OCl_2$ and the fragment of *m*/*z* 537 indicates removal of the Cl from the dimer.

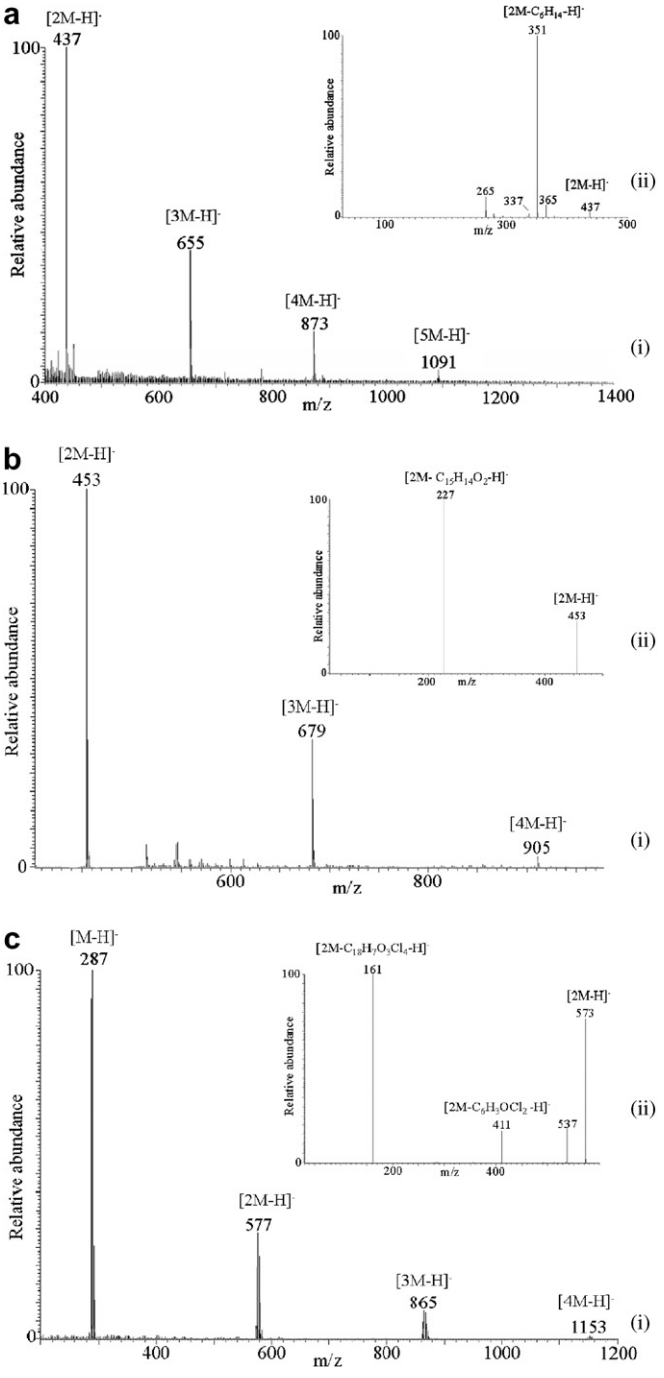

Fig. 5. ESI(-) (i) MS full scan spectrum of high molecular weight metabolites of (a) NP, (b) BPA and (c) TCS and (ii) MS^2 spectrum of the dimer produced by action of the enzyme preparation containing 50 U l^{-1} of laccase from *C. polyzona* at a pH of 5 and a temperature of 30 °C.

4. Discussion

The submerged fermentation of *C. polyzona* on leaves produces laccase and manganese peroxidase activity at a level up to 1900 U l^{-1} and 100 U l^{-1}, respectively, while no lignin peroxidase activity was detected under these conditions (Elisashvili et al., in press). Therefore, the major ligninolytic enzyme activity detected in the culture liquid under these fermentation conditions was laccase. The addition of catalase effectively removed any possibility of manganese peroxidase activity by eliminating hydrogen peroxide and ensuring that the only lignin modifying enzyme active in our system was laccase.

The ANOVA analysis highlighted the significant influences of temperature and pH on the enzymatic transformation of NP, BPA and TCS. This analysis allowed us to arrive at the best conditions for enzymatic transformation of NP, BPA and TCS under the conditions studied. It was

possible to conclude that 50 °C was the best temperature among the three temperatures considered for the disappearance of NP and TCS while results were not significantly different for temperatures of 40 °C and 50 °C in the case of BPA. A pH of 5 gave the best results for all compounds studied. These results are in agreement with a combination of stability produced by a higher pH and catalytic activity resulting from a higher temperature.

The removal at pH 3 and 4 is significantly lower ($\alpha = 0.05$). In fact, the half-life of laccase activity in the enzyme preparation using ABTS as substrate was estimated to be 4, 6 and 16 h at pH 3, 4 and 5 respectively and a temperature of 40 °C (results not shown). This could be due to the denaturation of this laccase at lower pH. Its isothermal inactivation rate constant (h^{-1}) at 40 °C was 0.35, 0.11 and 0.04 at respectively pH 3, 4 and 5 (results not shown). The optimum pH for a bioreactor based treatment of NP contaminated soil using laccase of *Trametes* sp. was approximately 5 (Tanaka et al., 2001). An optimum pH of 3 for the initial rate of BPA removal by laccase from *Coriolus versicolor* was previously found (Okazaki et al., 2002), but the time course of the transformation of BPA under this pH condition was not reported. Finally, the optimum temperature and pH for the elimination of BPA by commercial laccase from *T. versicolor* were recently reported as 45 °C and 5 respectively (Kim and Nicell, 2006).

In our study, the elimination of NP, BPA and TCS was best after a 4-h treatment under the conditions tested. In addition, based on the efficiency of elimination the laccase from *C. polyzona* had a decreasing affinity for NP, BPA and TCS in that order. Based on the catalytic coefficient k_{cat}, the laccase from a new fungus designated as I-4 had higher affinity for BPA than for NP (Saito et al., 2003).

In our work, the elimination of these phenolic substances was significantly enhanced by the use of the enzyme preparation/mediator system. The use of 1-HBT did not increase significantly the removal of NP, BPA or TCS compared to the treatment without mediators ($\alpha = 0.05$) under the conditions tested. For the TCS, no elimination of the substance under these conditions was observed except when ABTS was used as a mediator. In contrast, the removal of NP and BPA by the laccase of *T. versicolor* IFO7043 was enhanced by the presence of 0.2 mM of 1-HBT in solution (Tsutsumi et al., 2001).

Although the elimination of NP and BPA was quantified as above, we felt that it was also of great concern to address the elimination of the estrogenic activity associated with these compounds. Using the YES assay, we determined that the estrogenic activity of NP represented approximately 80% of BPA's estrogenic activity for the same concentration. These results are in agreement with those of Routledge and Sumpter (1996) who showed that BPA had a higher estrogenic activity than NP. The putative estrogenic activity of TCS could not be determined using this assay due to the antimicrobial action of TCS (Fig. 3).

The elimination of the estrogenic activity of NP and BPA solutions by the enzyme preparation from *C. polyzona* was significantly related to the elimination of the parent compound ($\alpha = 0.05$). These results indicate that the removal of these xenoestrogens does not create compounds able to bind to the hER present in the genetically modified *S. cerevisiae*. The elimination of the estrogenic activity of these solutions is associated with the loss of chemical structure similarity with human estrogens that allows these compounds to bind to the hER. Furthermore, the elimination of the estrogenic activity could be linked to the physical removal by precipitation of the oligomers produced. Our results differ from those previously reported which had shown that the elimination of NP and BPA using laccase or a laccase/1-HBT system was not directly linked to the elimination of the estrogenic activity of these xenoestrogens (Tsutsumi et al., 2001).

Laccase is widely applied to oxidize phenol-like chemicals to phenoxyl radicals (Durán and Esposito, 2000). These radicals react with phenolic substances to form polymers of the initial substance. Support for this polymerization mechanism comes from the full scan ESI(-) MS spectra of NP, BPA and TCS solutions treated with laccase: high molecular weight metabolites produced result from the polymerization mechanism of laccase oxidation. According to our results, the elimination of NP and BPA by the enzyme preparation from *C. polyzona* produces essentially dimers. These spectra show that this polymerization could occur at the level of C–C bond formation. This type of bond could be between phenol moieties of NP, BPA or TCS. This way of polymerization was previously proposed for BPA and the dimer produced by the laccase of *T. villosa* was identified as 5,5′-bis[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,2′-diol (Uchida et al., 2001). Recently, Huang and Weber (2005) proposed possible reaction pathways in which the polymerization mechanism of BPA also occurs through C–O bonds.

Our work is the first report of elimination of NP, BPA and TCS and of the associated estrogenic activity based on a clear understanding of the structure of the compounds resulting from the enzymatic treatment.

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