ÉLIMINATION DES PERTURBATEURS ENDOCRINIENS NONYLPHÉNOL, BISPHÉNOL A ET TRICLOSAN PAR L'ACTION OXYDATIVE DE LA LACCASE DE *CORIOLOPSIS POLYZONA*

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RÉSUMÉ

Les substances perturbatrices du système endocrinien sont des substances qui, de par leur capacité à induire des changements hormonaux chez les organismes vivants, génèrent des préoccupations dans le domaine de la qualité des eaux et, par extension, dans le domaine du traitement des effluents aqueux. Particulièrement, ce projet de recherche s'est attardé sur l'élimination des perturbateurs endocriniens phénoliques nonylphénol (NP), bisphénol A (BPA) et triclosan (TCS) en solution aqueuse à l'aide de la laccase (E.C. 1.10.3.2) sécrétée par la souche fongique *Coriolopsis polyzona*. Cette oxydase est une métalloprotéine pouvant catalyser l'oxydation d'une vaste gamme de substances phénoliques.

En premier lieu, l'impact du pH et de la température sur l'élimination de ces composés à l'aide de la laccase libre en utilisant un design factoriel. L'oxydation de ces composés produit des oligomères (dimère à pentamère) via le couplage des radicaux phénoxy produits par l'action de la laccase. Il s'avère que les substances produites suite à l'oxydation du NP et du BPA par la laccase ont perdu leurs similitudes structurales avec l'estrogène. Ainsi, l'élimination de l'activité estrogénique de ces substances est directement liée à la transformation des composés. Finalement, l'utilisation d'ABTS comme médiateur a permis d'augmenter le taux d'oxydation enzymatique de ces composés chimiques.

Puis, de façon à augmenter la possibilité d'utilisation de la laccase dans des biotechnologies environnementales, cette enzyme a été immobilisée sur un support siliceux et via la réticulation d'agrégats. L'impact des conditions d'immobilisation sur l'activité enzymatique, la stabilité du catalyseur et les propriété biocatalytiques apparentes a été déterminé pour différentes stratégies d'immobilisation. Globalement, l'immobilisation génère un biocatalyseur stable vis-à-vis les dénaturations chimique, physique et biologique. Particulièrement, l'immobilisation sur un support solide produit un biocatalyseur facile à utiliser ayant une faible activité massique et des propriétés cinétiques moindres que celle de l'enzyme libre. La formation de CLEAs de laccase a permis d'obtenir une activité massique élevée et des propriétés cinétiques supérieures à celle de l'enzyme soluble.

Ces biocatalyseurs solides ont étés utilisés pour éliminer en continu le NP, BPA et TCS dans différents types de bioréacteur. Le biocatalyseur sur silice a été utilisé pour éliminer ces substances dans un réacteur garni, tandis que les CLEAs ont été utilisés dans un réacteur à lit fluidisé et un réacteur à perfusion développé au cours de ce projet. Ces différentes configurations de bioréacteur ont permis d'éliminer efficacement ces différents perturbateurs endocriniens.

Globalement, les différents résultats obtenus, à l'échelle de laboratoire, au cours de ce projet de recherche démontrent que la laccase et particulièrement les biocatalyseurs formés via les différentes stratégies d'immobilisation testées représentent des approches extrêmement prometteuses pour le développement de biotechnologies environnementales vouées à l'élimination des perturbateurs endocriniens phénoliques.

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LISTE DES SYMBOLES ET ABRÉVIATIONS

Cette section reprend l'ensemble des abréviations utilisées dans ce travail. Il est à noter que la signification de ces abréviations est donnée dans la langue utilisée dans le texte. En ce sens, une abréviation provenant d'un chapitre écrit en français sera présentée en français dans la liste qui suit.

1-HBT, 1-hydroxy-benzotriazole;

a, impeller-dependant constant;

$\mathbf{A_{0,\ t,\ max,\ 24}}$, laccase activity, initial activity, activity at time *t*, maximum activity, activity after 24 h;

ABTS, 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid);

ANOVA, analysis of variance;

AOT, bis(2-ethylhexyl) sulfosuccinate sodium salt;

APTES, γ-aminopropyltriethoxysilane, aminopropyltriéthoxysilane;

BCCM^{TM}/MUCL, Mycothèque de l'Université Catholique de Louvain partner of the Belgian Coordinated Collection of Microorganisms;

BPA, bisphenol A, bisphénol A;

BR, perfusion basket reactor, réacteur à perfusion;

BSA, bovine serum albumin;

β-sit, β-sitosterol;

b, impeller-dependant constant;

C, constant of the 3-parameter inactivation model;

$\mathbf{C_{EDC}}$, molar concentrations of EDC in solution;

CLE, cross-linked enzyme;

CLEA, cross-linked enzyme aggregate;

> **CLEA-BSA-0.01(or 0.1 and 1)**, CLEA formed by the co-aggregation of laccase and 0.01 (or 0.1 and 1.0) mg of BSA per unit of laccase activity;

CLEC, cross-linked enzyme crystal;

COD, chemical oxygen demand;

CR633-GLU(or GLY), standard sequential immobilization procedure of laccase on Celite ® R-633 using GLU or GLY as cross-linking agent;

> **CR633-GLU (or GLY)-BSA-0.01(or 0.1 and 1.0)**, standard sequential immobilization procedure using a mass ratios of BSA to laccase activity of 0.01

(also used 0.1 and 1.0) mg per unit of laccase activity using GLU or GLY as cross-linking agent;

CR633-SIM-GLU(or GLY), simultaneous immobilization procedure of laccase on Celite ® R-633 using GLU or GLY as the cross-linking agent;

CR633-SIM-GLU(or GLY)-BSA-1, simultaneous immobilization procedure using different with the addition of BSA using different mass ratios of BSA to U;

CSDE, cross-linked enzyme dried by spray drying;

CSTR, continuous stirred tank reactor;

DBP, dibutylphthalate;

DEHP, di-(2-ethylhexyl)phthalate;

DEP, diethylphthalate;

D, diameter of the agitator;

E, [E], enzyme, concentration en enzyme;

E1, estrone;

E2, 17β-estradiol;

E3, estriol;

EDC, endocrine disrupting chemical;

EDTA, ethylenediamine-tetraacetic acid;

EE2, 17α-ethynylestradiol;

E·S, [E·S], complexe enzyme-substrat, concentration en complexe enzyme-substrat;

ESI(-), electrospray atmospheric pressure ionization (ESI) source in the negative ion monitoring mode;

$\mathbf{F_{in,out}}$, flowrates;

Fr, Froude number;

FBR, fluidized bed reactor;

G, G factor, velocity gradient;

G6PD, glucose-6-phosphate dehydrogenase;

GC, gas chromatography;

GEN, genistein;

GLU, glutaraldehyde, glutaraldéhyde;

GLY, glyoxal;

$\mathbf{H_{0,1}}$, null, alternative hypothesis;

^{1}**H-NMR**, proton nuclear magnetic resonance spectroscopy;

hER, human estrogen receptor;

i, level of the factor pH;

j,level of the factor temperature;

k, replication (chapter 4), constante cinétique (Annexe 2);

$\mathbf{k_{cat}}$, maximum rate of substrate transformation per mass of enzyme (catalytic coefficient);

$\mathbf{K_m}$, Michaelis-Menten constant;

$\mathbf{K_{ow}}$, water/octanol partition coefficient;

$\mathbf{K_t}$, impeller-dependant constant;

LiP, lignin peroxidase, lignine peroxydase;

LME, lignin modifying enzyme;

M, molar concentration, concentration molaire;

MnP, manganese peroxidase, manganèse peroxydase;

MS, $\mathbf{MS^2}$, mass spectra, tandem mass spectra;

MWCO, molecular weight cut-offs;

n, rotational speed of the agitator

NP, nonylphenol, nonylphénol;

p353NP, 4(3',5'-dimethyl-3'-heptyl)-phenol, a branched isomer of NP;

4nNP, 4-n-nonylphenol, a linear isomer of NP;

$\mathbf{N_p}$, power number;

NPE, nonylphenol ethoxylates;

O, [O], oxygène, concentration en oxygène;

P, power dissipated in the solution;

PAE, phthalic acid ester;

PBR, packed bed reactor, réacteur garnis;

PCP, personal care product;

PEG, polyethylene glycol;

PMSF, phenylmethylsulfonyl fluoride;

ρ, density of the reaction media;

Re, Reynold number;

r, $\mathbf{r_{EDC}}$, $\mathbf{r_s}$, $\mathbf{r_{ES}}$, reaction rate of transformation, of EDC, of substrate, of enzyme-substrate complex;

RM, reversed micelle;

rps, revolution per second;

S, [S], substrat, concentration en substrat;

SEM, scanning electron microscopy;

SIM, single ion monitoring mode, simultaneous (in abbreviations used to qualify immobilization procedures such as CR633-SIM-GLU);

STP, sewage treatment plant;

T_{av}, average temperature;

TCS, triclosan;

TEMPO, 2,2,6,6-tetramethoxypiperidine 1-oxyl;

U, unit of laccase activity;

UF, ultrafiltration;

VLA, violuric acid;

V, volume of the reactor;

$\mathbf{V_{max}}$, maximum rate of substrate transformation;

VP, versatil peroxidase, peroxydase versatile;

$\mathbf{W_0}$, degree of hydration;

WRF, white rot fungus/fungi;

WWTP, wastewaters treatment plant;

$\mathbf{X_{1,2}}$, independent variables, in coded form, used for the ANOVA analysis;

YES, Yeast Estrogenic Screen;

Z, produit de la réaction enzymatique;

α, the probability to reject the statistical null hypothesis (H_o) when this hypothesis is true (chapter 4), constant of the 3-parameter inactivation model (chapter 6);

β, effect of temperature (chapter 4), constant of the 3-parameter inactivation model (chapter 6);

$\mathbf{\Delta G_{transfert}}$, énergie libre de transfert;

ΔT, temperature difference;

ε, random error component;

ε_{max}, extinction coefficient;

μ, overall mean effect (chapter 4), viscosity of the reaction media (chapter 7);

τ, effect of pH (chapter 4), hydraulic retention time (chapter 7).

CHAPITRE 1

INTRODUCTION

La qualité de la vie sur Terre est inextricablement liée à la qualité de notre environnement. L'augmentation de la conscience collective à l'égard de cet élément a mené au développement de technologies alternatives permettant de diminuer l'impact de l'activité humaine sur notre milieu. Ces dernières s'attaquent à deux problèmes fondamentaux liés à nos modes de production. En premier lieu, elles tentent de diminuer la quantité de déchets produits et, en second lieu, elles s'attaquent aux polluants associées à différentes matrices environnementales.

Depuis une trentaine d'années, de nouvelles stratégies de bioremédiation basées sur la catalyse enzymatique ont été développées pour le traitement de matrices environnementales (eau, sol, boues) contaminées par différents xénobiotiques (colorants, HAP, explosifs, etc.). Plusieurs raisons peuvent expliquer ces efforts de développement :

(i) la vitesse d'introduction de ces xénobiotiques dans l'environnement est en augmentation et il devient de plus en plus difficile d'accomplir un haut degré d'élimination de ceux-ci par les processus biologique et chimique de traitement conventionnels. Il s'avère donc nécessaire de développer des méthodes de traitements alternatives qui sont plus rapides, moins chères, plus fiables et plus faciles à mettre en œuvre que les procédés conventionnels de traitement (Karam and Nicell, 1997) ;

(ii) la reconnaissance et le développement croissant de bioprocédés utilisant ces biocatalyseurs pour éliminer ces polluants cibles et cela pour de vastes gammes de concentrations (Karam and Nicell, 1997) ;

(iii) les avancées techniques récentes permettent la production plus rapide et à moindre coût d'enzymes grâce aux percées dans le domaine du génie génétique, à une meilleure isolement/sélection de souches et à de meilleures procédures de purification (Karam and Nicell, 1997) ;

(iv) les composés formés par biocatalyse peuvent s'avérer environnementalement plus acceptables que les produits formés par des procédés conventionnels (Couto and

Herrera, 2006; Ahuja et al., 2004) tels que, par exemple, les composés organochlorés formés suite à des traitements de chloration ;

(v) la spécificité de ces catalyseurs permet de limiter la production de composés « indésirables » résultant des réactions parallèles des procédés physico-chimiques traditionnels (Ahuja et al., 2004; Karam and Nicell, 1997) ;

(vi) les enzymes peuvent être utilisées pour traiter des rejets contenant des produits biologiques (Ahuja et al., 2004; Karam and Nicell, 1997) et

(vii) ces catalyseurs sont biodégradables (Karam and Nicell, 1997).

De plus, la biocatalyse enzymatique présente différents avantages comparativement à la catalyse microbienne. En ce sens, ces protéines sont caractérisées par la spécificité des substrats qu'elles peuvent utiliser, par leur fonction spécifique de catalyseur, par leur capacité d'être utilisées sur une large gamme de conditions environnementales (pH, température, salinité, concentrations, présence de composés toxiques), par leur faible sensibilité à la présence de prédateurs, d'inhibiteurs et de variation des conditions du milieu et par la simplicité et la facilité de contrôle des procédés utilisant des enzymes (Ahuja et al., 2004; Gianfreda and Rao, 2004; Karam and Nicell, 1997; Nannipieri and Bollag, 1991). De plus, certains facteurs spécifiques à un site peuvent favoriser l'utilisation d'enzymes pour la bioremédiation tels que l'absence ou la faible densité de micro-organismes capable d'éliminer la substance d'intérêt, la température, l'humidité, les conditions d'oxygénation, la présence d'éléments nutritifs, la biodisponibilité des contaminants et l'écologie microbienne du milieu (Ahuja et al., 2004).

L'utilisation de ces catalyseurs est limitée par différents éléments. En premier lieu, les coûts associés à la production, l'isolement et la purification des ces enzymes limitent leur utilisation. Toutefois, certains auteurs prétendent que les coûts associés à l'utilisation de ces protéines dans des biotechnologies environnementales peuvent s'apparenter aux coûts des technologies dites *ex situ* (Trombly, 1995). La connaissance des caractéristiques des souches produisant les enzymes ciblées, de leurs conditions optimales de culture, le développement de bioréacteurs favorisant la production d'enzymes d'intérêt, la modification génétique de différents micro-organismes menant à la création de mutants permettant de surexprimer ces protéines et le développement de nouvelles technologies de purification sont autant d'éléments qui peuvent diminuer ces coûts. De plus, la robustesse et les propriétés intrinsèques du biocatalyseur peuvent être améliorées via des stratégies

d'ingénierie des protéines (e.g. design rationnel d'enzymes, mutagénèse dirigée, etc.), l'immobilisation de ces biocatalyseurs, l'ajout de stabilisants chimiques, des modifications chimiques de la protéine, etc. (Couto and Toca-Herrera, 2007; Mateo et al., 2007; Ahuja et al., 2004; O'Fagain, 2003).

1.1 Les champignons responsables de la pourriture blanche du bois et leurs enzymes ligninolytiques

L'intérêt scientifique pour l'utilisation des champignons responsables de la pourriture blanche du bois (*white rot fungi*, WRF) et de leurs enzymes ligninolytiques dans des applications environnementales, de synthèse, bioénergétiques et analytiques a grandement augmenté au cours des dernières années (Couto and Herrera, 2006). Cet intérêt est motivé par la présence de ces microorganismes dans bon nombre d'environnements naturels et contaminés, par le système d'enzymes oxydatives uniques sécrétés par ces champignons, par la croissance des WRF qui peut être réalisée en utilisant des substrats peu onéreux et par les enzymes oxydatives sécrétées qui sont constitutives, i.e. qui sont sécrétées de façon constante par le WRF (Gianfreda and Rao, 2004). Les WRF sont des organismes uniques tant chez les eucaryotes que chez les procaryotes de par leur aptitude à sécréter un système d'enzymes oxydatives ayant la capacité d'oxyder la lignine. La lignine est un polymère hétérogène, complexe et stable composé de différents monomères aromatiques reliés entre eux par différents liens carbone-carbone et éthers. La structure hétérogène de la lignine implique que les enzymes ligninolytiques possèdent la capacité d'oxyder des substrats de structures chimiques variées possédant des potentiels redox élevés et ce, de façon non-spécifique. Cette capacité oxydative non-spécifique ainsi que leur potentiel redox élevé représentent des caractéristiques intrinsèques de ces protéines favorisant leur utilisation dans des biotechnologies environnementales.

Ce système enzymatique extracellulaire est généralement constitué de deux peroxydases, la lignine (LiP, E.C. 1.11.1.14) et la manganèse (MnP, E.C. 1.11.1.13) peroxydase et d'une phénoloxydase, la laccase (E.C. 1.10.3.2) (Pointing, 2001). Certaines souches de WRF sécrètent également de la peroxydase versatile (PV, E.C. 1.11.1.16) qui a la capacité d'oxyder les substrats types de la LiP et de la MnP (Ruiz-Duenas et al., 1999). Ce système

oxydatif est supporté par une machinerie enzymatique permettant de générer *in situ* le peroxyde d'hydrogène, composé de la glyoxal oxydase (E.C.1.2.3.5) et de la superoxyde dismutase (E.C. 1.15.1.1) (Leonowicz et al., 1999).

1.1.1 La laccase

Les laccases sont des enzymes catalysant l'oxydation d'un grand nombre de composés organiques tels que des méthoxyphénols, des phénols, des *o*- et *p*- diphénols, des aminophénols, des polyphénols, des polyamines, des molécules provenant de la lignine et certains ions inorganiques (Burton, 2003; Call and Mucke, 1997). De plus, ces enzymes peuvent catalyser la déméthylation de la lignine, des acides méthoxyphénols et des méthoxyaromatiques. Globalement, les laccases sont responsables de l'abstraction d'un électron du substrat et de la réduction de l'oxygène en eau (voir Figure 1.1).

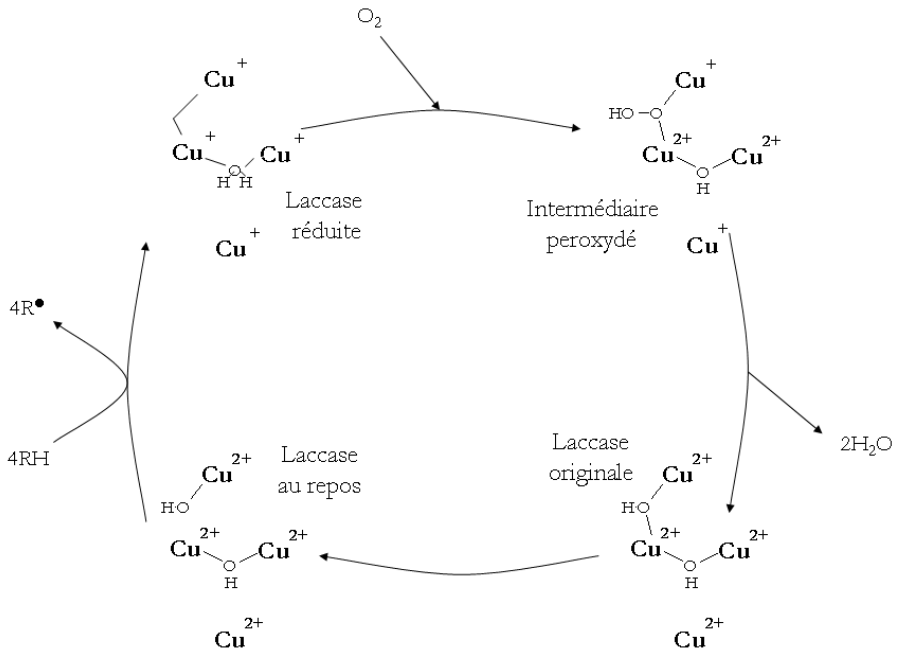


Figure 1.1. Cycle catalytique de la laccase. RH représente le substrat et R· représente le substrat oxydé (modifié de Wesenberg et al. (2003))

Les laccases sont des métalloprotéines contenant généralement 4 atomes de cuivre dans leur site catalytique. Ces atomes de cuivre sont caractérisés, entre autres, en fonction de leur action dans ce site. L'atome de cuivre de type 1 est responsable de la capture d'un électron provenant du substrat, l'atome de type 2 du transfert de cet électron vers son récepteur tandis que les 2 atomes de type 3 sont responsables de la liaison de l'oxygène à la protéine et du transfert des électrons vers ce récepteur. La masse moléculaire de ces enzymes N-glycolysées varie entre 60 et 390 kDa (Call and Mucke, 1997) et leur potentiel redox entre +500 et +800 mV (Wesenberg et al., 2003). Ces enzymes catalysent l'oxydation d'une grande variété de composés aromatiques donneurs d'hydrogène avec la réduction simultanée de l'oxygène moléculaire en eau (Figure 1.1).

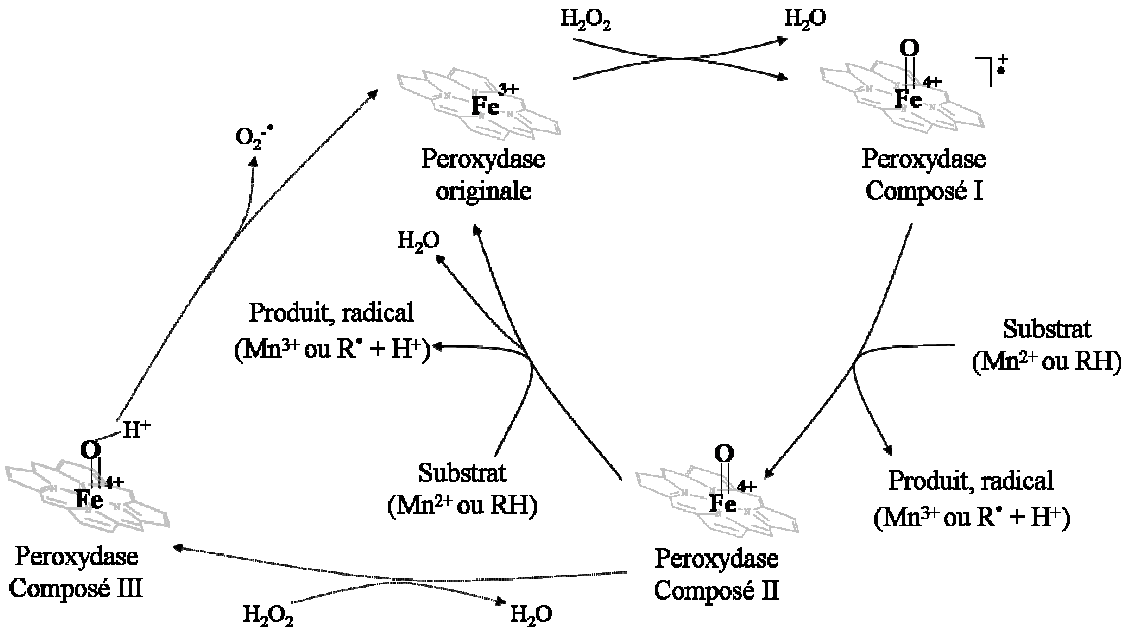
L'action de la laccase sur le substrat résulte en la formation de radicaux cationiques. Ces radicaux sont impliqués dans des réactions non-enzymatiques telles que la déprotonation, l'attaque nucléophile par les molécules d'eau du milieu, la formation d'oligomères, la rupture des liens C_α-C_β, l'oxydation du C_α, le clivage de type alkylaryle, la déméthylation, la décarboxylation et la déméthoxylation (Call and Mucke, 1997).

Ces enzymes ont été utilisées pour l'élimination de nombreux xénobiotiques de différentes matrices environnementales. En ce sens, elles ont été utilisées pour élimininer des colorants (Wesenberg et al., 2003), des HAPs (Pozdnyakova et al., 2006), des chlorophénols (Bollag et al., 2003), des alkylphénols (Tsutsumi et al., 2001), des phénols (Ryan et al., 2007), etc. De plus, ces enzymes ont été utilisées pour des fins de synthèse chimique (Burton, 2003), de production d'énergie (Tayhas and Palmore, 2004), de blanchiment biologique (Couto and Herrera, 2006), de clarification de breuvages (Minussi et al., 2002) et d'analyses (Couto and Herrera, 2006).

1.1.2 Les peroxydases

Pour leur part, les peroxydases sont des enzymes contenant un groupement prosthétique composé de protoporphyrine uni à un atome de fer (hème). Elles sont responsables de l'oxydation de nombreux substrats et elles utilisent le peroxyde d'hydrogène comme accepteur d'électron. La Figure 1.2 présente le cycle catalytique général de ces peroxydases. La peroxydase sous sa forme originale, avec son hème sous la forme ferrique (Fe(III)), se lie au peroxyde d'hydrogène pour former le composé I. Ce dernier est appauvri

de 2 électrons: l'un provenant de la formation du Fe(IV) et l'autre d'une liaison double formée avec un atome d'oxygène du peroxyde d'hydrogène. Cette disposition produit un composé radicalaire cationique. C'est ce composé I qui est responsable de l'oxydation d'une molécule de substrat (ce substrat varie selon le type de la peroxydase). Cette étape oxydative implique le transfert d'un électron et d'un proton et résulte en un site catalytique ayant la forme du composé II. Une seconde molécule de substrat est par la suite oxydée ce qui résulte en la production d'une molécule d'eau et la réduction de l'enzyme en sa forme originale.



$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2\ H_2O$$

Figure 1.2. Cycle catalytique général des peroxydases (modifié de Wesenberg et al. (2003))

La MnP est la peroxydase la plus sécrétée par la majorité des basidiomycètes colonisant le bois et responsable de la pourriture de ce dernier (Hofrichter, 2002). Cette enzyme a un poids moléculaire se situant entre 32 et 62.5 kDa et un potentiel redox typique de l'ordre de +1510 mV (Wesenberg et al., 2003). Cette enzyme oxyde préférentiellement le Mn(II) en Mn(III) qui est stabilisé par des chélateurs, tels que l'acide oxalique, générés *in situ* par ces microorganismes. Le Mn^{3+} ainsi stabilisé est un puissant oxydant. Ce composé peut facilement diffuser dans la structure de la lignine et agir comme médiateur redox. Ainsi, cette enzyme est impliquée dans la dépolymérisation de la lignine et peut être utilisée pour l'oxydation de xénobiotiques tels que des chlorophénols, des colorants, des composés

nitro-aromatiques, des HAPs, du Nylon, etc. (Hofrichter, 2002; Wesenberg et al., 2003). L'élimination de ces composés s'effectue via une minéralisation partielle des composés initialement présents (Hofrichter, 2002).

La LiP, quant à elle, est une peroxydase N-glycolysée dont la masse moléculaire varie typiquement entre 38 et 47 kDa. Le potentiel redox de cette enzyme est de l'ordre de +1450 mV (Wesenberg et al., 2003). Cette enzyme catalyse l'oxydation de nombreux composés aromatiques non-phénoliques présents dans la lignine. Cette abstraction d'un électron des substrats résulte en la formation de radicaux. Globalement, l'action de la LiP peut résulter en l'oxydation de l'alcool benzylique, le clivage de chaînes latérales, l'ouverture de cycles aromatiques, la déméthoxylation et la déchlorination (Gold and Alic, 1993). La LiP a été utilisée dans des applications bio-environnementales pour l'élimination de HAPs, de BPCs, du TNT et de colorants (Gianfreda and Rao, 2004; Conesa et al., 2002).

Finalement, la PV est une enzyme hybride ayant une masse moléculaire de 45 kDa (Moreira et al., 2006) pouvant oxyder le Mn(II), des composés aromatiques et non aromatiques. Cette enzyme a été utilisée pour la décoloration d'effluents (Sugano et al., 2006) et l'élimination de phénols (Moreira et al., 2006; Pozdnyakova et al., 2006).

1.1.3 Le système enzyme / médiateur

Étant donné la nature hétérogène du polymère formant la lignine et de la taille des enzymes ligninolytiques, les interactions substrat(s) / enzyme(s) s'avèrent limitées (Call and Mucke, 1997). Pour outrepasser cette limitation, ces enzymes interagissent avec des composés chimiques de faible masse moléculaire dont le potentiel redox est élevé (> +900 mV) ayant la capacité de diffuser facilement au travers du complexe ligno-cellulosique. Ces composés, nommés médiateurs, servent de transporteurs d'électrons entre l'enzyme et son substrat. La Figure 1.3 présente le schéma d'interaction des enzymes avec ces médiateurs. Ces substances peuvent être sécrétées par le champignon ou provenir de son environnement naturel comme l'alcool vératrylique, le Mn(III) ou l'acide 3-hydroxyanthranilique ou encore être des substances synthétiques et exogènes telles que

l'acide violurique, le 1-hydroxybenzotriazole ou le 2,2'azinobis (3-ethylbenzthiazoline-6-sulphonate).

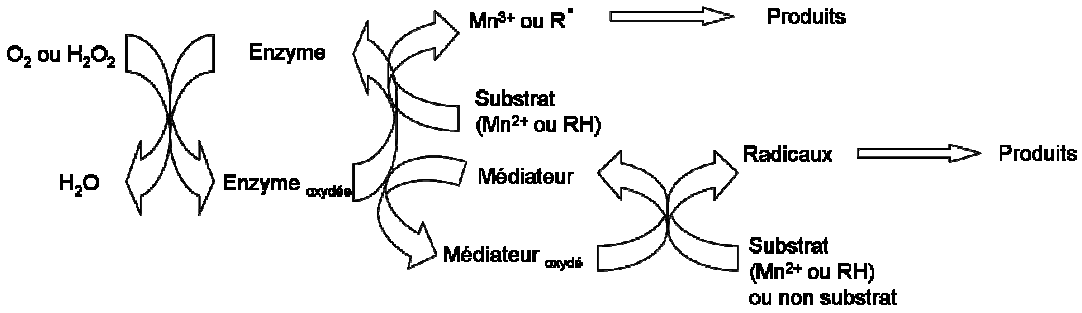


Figure 1.3. Représentation schématique des interactions entre les enzymes ligninolytiques leurs substrats et des médiateurs redox (modifié de Kurniawati et Nicell (2007))

L'emploi de ce système enzyme / médiateur permet, dans bien des cas, d'augmenter le taux d'oxydation du substrat d'intérêt (Call and Mucke, 1997) et d'oxyder des substances qui, à priori, ne sont pas des substrats de l'enzyme utilisée (Bourbonnais and Paice, 1990). Ce type de système a été utilisé dans des procédés de fabrication et de blanchiment biologique de la pâte à papier, dans des procédés de synthèse chimique et dans des biotechnologies environnementales (Burton, 2003; Call and Mucke, 1997).

1.2 Les substances perturbatrices du système endocrinien

La quantité et la qualité des publications scientifiques traitant des perturbations du système endocrinien d'espèces animales sauvages a grandement augmenté au cours des dernières années. Un grand nombre de perturbations associées à différentes substances chimiques a été répertorié tant chez des organismes vertébrés qu'invertébrés (Rodriguez et al., 2007; Sumpter, 2005; Tyler et al., 1998). Une substance perturbatrice du système endocrinien est un composé chimique exogène ou un mélange de composés qui altère une ou des fonctions du système endocrinien des organismes et qui, par conséquent, cause des effets néfastes sur la santé des organismes vivants, sur leur progéniture ou sur une partie de la population (Vos et al., 2000).

Les perturbations endocriniennes peuvent intervenir à différents niveaux physiologiques. En ce sens, elles peuvent i) altérer (inhiber ou stimuler) la sécrétion d'hormones, ii) interférer avec le récepteur hormonal (effet agoniste ou antagoniste) et iii) modifier le métabolisme de ces enzymes par exemple, en augmentant ou diminuant leur taux de sécrétion et/ou de biotransformation dans le pancréas, les reins ou dans tout autre organe (Mills and Chichester, 2005). Ces perturbations sont causées par une exposition prolongée à ces substances et ce, malgré leurs faibles concentrations dans les milieux récepteurs.

Un grand nombre de polluants ont été décrits comme étant perturbateurs endocriniens ; notons surtout des composés organiques tels que des alkyphénols, des BPCs, des pesticides chlorés, des herbicides et des composés médicamenteux (Singleton and Khan, 2003). Il a été démontré que ces composés peuvent présenter une activité estrogénique, androgénique, anti-androgénique et thyroïdienne (Sumpter, 2005). Le type de perturbation générée par ces composés est associé à la similitude structurale entre le composé et l'hormone mimée. À ce propos, Fang et al. (2001) ont identifié les éléments structuraux nécessaires à la liaison de composés présentant une activité estrogénique avec le récepteur humain d'estrogène. L'activité estrogénique associée à ces composés est, de loin, la perturbation endocrinienne la mieux documentée.

L'environnement aquatique est l'ultime récepteur pour la majorité des composés chimiques émis par des évènements naturels ou des activités anthropogéniques et ce, malgré les efforts d'épuration effectués à l'aide d'ouvrages de traitement des eaux. De nombreuses substances perturbatrices du système endocrinien ou des métabolites provenant de leur biodégradation se retrouvent dans les effluents et les boues des stations d'épuration des eaux usées (Tan et al., 2007; Auriol et al., 2006).

Parmi ces substances perturbatrices du système endocrinien se retrouvant dans les extrants des usines d'épuration des eaux usées, le nonylphénol (NP), le bisphénol A (BPA) et le triclosan (TCS) sont parmi les xénobiotiques organiques les plus détectés dans les zones urbanisées (Boyd et al., 2004; Boyd et al., 2003; Kolpin et al., 2002). Ces composés phénoliques sont responsables de perturbations endocriniennes variées. Le chapitre 2 présentera en détails les perturbations associées à ces composés ainsi que les concentrations de ces substances dans différentes matrices environnementales.

Ces substances sont caractérisées par la présence d'un groupement phénolique dans leur structure chimique. La présence de ce groupement rend possible l'utilisation de la laccase pour oxyder et bio-éliminer ces composés.

1.3 Objectif général

L'objectif général de cette thèse est de démontrer l'efficacité de transformation des perturbateur endocriniens nonyphénol, bisphénol A et triclosan à l'aide de la laccase sécrétée par la souche fongique *Coriolopsis polyzona* (voir Annexe 1 pour le choix de la souche fongique).

1.4 Objectifs spécifiques

- Quantifier et caractériser la capacité de transformation de ces perturbateurs endocriniens présents dans des solutions aqueuses par la laccase libre ;
- Identifier les produits résultant de l'action oxydative de la laccase ;
- Déterminer l'activité estrogénique des produits formés ;
- Former des biocatalyseurs solides robustes ;
- Caractériser les biocatalyseurs solides formés ;
- Démontrer la possibilité d'utiliser ces biocatalyseurs solides dans différentes configurations de bioréacteurs pour éliminer de façon continue ces perturbateurs endocriniens présents dans des solutions aqueuses.

1.5 Structure du manuscript

Les différents objectifs spécifiques de cette thèse sont traités dans les chapitres qui suivent. En premier lieu, le chapitre 2 contient une revue exhaustive de la littérature scientifique portant sur l'élimination de différents composés perturbateurs du système endocrinien par les WRF et leurs enzymes ligninolytiques. Le chapitre 3, quant à lui, présente les éléments théoriques des différentes stratégies d'immobilisation utilisées pour augmenter le potentiel d'utilisation de la laccase dans des procédés biotechnologiques. Puis, le chapitre 4 traite de

l'élimination du NP, BPA et du TCS à l'aide de la laccase libre provenant de *C. polyzona*. Les chapitres 5 à 7 présentent les résultats relatifs à l'immobilisation de la laccase de *C. polyzona*. Particulièrement, le chapitre 5 expose les résultats obtenus suite à l'immobilisation de cette enzyme sur un support inorganique, tandis que le chapitre 6 aborde l'immobilisation de la laccase via la formation d'agrégats d'enzymes réticulés (*cross-linked enzyme aggregates*, CLEAs). Ces deux chapitres traitent de la caractérisation des biocatalyseurs formés et de leur utilisation pour éliminer en continu le NP, le BPA et le TCS. Finalement, le chapitre 7 présente un design de réacteur novateur pour l'élimination des perturbateurs endocriniens ciblés et ce, via l'utilisation en continue des CLEAs précédemment formés.

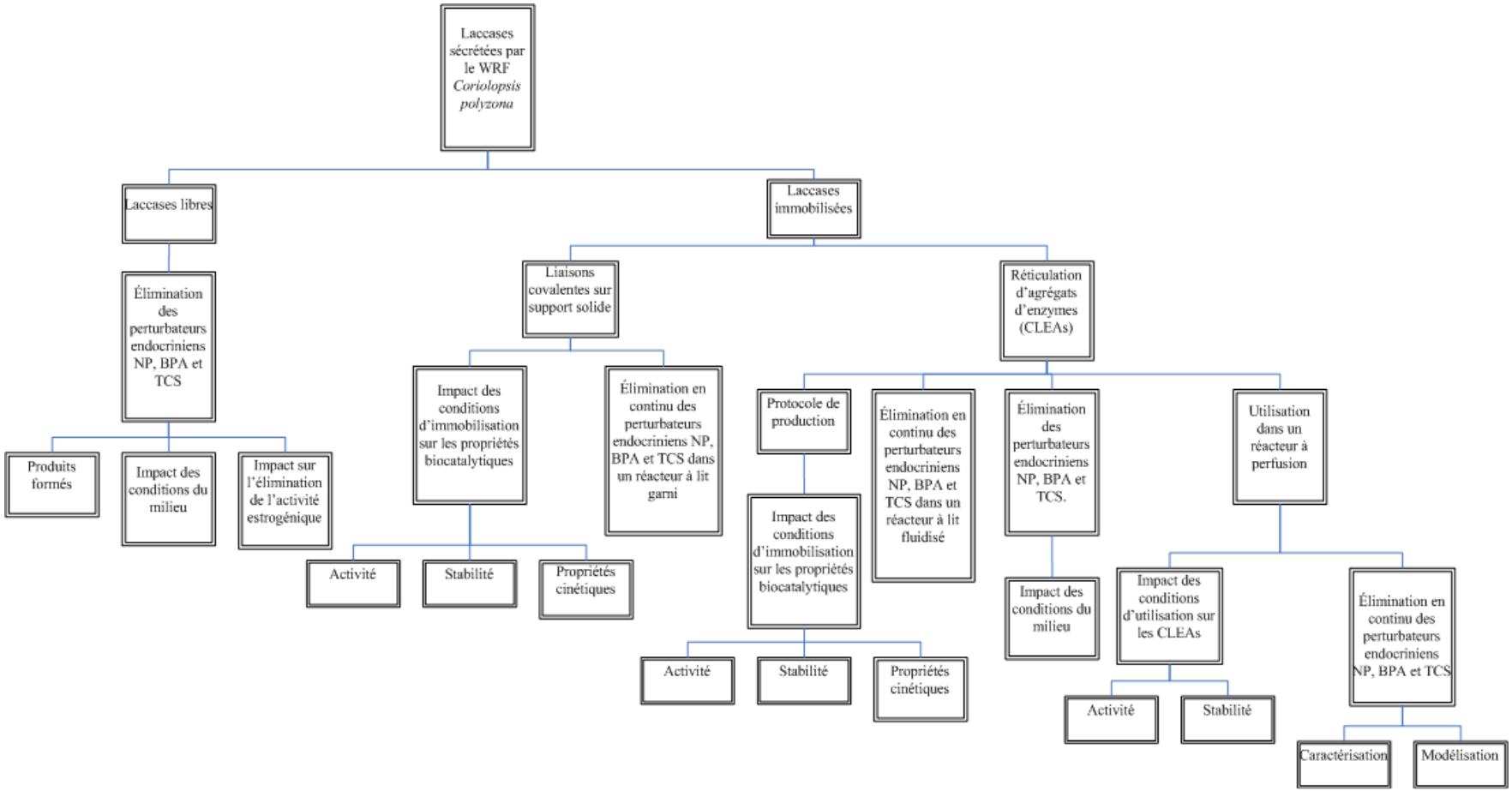


Figure 1.4. Représentation schématique de ce projet de recherche

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CHAPITRE 2

ÉLIMINATION DES SUBSTANCES PERTURBATRICES DU SYSTÈME ENDOCRINIEN À L'AIDE DES CHAMPIGNONS DE LA POURRITURE BLANCHE DU BOIS ET DE LEURS ENZYMES LIGNINOLYTIQUES : UNE REVUE DE LA LITTÉRATURE

ELIMINATION OF ENDOCRINE DISRUPTING CHEMICALS USING WHITE ROT FUNGI AND THEIR LIGNIN MODIFYING ENZYMES: A REVIEW

2.1 Avant-propos

Ce chapitre a préalablement été publié dans la revue *Engineering in Life Sciences*. La référence exacte de ce texte est :

H. Cabana, J. P. Jones, S. N. Agathos. Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: a review. *Engineering in Life Sciences* 2007. 7, 429-456.

2.2 Abstract / Résumé

The ability of white rot fungi (WRF) and their lignin modifying enzymes (LMEs) laccase and lignin- and manganese- dependent peroxidases to treat endocrine disrupting chemicals (EDCs) is extensively reviewed. These chemicals cause adverse health effects by mimicking endogenous hormones in receiving organisms. The elimination of the alkylphenolic EDCs nonylphenol, bisphenol A and triclosan, the phthalic acid esters dibutylphthalate, biethylphthalate and di-(2-ethyhexyl)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 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 hormone-mimicking activity associated. 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, that 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.

Dans cet article, la capacité d'élimination des substances chimiques perturbatrices du système endocrinien par des champignons responsables de la pourriture blanche du bois et de leurs enzymes ligninolytiques, à savoir la laccase, la manganèse et la lignine peroxydase, est revue. Ces substances chimiques peuvent avoir des effets négatifs sur la santé des organismes présents dans les milieux récepteurs de par leur capacité à imiter les hormones endogènes de ces êtres vivants. Particulièrement, les perturbateurs endocriniens de type alkylphénoliques (nonylphénol, bisphénol A et le triclosan), les esters d'acide phtalique (dibutylphtalate, diethylphtalate et le di-(2-éthylhexyl) phtalate), les œstrogènes naturelles et synthétiques (17β-estradiol, estriol et le 17α-éthynylestradiol) et les phytoestrogènes (β-sitostérol et la génistéine) ont été éliminés par différentes souches fongiques et enzymes ligninolytiques. Différents champignons ont démontré une grande efficacité d'élimination de ces substances dans des solutions aqueuses et dans des sols via des systèmes ligninolytiques et non-ligninolytiques. D'autre part, la laccase et la manganèse et la lignine peroxydase ont également été utilisés avec succès *in vitro* pour l'élimination de ces substances et de l'activité estrogénique qui leur est associée. La transformation de ces polluants par ces souches fongiques et leurs enzymes ligninolytiques est supportée par des connaissances émergentes en biochimie et en physiologie microbienne relatives à ces organismes et aux propriétés biocatalytiques de leurs enzymes. Finalement, compte tenue de la diversité de conditions pouvant être rencontrées dans des matrices environnementales réelles et ce, comparativement aux conditions testées en laboratoire, il est impératif de développer des biotechnologies robustes et fiables pour le traitement de ces matrices contaminées par ces substances perturbatrices du système endocrinien.

2.3 Introduction

There is extensive scientific evidence that low-dose man-made chemicals (xenobiotics) released in 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 [2-4].

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.

2.4 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 and the low molecular weight mediators that enlarge the spectrum of compounds they are able to oxidize [5]. Furthermore, 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. Moreover, 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 are essential for lignin degradation even if the mineralization of this recalcitrant biopolymer requires the combination of their actions 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 cosubstrate 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 mediators 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 substrates specificity of the laccase employed. These features are important in the process design and optimization of fungal and LME treatment of EDC-containing effluents. Table 2.1 reports fungal and LMEs eliminations of EDCs, the type of treatment, the matrix in which the treatment was performed and the removal rate are specified.

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Bjerkandera spp	WRF mycelium	NP	45 mg l^{-1}	Aqueous	95% (at 9.7 mg l^{-1} day^{-1}) after 5 days of incubation.		The rate of NP removal was favorised by agitated culture.	[106]
	WRF mycelium	NP	430 mg kg^{-1}	Soil	98% after a 5-week incubation period.		Grew at soil's surface.	[107]
	WRF mycelium	β-sit	0.21 mg g^{-1} 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 constituent. 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 mycelium	β-sit	0.16 mg g^{-1} oven dry wood	Scots pine sapwood matrix	69% after 6-week incubation.			[110]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	WRF mycelium	β-sit	0.27 mg g^{-1} oven dry wood	Scots pine heartwood matrix	18% after a 6-week period.			[110]
Clavariopsis aquatica	Fungi	NP	100-250 µM	Aqueous	50% and 60% of a 250 µM 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% s of 4nNP and t-NP after a contact time of 24 hours.		The laccase/ABTS (1mM) increases the elimination of t-NP to 97.2% after 24 hours.	[112]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Coriolopsis polyzona	Free laccase	BPA	5 mg l^{-1}	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 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]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free laccase	NP	5 mg l^{-1}	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^{-1}	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 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Insolubilized as CLEA	BPA	5 mg l^{-1}	Aqueous	50% and 90% after a hydraulic residence time of 40 and 150 minutes.		Utilization in a fluidized bed reactor. K_m and k_{cat} kinetic constant were respectively 0.23 mM and 0.16 µmol s^{-1} mg^{-1}.	[114]
	Insolubilized as CLEA	NP	5 mg l^{-1}	Aqueous	80% and >95% after a hydraulic residence time of 40 and 60 minutes.		Utilization in a fluidized bed reactor. K_m and k_{cat} kinetic constant were respectively 0.45 mM and 0.8 µmol s^{-1} mg^{-1}.	[114]
	Insolubilized as CLEA	TCS	5 mg l^{-1}	Aqueous	80% and >95% after a hydraulic residence time of 40 and 60 minutes.		Utilization in a fluidized bed reactor. K_m and k_{cat} kinetic constant were respectively 0.12 mM and 0.24 µmol s^{-1} mg^{-1}	[114]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
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]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Confined laccase	BPA	2 mM	Aqueous	50 - 90% and 100% for contact time of 24 and 96 hours.		Extend of removal depends of the MWCO of the dialysis membrane.	[116]
	Free laccase	NP	3 µmol g^{-1} soil	Soil	65% and 90% after a contact time of 0.25 and 1 day.		Impact of pH on the rate of elimination.	[117]
	Free laccase	BPA	3 µmol g^{-1} 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 cultures of transgenic tobacco plant secreting laccase.	[118]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Cunninghamella spp	WRF mycelium	NP	11 mg l^{-1}	Aqueous	Half-lives: 1 day (4nNP), 2 days (NP).			[119]
Daldinia concentrica	WRF mycelium	DBP	28 mg l^{-1}	Aqueous	94% and 100% after 1 and 6 days 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^{-1}.	[120]
Funalia trogii	WRF mycelium	β-sit	0.16 mg g^{-1} oven dry wood	Scots pine sapwood matrix	50% after 6-week incubation.		This strain was able to remove other wood pitch constituent.	[110]
	WRF mycelium	β-sit	0.27 mg g^{-1} oven dry wood	Scots pine heartwood matrix	41% after 6-week incubation.		This strain was able to remove other wood pitch constituent.	[110]
Fusarium spp	WRF mycelium	NP	11 mg l^{-1}	Aqueous	Half-lives: 1-2 days (4nNP), >8 days (NP).		Strains isolated from a soil/sludge mixture contaminated by NP.	[119]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Heterobasidium insulare	WRF mycelium	BPA	200 mg l^{-1}	Aqueous	77% and 100% after 3 and 14 days of incubation.	100% after 1 day incubation.	High resistance of the WRF strain to BPA (up to 100 mg l^{-1}).	[121]
Ischnoderma benzoinum	WRF mycelium	β-sit	0.24 mg g^{-1} oven dry wood	Scots pine sapwood matrix	79.6% after 4-week incubation.		This strain was able to remove other wood pitch constituents. Allowed a 6 fold detoxification of the wood pitch.	[109]
Mucor spp	WRF mycelium	NP	11 mg l^{-1}	Aqueous	Half-lives: 1.5-2 days (4nNP), 3-5 days (NP).		Strains isolated from a soil/sludge mixture contaminated by NP.	[119]
Mycelyopthora sp	Immobilized laccase	E2	4.9 g l^{-1}	Organic solvent			Production of E2-dimers. Identification of the dimers structures.	[122]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Peniophora pini	WRF mycelium	β-sit	0.23 mg g^{-1} oven dry wood	Scots pine sapwood matrix	76.9% after 4-week incubation.		This strain was able to remove other wood pitch constituent. Allowed a 4 fold detoxification of the wood pitch.	[109]
Phanerochaete chrysosporium	WRF mycelium	NP	11 mg l^{-1}	Aqueous	Half-lives: 6 days (4nNP), 3 days (NP).			[119]
	WRF mycelium	DBP	28 mg l^{-1}	Aqueous	85% after 6 and 20 days of incubation.		Growth inhibited at a DBP concentration of 500 mg l^{-1}.	[120]
	Free MnP	BPA	0.22 mM	Aqueous	90% and 100% after contact times of 30 and 60 minutes.	40% and 90% after contact time of 4 and 6 hours for a 0.88 mM solution.		[123]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free MnP	NP	0.23 mM	Aqueous	90% and 95% after contact times of 30 and 60 minutes.	60% and 80% after contact time 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]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	LiP / reversed micelles	BPA	0.1 mM	Organic solvent	40% and 50% after contact time 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 / reversed micelles	NP	0.1 mM	Organic solvent	70% and 85% after contact time of 30 and 120 minutes.			[125]
Phanerochaete sordida	WRF mycelium	E1	10^{-5} M	Aqueous	70% and 100% after 3 and 6 days of incubation.		2 days lag period.	[126]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	WRF mycelium	GEN	10^{-4} M	Aqueous	50% and 95% after 3 and 4 days of incubation.		2 days 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 mycelium	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-h treatment.		Identification of metabolites.	[128]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free laccase	NP	3 µmol g^{-1} soil	Soil	35% and 55% after a contact time of 1 and 3 days.		Elimination in test tube.	[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 mycelium	TCS	0.25 mM	Aqueous			Identification of metabolites produced. Metabolites less toxic than TCS.	[130]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Pycnoporus coccineus	Free laccase	BPA	3 µmol g^{-1} soil	Soil	15% after a contact time of 5 hours.		Elimination in test tubes.	[131]
	Free laccase	EE2	3 µmol g^{-1} 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^{-1} 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^{-1} 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^{-1}	Organic solvent			The oxidation of estrogens was inhibited by KCN.	[132]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Stereum hirsutum	WRF mycelium	BPA	200 mg l^{-1}	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^{-1}).	[121]
	WRF mycelium	β-sit	0.21 mg g^{-1} oven dry wood	Scots pine sapwood matrix	100% after 4-week incubation.		This strain was able to remove other wood pitch constituent. This strain caused a 7% woody mass losses in 4 weeks. Allowed a 13.5 fold detoxification of the wood pitch.	[109]
Stereum sanguinolentum	WRF mycelium	β-sit	0.23 mg g^{-1} oven dry wood	Scots pine sapwood matrix	53.2% after 4-week incubation.		This strain was able to remove other wood pitch constituent. Allowed a 2.5 fold detoxification of the wood pitch.	[109]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Trametes spp	Combination laccase / activated sludge	BPA	5 - 100 mg l^{-1}	Aqueous			The treatment efficiency depends of 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 of the water content (1-4% v/v).		Determination of optimal type of organic solvent and water content. Identification of BPA products after laccase oxidation. Treatment of chlorophenols.	[134]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	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's performances, in regard of BPA removal, were maintained over a 10-day period.	[135, 136]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free laccase	NP	3 μmol g^{-1} 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]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free laccase	BPA	3 µmol g^{-1} soil	Soil	85% after a contact time of 5 hours in test tube. 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]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free laccase	EE2	3 µmol g^{-1} 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 rotating reactor.	[131]
Trametes hirsuta	Pilot-scale fungal/UF system	NP	2.9 µg l^{-1}	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^{-1}	Aqueous	45% after an overall cycle of 1.5 day.		Sequencing batch mode reactor.	[138]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Trametes pubescens	Free laccase / emulsion system	E2	5 g l^{-1}	Organic solvent			Production of E2-dimers. Identification of the dimers structures.	[122]
Trametes versicolor	WRF mycelium	NP	45 mg l^{-1}	Aqueous	90% (at 2.8 mg l−1 day−1) after 15 days of incubation.		The rate of NP removal was favorised by static culture.	[106]
	WRF mycelium	NP	11 mg l^{-1}	Aqueous	half-lives : 1 day (4nNP), < 1 day (NP).			[119]
	WRF mycelium	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 mycelium	DBP	28 mg l^{-1}	Aqueous	83% and 100% after 1 and 6 days of incubation.		Growth inhibited at a DBP concentration of 500 mg l^{-1}.	[120]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	WRF mycelium	NP	430 mg kg^{-1}	Soil	98% after a 5-week incubation period.		Grew diffusely through the soil.	[107]
	WRF mycelium	β-sit	0.24 mg g^{-1} oven dry wood	Scots pine sapwood matrix			This strain was able to remove other wood pitch constituent. 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 estrogenic activity removal.	[123]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	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]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free laccase	NP (4nNP)	5 mg l^{-1}	Aqueous	90% and 100% after a contact time of 5 and 90 minutes.		The NP-transformation rate was pH dependent.	[119]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free laccase	TCS	20 µM	Aqueous			Determination of optimal conditions of treatment in regard to the controllable parameters (temperature, pH, mediator) and impact of wastewater constituents on the TCS transformation. K_m and V_{max} were respectively 24 µM and 0.92 µmol l^{-1} min^{-1} in the presence of 3 000 U $l^{-1}$of laccase activity. V_{max} was improved 65-fold when using ABTS at a concentration of 20 µM as a redox mediator.	[140]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	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]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	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 respectively from 0.10 - 1.20 mM and 1.8 and 3.4 µmol min^{-1} as a function of T_{av} and ΔT.	[141]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
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 respectively of 14.1 mM and 59.1 µmol min^{-1} mg^{-1} protein.	[142-144]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
Mitosporic strain UHH 1-6-18-4	WRF mycelium	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% s of 4nNP and t-NP after a contact time of 24 hours.		The laccase/ABTS (1mM) increase 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 respectively of 10 000 µM and 14 s^{-1}. The laccase/1-HBT (1mM) did not improve the transformation rate.	[145, 146]

TABLE 2.1. EDCs ELIMINATION BY WRF AND THEIR LMEs

Fungal strain	Mode of treatment	EDC	EDC concentration	Matrix	EDC removal rate	Estrogenic activity removal rate	Comments	Reference
	Free laccase	NP	5mM	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 respectively of 5 000 µM and 1 s^{-1}.	[145, 146]

2.5 Endocrine disrupting chemicals

Over the last 50 years, a lot of scientific data concerning the hormone-like effects of anthropogenic chemicals in the environment has 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, we 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 behaviour of aquatic organisms at environmentally relevant concentrations [16].

Figure 2.1 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]. Table 2.2 presents some of the endocrine disruptions brought about by these chemicals in different living organisms.

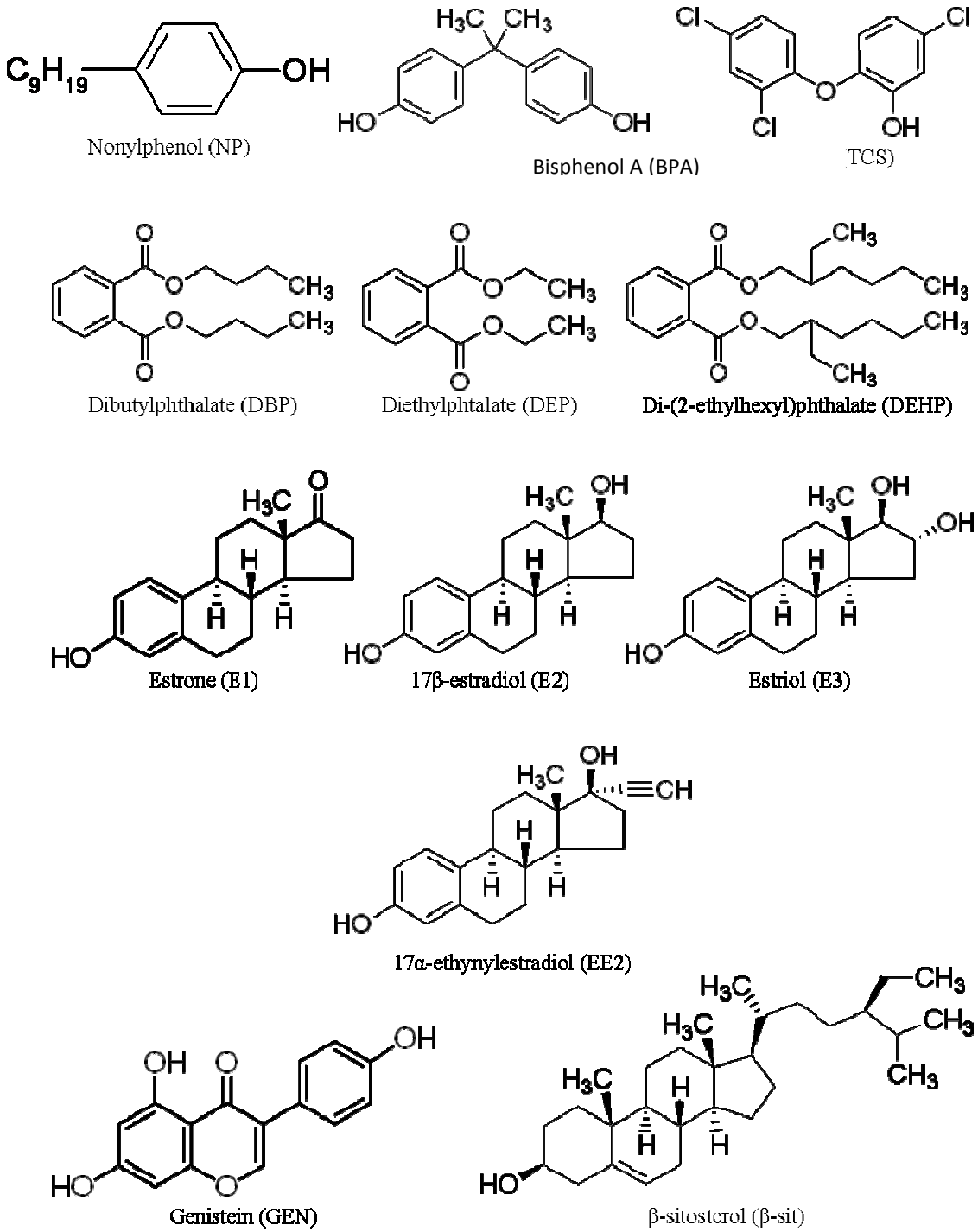

Figure 2.1. Chemical structure of endocrine disrupting substances eliminated from various environmental matrices by several white rot fungi and their lignin modifying enzymes

TABLE 2.2. SOME ENDOCRINE DISRUPTIONS ASSOCIATED WITH ENDOCRINE DISRUPTING CHEMICALS ELIMINATED BY LMEs

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
Bisphenol A (BPA)	10^{-10} - 10^{-6} M	Sensitive human breast cancer cells	Stimulation of G6PD activity.	[147]
	20 - 500 mg kg^{-1}	Female immature Sprague-Dawley rats	Uterine weight increase. Stimulation of glutathione peroxidase activity.	
		Cell line from fruit fly (*Drosophila melanogaster*)	Antagonistic activity on 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^{-1}	Aquatic snail (*Potamopyrgus antipodarum*)	Affect growth of snails Affect snail embryo production Interactions with estrogen receptor.	[42]
	500 mg kg^{-1}	Immature mouse	Progestogenic and estrogenic properties	[150]
	0.1, 10 or 100 µg l^{-1}	Medaka (*Oryzias latipes*)	Induction of female specific proteins in male fish.	[151]
	10 - 200 µg l^{-1}	Medaka (*Oryzias latipes*)	Induction of choriogenin subunits	[152]
	10^{-9} - 10^{-3} M	Frog (*Xenopus laevis*)	Binding to liver estrogen	[153]

TABLE 2.2. SOME ENDOCRINE DISRUPTIONS ASSOCIATED WITH ENDOCRINE DISRUPTING CHEMICALS ELIMINATED BY LMEs

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
			receptor.	
	10^{-10} - 10^{-5} M	Frog (*Xenopus laevis*)	Induction of the estrogenic biomarker vitellogenin in primary cultured hepatocytes of Male.	[154]
	500 µg day^{-1}	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 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^{-1}	Daphnia (*Daphnia magna*)	Multi-generational reproductive process disruptions.	[158]
	10^{-10} - 10^{-6} M	Sensitive human breast	Stimulation of glucose-6-	[147]

TABLE 2.2. SOME ENDOCRINE DISRUPTIONS ASSOCIATED WITH ENDOCRINE DISRUPTING CHEMICALS ELIMINATED BY LMEs

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
		cancer cells	phosphate dehydrogenase activity.	
	20 - 500 mg kg^{-1}	Female immature Sprague-Dawley rats	Uterine weight increase Stimulation of glutathione peroxidase activity.	
	250 - 500 mg kg^{-1}	Immature mouse	Progestogenic and estrogenic properties.	[150]
	0.1, 10 or 100 µg l^{-1}	Medaka (*Oryzias latipes*)	Induction of female specific proteins in male fish.	[151]
	5 - 500 µg l^{-1}	Medaka (*Oryzias latipes*)	Induction of choriogenin subunits.	[152]
	10^{-9} - 10^{-3} M	Frog (*Xenopus laevis*)	Binding to 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^{-1}	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^{-1}	Rainbow trout (*Oncorhynchus mykiss*)	Increased synthesis of vitellogenin.	[160]

TABLE 2.2. SOME ENDOCRINE DISRUPTIONS ASSOCIATED WITH ENDOCRINE DISRUPTING CHEMICALS ELIMINATED BY LMEs

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
			Reduction of plasma follicle stimulating hormone levels and gene expression in the pituitary. Inhibition of gonadal development and steroidogenesis.	
	$10^{-12} – 10^{-8}$ M	Clonal rat prolactinoma cell line GH3/B6/F10	Dose-responses for extracellular regulated kinase activation.	[161]
	0.01 – 100 µg l^{-1}	Shrimp (*Neomysis integer*)	Induction of vitellin synthesis	[162]
Triclosan (TCS)	0.3 – 30 µg l^{-1}	Bullfrog (*Rana catesbeiana*)	Exposure to triclosan disrupt gene expression associated with the presence of thyroid hormones	[163]
	1 – 100 µg l^{-1}	Medaka (*Oryzias latipes*)	Weak Androgenic activity	[164]
	20 – 200 µg l^{-1}	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	Activation of	[166]

TABLE 2.2. SOME ENDOCRINE DISRUPTIONS ASSOCIATED WITH ENDOCRINE DISRUPTING CHEMICALS ELIMINATED BY LMEs

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
		hepatoma cell line (HuH7)	human pregnane X (PRX) receptor by TCS resulting in an alteration the metabolism regulated by PRX.	
Dibutylphthalate (DBP)	0 – 10 000 mg l^{-1}	Rats	Developmental exposure to DBP affected both male and female sexual development	[167]
	10^{-6} – 10^{-3} M		Interference with the endocrine functions of steroid-binding protein	[168]
Diethylphthalate (DEP)	0.1 - 20 mg l^{-1}	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^{-1} d^{-1}	Rats	Perinatally exposed males present testicular atrophy, reduced weights of reproductive	[170]

TABLE 2.2. SOME ENDOCRINE DISRUPTIONS ASSOCIATED WITH ENDOCRINE DISRUPTING CHEMICALS ELIMINATED BY LMEs

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
			organs, reduced anogenital distance and an increased number of nipples/areolas.	
Estrone (E1)	10 – 1000 ng l^{-1}	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^{-1}	Zebrafish (*Danio rerio*)	Alteration of sex ratio in the population. Induction of vitellogenin synthesis.	[172]
17α-ethynyestradiol (EE2)	1 – 100 ng l^{-1}	Zebrafish (*Danio rerio*)	EE2 showed toxic effects on larvae, embryo and juveniles. Production of vitellogenin into male adult fishes.	[173]
β-sitosterol (β-Sit)	200 µg g^{-1}	Goldfish (*Carassius auratus*)	Reduction of steroidogenic acute regulatory protein transcript abundance with decreased plasma components resulting from anon-estrogenic effect.	[174]
	50 mg kg^{-1} d^{-1}	Mink (*Mustela*	Disruption of	[175]

TABLE 2.2. SOME ENDOCRINE DISRUPTIONS ASSOCIATED WITH ENDOCRINE DISRUPTING CHEMICALS ELIMINATED BY LMEs

Endocrine disrupting chemicals	Exposure	Organism used	Effect	Reference
		vison)	hormonal balance and development of sexual organs	
Genistein (GEN)	50 mg kg^{-1} d^{-1}	Mink (*Mustela vison*)	Disruption of hormonal balance and development of sexual organs	[175]

2.5.1 Bisphenol A

Bisphenol A (BPA) is used as a raw material for the production of polycarbonates and epoxy resins. Its discharges in 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 waters can reach up to 21 000 µg l^{-1} [22-31] and up to 191 ng l^{-1} 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^{-1} [24, 28, 31, 34-38] in sewage effluents and up to 2.89 µg g^{-1} [35] in sewage sludges have been reported. Bacteria able to degrade BPA have been isolated from a sewage treatment plan (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]. Table 2.2 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 mammary glands [47]. Structure-activity investigations highlighting the ability of BPA to act as estrogen derive from the presence of a phenolic group on the molecule [17].

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.

2.5.2 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^{-1} to 1000 µg l^{-1} [23, 37, 38, 52-62]. Concentrations of NP up to 500 mg kg^{-1} in sludges [35, 63] and up to 6.86 µg l^{-1} 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] able to use NP as the sole source of carbon and energy have been described. 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 1 430 ng g^{-1} [32, 76]. Furthermore, the reduction of the available concentration of these chemicals in the aquatic environment is essentially 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 Table 2.2). According to Fang et al. (2001) [17], the xenoestrogenic activity of NP is linked to the phenolic group in the chemical structure and the high hydrophobicity of the substance.

2.5.3 Triclosan

Triclosan (TCS) is a broad spectrum antimicrobial agent. It has been incorporated into a wide range of 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^{-1} [36, 52, 78-86], in sludges up to 55 mg kg^{-1} [78, 81, 83, 85, 87] and in surface water from <0.2 to 431 ng l^{-1} [80, 88-90]. 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 [91]. Attention has been drawn to TCS due to its structural similarity to those 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 Table 2.2, TCS is suspected to induce weak estrogenic or androgenic activity or to disrupt gene expression linked with the thyroid hormone. Until now, there has been relatively little research on the removal of TCS using WRF and their LMEs.

2.5.4 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 WWTP effluents are reported at several tens of µg l^{-1} [34, 93-95], whereas in sediments and activated sludge biosolids these same phthalates attain concentrations of tens of mg kg^{-1} [93, 96, 97]. The concern with PAEs at such levels is their strong potential interference with reproduction and development of many organisms

in the food chain by endocrine or antiandrogenic-mediated processes. Table 2.2 presents some of the endocrine disruption processes linked to DBP, DEP and DEHP.

2.5.5 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 occurence of these EDCs in the aquatic environment is a result of their discharge by municipal WWTP effluents. Wastewater treatment installations have shown low elimination efficiency for this kind of EDCs [34]. The concentrations of E1, E2, E3 and EE2 in WWTPs effluents are typically 1-147, 0.2-158, 10-30 and 10-78 ng l^{-1} [34, 98].

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 WWTP effluents could be up to 10 000 ng l^{-1} [34, 103, 104]. The isoflavanoid GEN has been detected in municipal sewage at concentrations of 83 ng l^{-1} [28], up to 10.5 µg l^{-1} in bleach kraft pulp mill effluents [104, 105] and 7 ng l^{-1} in surface water [28]. Such environmental concentrations of these naturally-occurring EDCs exert unwanted effects on organisms present in receiving environments (see Table 2.2).

2.6 Elimination of EDCs in aqueous solution using fungal mycelium

BPA was removed using mycelia of 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^{-1} 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. (2005) [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 enzyme(s) may be involved in the transformation of BPA. This observation was corroborated 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, respectively, dehydroxylation, carboxylation, and hydroxylation 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 1-d incubation. All of the estrogenic activity was eliminated after 3 d. In comparison, all of the estrogenic activity of the solution was removed after a 1-d culture 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 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 by *T. versicolor* was favoured by agitated culture conditions, while the biotransformation carried out by *Bjerkandera* sp. was favoured by static conditions [106]. These results support the more-or-less general statement that the production of laccase is favoured by agitated culture conditions [14].

Junghanns et al. (2005) [112] and Moeder et al. (2006) [111] suggested that the metabolic pattern produced during the removal of NP by each of the two cultures, respectively the strain UHH 1-6-18-4 and the aquatic hyphomycete *C. aquatica*, 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-labelled 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 respectively as 2-O-(2,4,4'-trichlorodiphenyl ether)-β-D-xylopyranoside and 2-O-(2,4,4'-trichlorodiphenyl ether)-β-D-glucopyranoside. 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. (2000) [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. (2004) [120] presented the time course of the transformation of a 28 mg l^{-1} solution of DBP at a temperature of 30°C. After 1-d incubation, *T. versicolor* and *D. concentrica* eliminated respectively 83 and 94% of the DBP initially present in solution. A similar extent of reaction was achieved by *P. chrysosporium* after 6-d 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 shaken cultures (150 rpm) of the WRF *Phanerochaete sordida* YK-624 at 30°C [126, 127]. After a 2-d lag period, 70% and 100% of E1 was removed after respectively 3- and 6-d incubations and the GEN concentration decreased by 90% between days 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 d. 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].

2.7 Elimination of EDCs in soil by WRF

Soares et al. (2005) [107] tested the ability of *T. versicolor* and *Bjerkandera* sp. BOL13 to eliminate NP from a contaminated soil (430 mg kg^{-1} 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% removal of NP had been achieved by both WRF [107].

2.8 Elimination of β-sit in wood matrices using WRF mycelium

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, complete conversion was achieved by strains

Bjerkandera sp. and *S. hirsutum* [109]. The strains *T. versicolor*, *I. benzoinum*, *P. pini* and *S. sanguinolentum* reached, respectively, levels of 86.5, 79.5, 76.7 and 53.2% transformation 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 week by *Bjerkandera* sp. and after less than a week by *T. versicolor* [108].

2.9 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^{-1} 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-h 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 cosubstrate 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^{-1} of MnP and 50 µM of $MnSO_4$ and resulted in a complete transformation of both EDCs after one hour.

A 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^{-1} of MnP from *P. chrysosporium* ME-446 and *P. sordida*

YK-624 [124, 126]. After a 1-h treatment none of the three hormones were detected 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: *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^{-1}, 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 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, Table 2.3 presents kinetic constants (see Annexe 2 for details about Michaelis-Menten kinetic) of laccase from strain I-4 for several substrates.

TABLE 2.3. 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})	$k_{cat} K_m^{-1}$ (µmol s^{-1} l^{-1})
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

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 and *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 laccase activity of 1 U l^{-1} [113]. The various laccases secreted by WRF were able to achieve 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 laccase from *C. polyzona* and *T. versicolor* [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].

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. (2006, 2005) [126, 127] and Suzuki et al. (2003) [124]. A 10 μkat l^{-1} activity of laccase from the WRF *T. versicolor* IFO-6482 was used in solution at pH 4.5 and 30°C. After a 1-h 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-h 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 *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. 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 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-catalysed 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 denaturants methanol (5 and 10% w/w), acetone (5 and 10% w/w) and formaldehyde (1 and 2 % w/w) decreased significantly 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 concentration of 50 mg l^{-1} 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-catalysed 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 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 reaction rate [181, 185]. Kim and Nicell (2006) [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: 3 000 U l^{-1} for the treatment of a 20 µmol l^{-1} solution of TCS compared to 150 U l^{-1} for a 120 µmol l^{-1} 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^{-1} 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^{-1}) system and the laccase/ABTS-mediated system represented 21, 41 and 1 200% 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 enhanced solubility of the polymeric metabolites produced. 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 laccase source, 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. (2001) [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 examples see Figures 2.2 and 2.3).

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 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-h 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.

2.10 Elimination of EDCs in aqueous solution using immobilized/insolubilized LMEs

In order to enhance the industrial utility 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].

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^{-1} in the prototype unit resulted in an elimination proportional to the hydraulic residence time [135]. For a flow rate of 2 ml min^{-1}, 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 determined 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 by 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. (2003) [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 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 (CLEA). These were used in a fluidized bed reactor for the elimination of the EDCs BPA, NP and TCS from solutions containing 5 mg l^{-1} of each at pH 5 and room temperature [114]. At a 150 min hydraulic residence time, a 90% conversion of BPA was achieved. The complete elimination of NP and TCS was obtained with a residence time of 60 minutes. This solid biocatalyst had lower affinity for, respectively TCS, BPA and NP 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].

2.11 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].

A 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^{-1} g^{-1} when W_o was 15 down to 150 µmol min^{-1} g^{-1} 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. (2005)

[134] obtained a complete removal of BPA using the same concentration of laccase in a RM system.

The treatment of a 0.33 g l^{-1} 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 (1941) [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-h treatment, five unidentified metabolites were detected by thin layer chromatography, but none of them showed any estrogenic activity as measured by 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 3 unidentified compounds for each EDC in the above emulsion system [129].

2.12 Elimination of EDCs in soil using LMEs

The removal of the EDCs BPA, NP, E1 and EE2 (3 μmol g^{-1}) 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 EDCs transformations were 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 decreasing affinity for NP as seen 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^{-1} was removed 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 occurence 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 of the dilution used (maximum concentrations of NP of 100 or 300 µg l^{-1}) 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 their ability to remove BPA from artificially contaminated soil (spiked with BPA) [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].

2.13 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 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. (2001) [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 have been reported [142]. The formation of low molecular weight metabolites such as phenol, 4-isopropenylphenol, 4-isopropylphenol and hexestrol has also 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. (2004) [142] and a modeled mechanistic one was proposed by Huang and Weber (2005) [177].

Figure 2.2 shows schematically the reactions undergone by BPA as a result of laccase-catalyzed oxidation.

Lugaro et al. (1973) [129] and Nicotra et al. (2004) [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 *Mycelyopthora* laccase. The other form of dimer, occurring via C-C bonds was also a mixture of stereoisomers in a 1:1 ratio [122]. Figure 2.3 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].

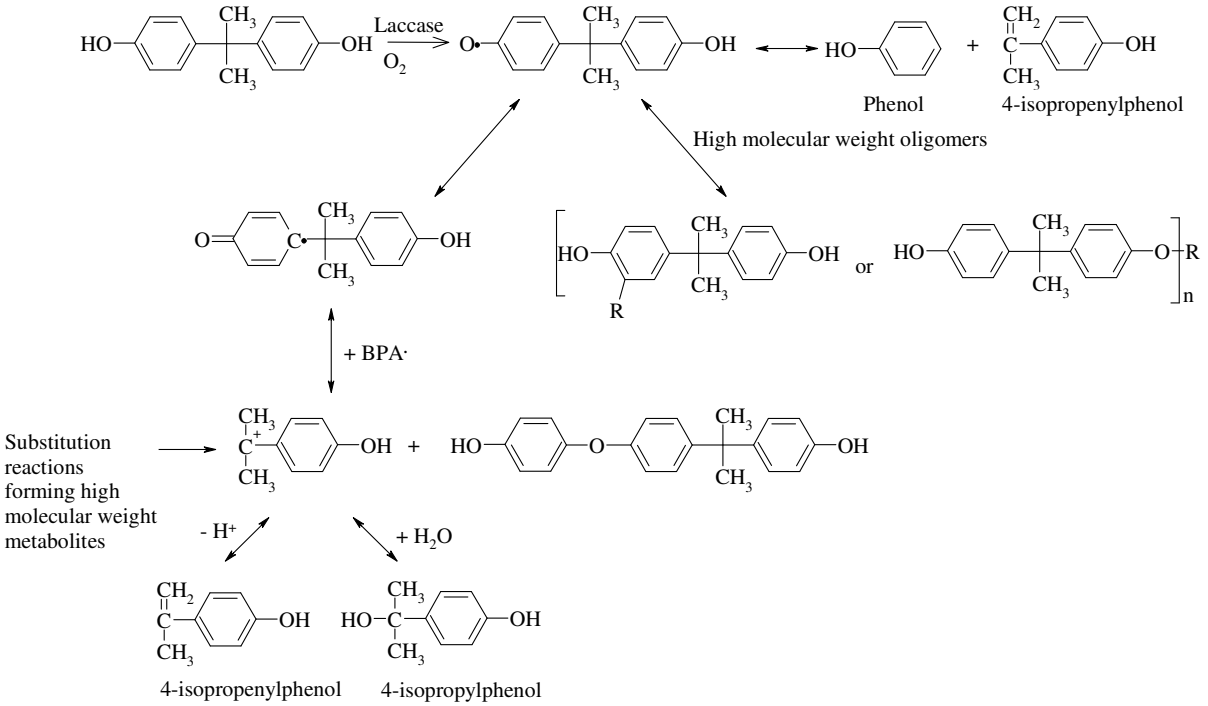

Figure 2.2. Reaction pathway of laccase-oxidized BPA (modified from Huang and Weber (2005) [177])

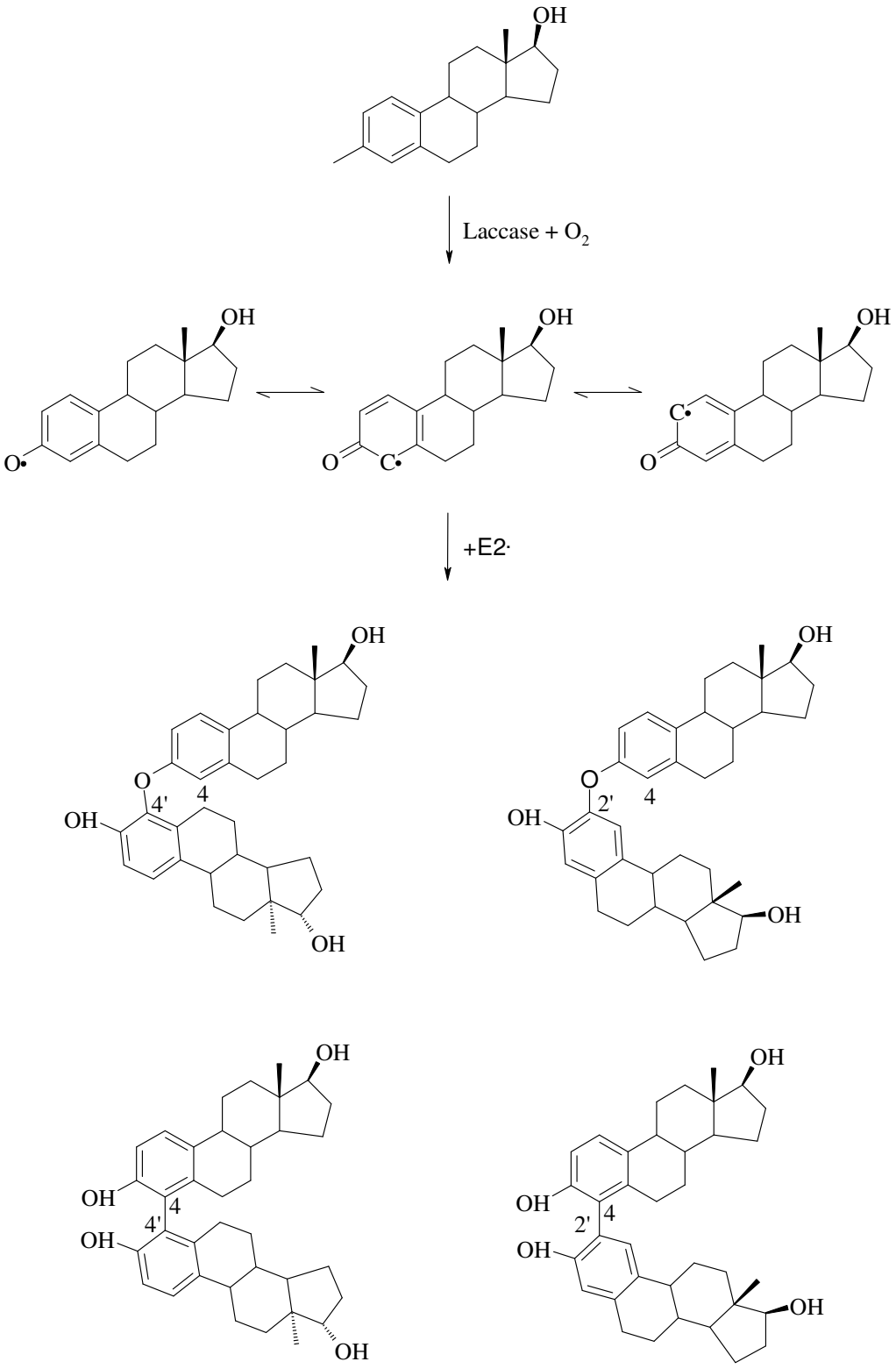

Figure 2.3. Reaction pathway of E2 oxidized by laccase and structures of dimers produced (modified from Nicotra et al. (2004) [122])

2.14 General Discussion

2.14.1 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. It has been recently demonstrated that the *in vivo* decolourization capacity of the WRF *Pycnoporus sanguineus* and *P. cinnabarinus* is brought about by 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 determined 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 LME that 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. (2002) [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].

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 appropriate enzyme spectrum) or by the addition of exogenously grown strains, a more challenging prospect. Several strategies for 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 cultivations (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.

2.14.2 Free and immobilized/insolubilized LMEs

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 matrices. 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 forms will likely involve improved WRF cultivation methods. 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 physico-chemical and stability properties of the enzyme preparation which are crucial for the treatment of xenobiotics. Furthermore, the economic dimension of the LMEs 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 [204]. In addition, 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 biotreament 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 (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 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 (CLEA) exhibit high activity/mass ratio. 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 physico-chemical processes in complex matrices and the endocrine disruption potential of such newly formed chemicals in 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 favourable 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 favourably the hydrophobic EDCs in the vicinity of the immobilized biocatalyst.

2.15 Concluding remarks

The elimination of EDCs by WRF cultures or their LMEs are promising processes for the treatment of different contaminated environmental matrices. However, several issues must be considered before 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.

2.16 Acknowledgements

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CHAPITRE 3
IMMOBILISATION ENZYMATIQUE

3.1 Introduction

L'élimination des perturbateurs endocriniens NP, BPA et TCS à l'aide de la laccase libre de *C. polyzona* a été étudiée (voir Chapitre 4). Ce biocatalyseur élimine efficacement ces substances ainsi que l'activité estrogénique associée au BPA et au NP. Malgré ces résultats encourageants, l'utilisation d'enzymes libres dans des applications de biotechnologie environnementales s'avère très limitée. En effet, une telle approche nécessite l'utilisation d'une grande quantité d'enzymes libres pour le traitement d'effluents liquides. De plus, ces enzymes, sous leur forme soluble, sont sensibles à l'effet de différents dénaturants physiques, chimiques et biologiques présents dans ces effluents réels et s'avèrent impossibles à confiner dans un espace restreint de façon à les utiliser de manière continue.

Néanmoins, ces contraintes peuvent être contournées via l'immobilisation de l'enzyme sur un support solide ou via la réticulation des agrégats d'enzymes. Cette immobilisation permet de générer des biocatalyseurs réutilisables et, par extension, de les utiliser en continu dans des bioréacteurs. De plus, l'immobilisation d'enzymes permet de stabiliser ces protéines contre l'effet dénaturant de différents sels, solvants et enzymes protéolytiques (O'Fagain, 2003). Par conséquent, cette approche s'avère avantageuse pour des applications bio-environnementales.

De prime abord, ce chapitre traitera de l'immobilisation enzymatique de façon générale puis il sera ensuite question des deux méthodes utilisées dans le cadre de cette dissertation de thèse : l'immobilisation de la laccase sur un support siliceux par la formation de liens covalents et par la réticulation des agrégats de laccase obtenus suite à une étape de précipitation.

3.2 Immobilisation enzymatique

L'immobilisation enzymatique est le processus par lequel des protéines sont confinées physiquement de façon synthétique dans une quelconque matrice à des fins de catalyse continue. Ces biocatalyseurs doivent rencontrer deux fonctions essentielles à savoir la fonction non catalytique qui permet une séparation du catalyseur de la solution et la fonction catalytique qui sert à convertir le substrat d'intérêt en un produit désiré et ce, en un lieu et un temps donnés. Cette utilisation d'enzymes immobilisées offre les possibilités suivantes :

1. Facilité d'utilisation comparativement aux enzymes libres ;
    a. Réutilisation des enzymes ;
    b. Élimination de la contamination du produit par les enzymes ;
    c. Flexibilité dans le design du procédé ;
    d. Contrôle aisé de la réaction biocatalysée ;
2. Possibilité de modifier des propriétés physico-chimiques de l'enzymes ;
3. Augmentation de la stabilité de l'enzyme ;
4. Modélisation des conditions rencontrées dans des processus biologiques (e.g. enzymes liées à des membranes cellulaires).

La sélection du mode d'immobilisation, et par conséquent le design des fonctions catalytiques et non catalytiques, dépendent globalement du type de procédé rencontré, du type de réaction, des conditions du milieu, des procédés de séparation, etc. La méthode d'immobilisation utilisée doit impérativement rencontrer les attentes catalytiques (productivité, conversion, etc.) et non catalytique de l'enzyme permettant une séparation de l'enzyme du milieu réactionnel. L'enzyme immobilisée sera qualifiée de robuste lorsqu'elle rencontrera ces différentes fonctions (Cao, 2005).

Diverses formes d'immobilisation sont possibles. Ces modes d'immobilisation peuvent être divisés en fonction du type de liens impliqués. En ce sens, il y a des moyens chimiques et physiques. Les méthodes d'immobilisation physique reposent sur des procédés ne faisant pas intervenir de liens covalents entre l'enzyme et son support. Ce type d'immobilisation fait

intervenir des forces physiques telles que les interactions électrostatiques, les forces de Van der Waals, les interactions protéines-protéines (résultant d'interactions électrostatiques, de Van der Waals, hydrophobes, entre ponts hydrogènes, etc). De plus, le confinement de protéines dans des matrices définies par des procédés tels que le piégeage enzymatique, sont également des méthodes physiques d'immobilisation. En principe, l'immobilisation résultant de la formation de liens physiques produit des interactions protéine/support qui sont réversibles. Ainsi, il est possible de varier la constante d'équilibre de ces systèmes de façon à rendre le processus d'immobilisation réversible sous les conditions du milieu. Il est toutefois possible de contrôler les éléments du milieu de façon à les rendre irréversibles (sous des conditions précises). Toutefois, la variabilité des matrices traitées dans des procédés de environnementaux fait en sorte qu'il est possible de rencontrer des conditions ponctuelles permettant de favoriser l'état soluble de ces catalyseurs et d'ainsi d'éliminer les enzymes de la matrice impliquée dans leur immobilisation.

Les méthodes chimiques, quant à elles, reposent sur la formation de liens covalents. Ces liens peuvent être formés entre les protéines ou entre un groupement fonctionnel présent sur le support solide et les protéines. Il est à noter que la méthode d'immobilisation covalente choisie aura une influence significative sur les propriétés du biocatalyseur formé.

Les méthodes chimiques et physiques offrent des avantages et des désavantages qui dépendent de différents facteurs. En ce qui concerne les méthodes physiques d'immobilisation par piégeage et adsorption, elles perturbent beaucoup moins la structure tridimensionnelle de la protéine et offrent la rétention des propriétés enzymatiques ressemblant à celles de la molécule en solution (Zaborsky, 1974). En contrepartie, la stabilité de l'activité enzymatique est typiquement moindre que celle observée lorsque des liaisons covalentes interviennent dans le procédé d'immobilisation.). Toutefois, d'un point de vue industriel, ce type d'immobilisation permet, en modifiant les conditions du milieu dans lequel se trouve l'enzyme immobilisée, de récupérer le support solide (suite à la désorption de l'enzyme) dépourvu d'enzymes. Ainsi, ce type d'immobilisation permet une regénération du support utilisé (Mateo et al., 1999).

En général, les méthodes d'immobilisation chimique tendent à réduire l'activité de l'enzyme. Cette diminution de l'activité peut être expliquée par un problème de diffusion du substrat vers le site actif de l'enzyme. Ce problème résulte de l'encombrement stérique résultant de l'immobilisation. De plus, cette diminution de l'activité peut également être associée à une torsion de la structure tridimensionnelle de l'enzyme (Durante et al., 2004). De plus, ces modifications de la structure de l'enzyme peut également se traduire par une diminution de l'affinité de l'enzyme pour le substrat.

Par contre, de telles liaisons covalentes fournissent un attachement stable à l'enzyme et peuvent réduire le taux de désactivation et altérer favorablement les propriétés biocatalytiques de l'enzyme. Le Tableau 4.1 présente les caractéristiques des différentes techniques d'immobilisation. Un choix entre des méthodes chimiques et physiques dépend donc de différents facteurs. Habituellement, un biocatalyseur avec une faible activité enzymatique, mais dont cette dernière s'avère stable sur une longue durée est préférable à un catalyseur présentant une haute activité initiale mais dont l'activité est manifeste seulement sur une courte durée (Duran et al., 2002).

Les performances catalytiques d'un système biphasique composé d'enzymes immobilisées (phase solide) et de la matrice contenant le substrat (ex : aqueuse) peuvent être limitées par le transfert de masse. Ces limitations sont de différents ordres. En effet, le substrat doit migrer (par diffusion et/ou convection) du milieu vers la couche limite autour du catalyseur insoluble, puis à travers cette couche limite. Par la suite, il doit pénétrer dans les pores à la surface du catalyseur et diffuser dans ces derniers (présentant des tortuosités variables en fonction du matériau) jusqu'à atteindre l'enzyme. Puis, le produit formé doit également passer par ces différentes étapes. Ces différentes étapes de transfert de masse peuvent, dans certains cas, limiter la cinétique globale du procédé. Toutefois, cet inconvénient peut être réduit en appliquant un design approprié au réacteur (Krajewska, 2004). De plus, en référant spécifiquement au mécanisme réactionnel de la laccase, les oligomères formés par l'action des biocatalyseurs peuvent s'adsorber à la surface du support et altérer l'activité enzymatique de l'enzyme (par modification de la structure tridimensionnelle de l'enzyme, par encombrement stérique, par limitation du transfert de masse, etc.). Ce phénomène peut être atténué en

choisissant des supports ayant des capacités d'adsorption limitées (par exemple, des composés inorganiques) ou développant un protocole de nettoyage adapté (Duran et al., 2002).

TABLEAU 3.1. CARACTÉRISTIQUES DES DIFFÉRENTES TECHNIQUES D'IMMOBILISATION (ADAPTÉ DE KENNEDY ET MELO (1990))

	Types d'immobilisation					
	Immobilisation par liaisons covalentes		**Immobilisation physique**			
	Réticulation d'agrégats	Liaison à un support solide	Force ionique	Chélata-tion	Adsorption	Encapsu-lation
Préparation	Intermédi-aire	Difficile	Simple	Simple	Simple	Difficile
Force de liaison	Forte	Forte	Intermédi-aire	Intermé-diaire	Faible	Intermé-diaire
Activité de l'enzyme immobilisée	Élevée	Intermédiaire / faible	Élevée	Élevée	Élevée	Faible
Régénération du support	N/A	Difficile	Élevée	Intermé-diaire	Élevée	Intermé-diaire
Coût de l'immobilisa-tion	Intermédi-aire	Élevé	Faible	Intermé-diaire	Faible	Intermé-diaire
Stabilité	Élevée / intermédi-aire	Élevée	Intermédi-aire	Intermé-diaire	Faible	Élevée

Globalement, de nombreuses méthodes sont disponibles pour immobiliser une enzyme. Les procédures d'immobilisation influencent grandement les propriétés du biocatalyseur solide résultant. La sélection d'une stratégie d'immobilisation détermine donc les spécifications de procédé du catalyseur, c'est-à-dire différents paramètres comme l'activité catalytique, l'efficacité de l'utilisation du catalyseur, les cinétiques de désactivation et de régénération, et le coût (Duran et al., 2002). Toutefois, il n'y a pas de concept systématique pour trouver la méthode d'immobilisation la plus appropriée pour les différents biocatalyseurs.

Malgré cette absence de concept systématique, certains éléments doivent être considérés lors du choix du support à utiliser et de la méthode d'immobilisation. En ce sens, pour des applications enzymatiques où la valeur ajoutée du produit est faible, telles que rencontrées dans les applications environnementales, il est impératif d'utiliser un support simple, peu onéreux, facile à manipuler, stable chimiquement et mécaniquement, non altérable par l'action

de micro-organismes, avec une surface spécifique élevée (pores de grande taille et large distribution des pores) (Cao, 2005) et pouvant être recyclé. Les supports utilisés peuvent être d'origine organique ou inorganique, et être une substance naturelle ou synthétique. Les supports inorganiques conviennent mieux à des utilisations industrielles grâce à leurs propriétés physiques. Ils offrent certains avantages tels qu'une résistance mécanique élevée, une stabilité thermique, une résistance aux solvants organiques et à l'action microbienne et une facilité à être régénérés (Cabral and Kennedy, 1991). De plus, la structure de ces supports est souvent résistante aux conditions des milieux telles que le pH, la température et la pression. Idéalement, le support devrait induire des changements structurels à la protéine permettant i) d'augmenter la spécificité enzymatique ($k_{cat} K_m^{-1}$) ii) de stabiliser cette dernière, iii) de déplacer le pH optimal d'opération de ce catalyseur jusqu'à celui désiré pour le processus et iv) de diminuer l'adsorption non spécifique (Colowick and Kaplan, 1976). Les supports inorganiques souvent utilisés sont le verre à porosité contrôlée, la silice, l'alumine, des céramiques, le titane, etc. Des études ont démontré que la quantité d'enzymes immobilisées et l'expression de l'activité de ces dernières dépend largement de la taille des pores et de la surface disponible du support (Blanco et al., 2004). En ce sens, plus la taille des pores est grande, plus il est possible d'immobiliser des enzymes (Ferreira et al., 2003). Toutefois, un accroissement de cette taille résulte en une diminution de la surface spécifique du support. De plus, l'agent réticulant choisi doit être également peu onéreux, disponible, facile à manipuler, provenir de ressources renouvelables et ne pas présenter de risque pour la santé humaine et la santé environnementale.

Le choix d'un support siliceux pour l'immobilisation de laccase peut être justifié par le fait que l'utilisation de ce type de support est favorisée dans des conditions acides (Cabral and Kennedy, 1991). En effet, ces conditions avoisinent le point isoélectrique de la silice qui est normalement aux environs de pH 2 (Frank, G., 2002). Sous ces conditions, la charge nette de la silice est nulle, ce qui résulte en une diminution des interactions électrostatiques entre le support et l'enzyme. Ces interactions peuvent induire une alterration de la structure 3D de la protéine et par extension en une variation de l'activité biocatalytique. Une diminution de la charge de surface favorise donc la conservation d'une structure s'apparentant à celle de

l'enzyme en solution. De plus, ce type de support s'avère stable à ds pH acides. Finalement, ces conditions acides favorisent l'activité de la majorité des laccases (Wesenberg et al., 2003).

Cependant, un des inconvénients des méthodes d'immobilisation des enzymes sur des supports solides réside en la faible activité massique du biocatalyseur formé. En effet, la masse de l'enzyme immobilisée représente tout au plus 10% de la masse totale du biocatalyseur formé (support + enzyme) (Cao, 2005). Il est possible de former des biocatalyseurs présentant un ratio activité/masse élevé en réticulant des agrégats d'enzymes (Cao, 2005). Ces biocatalyseurs sont formés uniquement d'enzymes, ce qui résulte justement en ce ratio élevé.

3.3 Immobilisation par liaisons covalentes

L'attachement d'enzymes sur des supports fonctionnalisés par des résidus d'acides aminés non-essentiels est la méthode la plus utilisée pour immobiliser des enzymes. Elle permet un attachement de la protéine plus stable que les autres méthodes d'immobilisation (Cabral and Kennedy, 1991). Les résidus d'acides aminés qui ne sont pas impliqués dans le site actif peuvent être utilisés pour former des liaisons covalentes avec soit différentes molécules présentes à la surface du support, soit d'autres résidus présents à même la structure primaire d'enzymes liées au support (Aehle, 2004). Il est également possible d'immobiliser les enzymes sans utiliser de tels supports (Cao et al., 2003) en réticulant les agrégats ou des cristaux d'enzymes. Encore une fois, des liaisons covalentes intra/inter-moléculaires interviennent entre différents acides aminés de la protéine ou entre différentes molécules de protéines.

Lors de l'immobilisation de l'enzyme par la formation de liens covalents entre l'enzyme et le support ou entre les enzymes, il est impératif que la protéine maintienne sa conformation active. L'activité enzymatique est assurée par la structure tridimensionnelle de la protéine. Cette structure, quant à elle, dépend de la structure primaire de la molécule. En effet, les liens définissant les structures secondaires, tertiaires et quaternaires de la molécule dépendent de la

structure en acides aminés de la protéine. Cette structure tridimensionnelle est assurée par des interactions non covalentes à l'exception des liaisons disulfures.

Les liaisons covalentes sont assurées par les groupes fonctionnels des acides aminés formant la structure primaire de l'enzyme. Le Tableau 4.2 présente les différents groupes fonctionnels pouvant être impliqués dans des liaisons covalentes entre différents acides aminés de l'enzyme et le support. En plus de la présence de groupements fonctionnels réactifs, la concentration relative des différents acides aminés a un impact significatif sur la réactivité d'un enzyme avec un support. La réactivité des chaînes latérales est déterminée par leur niveau de protonation. Ainsi, la protonation de ces chaînes est fonction des résidus et leur réactivité varie ainsi : $-S^- > -SH > -O^- > -NH_2 > -COO^- > -OH >> -NH_3^+$. Ces charges peuvent être estimées en connaissant le pK_a des groupes ionisés et le pH de la solution. La lysine est le résidu le plus utile pour établir des liens covalents avec des supports insolubles. En effet, il présente une large surface d'exposition et une grande réactivité spécialement dans des solutions légèrement alcalines. Elle est aussi rarement présente dans les sites actifs des enzymes. De plus, l'hydrophobicité des différents acides aminés a un impact significatif sur la disponibilité des groupements fonctionnels pour la liaison avec le support. En effet, plus un acide aminé est hydrophobe, plus il aura tendance à se situer à l'intérieur de la protéine. De ce fait, un acide aminé hydrophobe sera moins disponible pour la création de liaisons covalentes. L'hydrophobicité des acides aminés est déterminée par la variation de l'énergie libre de transfert ($\Delta G_{transfert}$) entre l'eau et l'éthanol ; plus l'énergie libre de transfert est négative, plus l'acide aminé est hydrophobe. Ainsi, les groupements fonctionnels de la tyrosine, de la méthionine, de la sérine et de la thréonine seront moins disponibles pour la création de telles liaisons. Il est extrêmement difficile de comparer la réactivité de chacun des groupes fonctionnels des acides aminés sans spécifier les conditions du milieu. Certains auteurs précisent toutefois que la lysine est l'acide aminé dont le groupe fonctionnel ($-NH_2$) a le plus grand potentiel de réagir, suivi de la cystéine, de la tyrosine, de l'histidine (à égalité avec l'aspartate et le glutamate), de l'arginine, de la tryptophane (à égalité avec la sérine et la thréonine) et, finalement, de la méthionine (Srere and Uyeda, 1976). Cette ordination est basée sur la concentration relative moyenne de ces acides aminés dans les protéines, de leur hydrophobicité et de la réactivité de leur groupe fonctionnel.

L'immobilisation d'enzymes sur des supports insolubles peut être effectuée via deux stratégies : i) le support est activé de façon à réagir directement avec les groupes fonctionnels de la protéine ou ii) des agents réticulant sont utilisés pour lier la protéine au support.

TABLEAU 3.2. CARACTÉRISTIQUES DES ACIDES AMINÉS IMPLIQUÉS DANS DES LIAISONS COVALENTES

Groupe fonctionnel	Acides aminés	Composition moyenne des protéines (%)[a]	Commentaire[b]
$-NH_2$	Lysine	7	Hydrophile
-SH	Cystéine	3,4	Hydrophile
-COOH	Aspartate	4,8	Hydrophile
	Glutamate	4,8	Hydrophile
Phénol	Tyrosine	3,4	Hydrophobe
Guanidine	Arginine	3,8	Hydrophile
Imidazole	Histidine	2,2	Hydrophile
Indole	Tryptophane	1,2	Hydrophile
CH_3-S-	Méthionine	1,6	Hydrophobe
$-CH_2OH$	Sérine	7,8	Hydrophobe
	Thréonine	6,5	Hydrophobe

[a] Cabral and Kennedy, 1991
[b] Tanford, 1962

3.3.1 Immobilisation sur des supports solides fonctionnalisés à l'aide d'un agent réticulant

Beaucoup d'efforts ont été déployés pour immobiliser différentes enzymes sur des supports solides par des liaisons covalentes. Il est toutefois à noter qu'il existe un grand nombre de techniques faisant intervenir différents supports et agents réticulants et par conséquent différents types de liaisons covalentes. Spécifiquement, une revue détaillée des différents supports et techniques d'immobilisation de laccases de diverses souches de WRF est présentée par Duran et al. (2002).

Toutefois, la stabilité des supports inorganiques fait en sorte qu'il est difficile de directement lier une protéine à ces supports. Il s'avère donc nécessaire que leur surface soit activée avec

différents types de recouvrements organiques et/ou inorganiques (Cabral and Kennedy, 1991). La méthode de dérivation la plus utilisée consiste en l'utilisation de dérivés alcoxylés de silane (voir Figure 4.1). Dans bien des cas, les groupements organiques de cet agent sont modifiés de façon à produire des réactifs capables de se lier aux résidus d'acides aminés situés à la surface de la protéine. Bon nombre d'agents réticulants peuvent être utilisés tels que des dialdéhydes (Cabral and Kennedy, 1991).

Pour l'immobilisation de laccases sur des supports solides inorganiques, la surface du support est dans la majorité des cas modifiée par réaction de l'aminopropyltriéthoxysilane (APTES) avec les groupements hydroxyle présents à la surface de ces supports. Par la suite, les groupements amino de la surface silanisée sont dérivatisés en groupements aldéhydes en utilisant du glutaraldéhyde (GLU) (Messing, 1975). Le groupement aldéhyde libre peut alors réagir avec des résidus de l'enzyme à immobiliser, tels que des groupements contenant des groupes fonctionnels NH_2. La combinaison de l'APTES et du GLU confère une grande stabilité chimique et mécanique à l'enzyme immobilisée. La Figure 4.1 schématise les étapes d'immobilisation d'une protéine sur un support siliceux activé à l'APTES et réticulée à l'aide de GLU. Cet agent réticulant est utilisé car il est facilement disponible et peu onéreux.

Il faut savoir qu'il existe un bon nombre d'autres techniques permettant une immobilisation covalente d'enzymes sur des supports solides. Parmi celles-ci, il existe des méthodes impliquant la formation de liens diazo, alkyle, aryle, etc. entre la surface (ou la surface activée) et résidus des acides aminés (Cabral and Kennedy, 1991). Le choix de la technique utilisée influence les propriétés biocatalytiques de l'enzyme immobilisée.

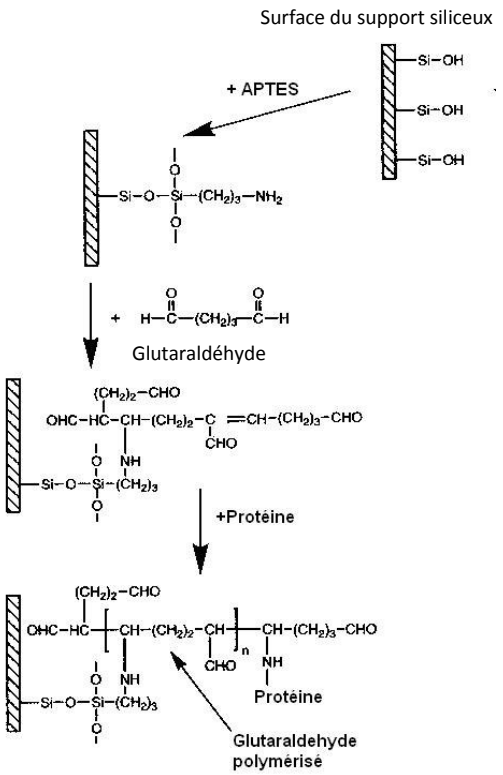

Figure 3.1. Immobilisation d'une enzyme sur une surface silanisée à l'aide d'APTES et réticulée avec du glutaraldéhyde (Modifiée (Singh et al., 1999))

3.3.2 Immobilisation sans support solide: immobilisation par réticulation inter- et intra-enzymatique

Les enzymes immobilisées sans support peuvent atteindre une grande activité massique résultant de l'absence d'un support inerte. Ces biocatalyseurs solides sont préparés par réticulation directe des enzymes par des agents bi- ou poly-fonctionnels tels le GLU ou le dextran polyaldéhyde (Mateo et al., 2004), avec des enzymes préalablement conditionnées pour être sous forme insoluble. Les types d'immobilisation sans support sont caractérisés par le procédé utilisé préalablement à la réticulation. Ces différents procédés sont présentés à la Figure 4.2. De façon générale, les enzymes en solution sont insolubilisées par différentes méthodes physico-chimiques à savoir par cristallisation (formation de CLEC), agrégation (formation de CLEA) et séchage (formation de CLSD). Ces enzymes sont par la suite réticulées de façon à conserver leur caractère insoluble. De plus, certains auteurs ont

également directement réticulé les enzymes en solution et ce, sans étape préalable de précipitation (formation de CLE).

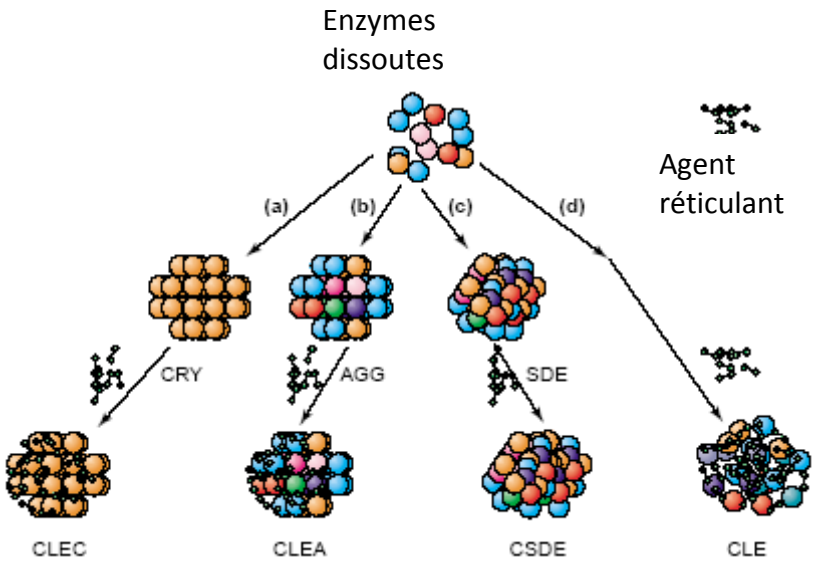

Figure 3.2. Les différentes approches pour la production d'enzymes immobilisées sans support : préparation par a) cristallisation (CLEC), b) agrégation (CLEA), c) séchoir atomiseur (CSDE), d) réticulation directe (CLE). Adaptée de (Cao et al., 2003)

Le mode d'immobilisation sans support utilisé influence grandement les différentes propriétés du biocatalyseur solide formé. De façon générale, ce type d'immobilisation augmente la stabilité thermique et au pH des enzymes. Il y a toutefois des différences au niveau de la rétention de l'activité enzymatique et de la stabilité mécanique des biocatalyseurs formés. En ce sens, les CLE et les CLSD présentent une rétention de l'activité enzymatique plus faible que celles observées sur les CLEC et les CLEA. De plus, la stabilité mécanique des CLEC et des CLEA est supérieure à celle des biocatalyseurs formés par réticulation (intra et extra moléclaire) directe entre les enzymes en solution (formation de CLE). Les propriétés des différents biocatalyseurs formés par immobilisation d'enzymes sans supports sont présentées au Tableau 3.3.

TABLEAU 3.3. PROPRIÉTÉS DES DIFFÉRENTS BIOCATALYSEURS SOLIDES OBTENUS SANS SUPPORT SOLIDE

	Mise en forme	Thermostabilité	Stabilité mécanique	Stabilité pH	Rétention de l'activité enzymatique	Manipulation	Commentaires	Références
CLE	Réticulation de l'enzyme libre	Augmentation	Faible		< 50%	Difficile car gélatineux		Broun et al., 1969; Cao et al., 2003
CLEC	Cristallisation + réticulation	Augmentation	Augmentation	Augmentation	30-70%	Facile	Taille du cristal déterminante. Nécessite des solutions enzymatiques "pures".	Cao et al., 2003; Quiocho F.A. and Richards F.M., 1966
CLEA	Précipitation + réticulation	Comparable à CLEC	Comparable à CLEC	Comparable à CLEC	Comparable à CLEC	Facile	Semblable à celle du CLEC. Impact du précipitant. Ne nécessite pas de solution enzymatique "pure"	Cao et al., 2001; Schoevaart et al., 2004
CLSD	Séchoir atomiseur + réticulation				Faible		Inactivation des enzymes par séchage	Cao et al., 2003

La formation de CLEA repose sur une technique d'immobilisation simple : la précipitation d'enzymes par l'ajout de différents précipitants (sels, surfactants, solvants organiques, etc.) est combinée à la réticulation (simultanée ou non) des enzymes présents (Mateo et al., 2004). Cette approche peut être utilisée pour co-immobiliser différentes enzymes d'intérêt.

De façon générale, le GLU a été utilisé comme agent réticulant. Ce dialdéhyde permet de lier deux groupements NH_2 présents dans la structure des acides aminés formant la protéine. Toutefois, il a été démontré que pour la formation de CLEAs de certaines enzymes telles les nitrilases, l'utilisation de GLU produits des agrégats dépourvus d'activité enzymatique (Mateo et al., 2004). Cette inactivation des CLEAs par le GLU serait imputable à la pénétration du GLU dans la structure interne de la protéine et à la réaction de cette substance avec des acides aminés essentiels à l'activité enzymatique. Ainsi, Mateo et al. (2004) ont développé du dextran polyaldéhyde qui est un agent réticulant volumineux ne pouvant pas migrer dans la structure interne de l'enzyme.

Les catalyseurs ainsi formés présentent des caractéristiques semblables à celles des CLEC en termes de rétention de l'activité enzymatique et de la stabilité au pH, à la température et des propriétés mécaniques. Cette technique d'immobilisation ne nécessite pas le développement d'un protocole de cristallisation qui s'avère bien souvent onéreux et laborieux (Lopez-Serrano et al., 2002). En ce sens, la précipitation enzymatique est une technique simple qui est utilisée dans la majorité des protocoles de purification des enzymes. Ainsi, il est possible d'utiliser des solutions protéiques non purifiées et de récupérer des biocatalyseurs composés uniquement de protéines. De plus, il est possible de co-réticuler entre elles plusieurs protéines, présentes initialement en solution, et ainsi former des CLEAs pouvant être utilisés dans des réactions en cascade.

La rétention de l'activité enzymatique et l'activité du biocatalyseur formé dépendent des conditions du milieu lors de la précipitation et de la réticulation. Par exemple, les conditions de précipitation influencent la rétention de l'activité enzymatique (Cao et al., 2003). En ce sens, l'activité enzymatique récupérée sera maximale lorsque l'ensemble des protéines seront précipitées tandis qu'elle sera minimale lorsqu'aucun précipitant ne sera utilisé (formation de CLE) (Tomimatsu et al., 1971). De plus, le type de précipitant utilisé peut influencer les propriétés catalytiques du CLEA formé (Cao et al., 2000). En plus des

conditions de précipitation, il est possible de modifier le CLEA formé en ajoutant des additifs tels que l'albumine bovine, des surfactants et des polymères à la solution enzymatique lors de l'immobilisation des enzymes de façon à varier les propriétés du biocatalyseur formé (par exemple, améliorer la stabilité ou la stéréosélectivité) (Shah et al., 2006; Sheldon et al., 2005; Wilson et al., 2004). Ces additifs peuvent permettre à l'enzyme d'adopter une conformation tridimensionnelle la rendant mieux adaptée à son application. Cette conformation est maintenue suite à la réticulation de l'enzyme (Lopez-Serrano et al., 2002).

Globalement, ces biocatalyseurs présentent plusieurs attraits pour le développement d'applications industrielles. Ainsi, la formation de CLEAs repose sur une technologie simple et facilement optimisable, ce qui se traduit par des coûts de développement faibles et une utilisation rapide, elle est applicable sur de nombreuses enzymes, incluant des solutions enzymatiques non purifiées et elle permet de produire des biocatalyseurs stables, réutilisables présentant des activités massiques élevées (Sheldon et al., 2005).

3.3.3 L'utilisation de nanomatériaux : une perspective novatrice pour l'immobilisation enzymatique

Les avancées récentes dans le développement de nanomatériaux a permis de produire une vaste gamme de ces matériaux et ce, à des prix beaucoup plus abordables (Kim et al., 2006; Asuri et al., 2006a). Cette disponibilité a permis d'utiliser ces matériaux pour le développement de nouvelles stratégies d'immobilisation enzymatique. Ces matériaux ont été utilisés de façon à amélioration l'efficacité biocatalytique et la stabilité du catalyseur solide formé.

L'utilisation de matériaux traditionnels (par exemple : alumine, silice, billes de verre, etc.) résulte en une faible activité volumique du biocatalyseur. Ces faibles activités peuvent être imputées 1) à la faible surface spécifique de ces matériaux et 2) à la présence de pores qui limitent le transfert de masse et par extension limite la constante cinétique globale du biocatalyseur solide (Kim et al., 2006). L'utilisation de nanomatériaux permet d'offrir une grande surface d'attachement aux protéines (Jia et al., 2003). En ce sens, la masse d'enzymes immobilisés sur ce type de particules peut atteindre jusqu'à 10% (masse :

masse) de la masse du support (Chen and Su, 2001). De plus, l'utilisation de nanomatériaux non poreux permet de limiter les transfert de masse ce qui globalement augmente l'efficacité du biocatalyseur formé (Kim et al, 2006). En effet, ces nanocatalyseurs présentent des propriétés à mi-chemin entre la catalyse homogène et hétérogène et ce, selon la taille des particules utilisée et la viscosité du milieu (Jia et al., 2003). Globalement, l'utilisation de nanoparticules pour l'immobilisation enzymatique permet de passer outre les limitations traditionnelles liées à la formation de biocatalyseurs en utilisant des matériaux traditionnels : transfert de masse, faible surface spécifique et masse d'enzyme liée au support.

En plus de l'amélioration de l'efficacité biocatalytique, l'utilisation de nanoparticules permet une stabilisation de l'enzyme comparativement à sa forme libre (Asuri et al., 2006b; Roach et al., 2006; Hong et al., 2004). Cette stabilité de l'enzyme est fonction de la forme et de la dimension de la nanoparticule utilisée. Ces éléments ont une influence sur la structure secondaire de la protéine et, par extension, sur l'activité et la stabilité (Lundqvist et al., 2004). Ces influences peuvent être dues à la courbure de la nanoparticule utilisée. En ce sens, une courbure plus prononcée (sur des particules plus petites) augmenterait la distance entre le centre de deux protéines situées côte à côte, ce qui diminuerait les interactions latérales non-favorables entre ces protéines Asuri et al., 2006a).

Différents nanomatériaux ont été développé et utilisé pour l'immobilisation enzymatique : nanoparticules (silice, oxyde de zinc, oxyde de fer, nanotubes de carbone, etc.) (Kim et al., 2006; Asuri et al., 2006b, Kumar and Chen, 2008), nanofibres (Kim et al., 2005; Jia et al., 2002) et des silices mésoporeuses (Takahashi et al., 2001). De plus, la formation de nanostructures permettant l'encapsulation d'enzymes par des procédés sol-gel a été rapportée (Luckarift et al., 2004). Finalement, la formation d'un réseau poreux organique et inorganique nanoscopique autour d'une enzyme permettant la stabilisation de cette dernière a récemment rapportée (Kim and Grate, 2003.)

3.3.4 Impact de l'immobilisation de laccases par liaisons covalentes

L'immobilisation de laccases, via des liaisons covalentes, provenant de différentes souches de WRF a permis d'augmenter grandement la stabilité de ces enzymes (voir Tableau 4.4).

C’est ainsi que la stabilité thermique, au pH et aux agents dénaturants chimiques, physiques et biologiques ont pu être améliorées par différentes méthodes d’immobilisation covalentes. Cette augmentation de la résistance à la dénaturation s’avère un avantage dans les applications de traitement d’effluents liquides où certaines substances chimiques dénaturantes peuvent être présentes et où l’action bactérienne peut également dénaturer l’enzyme par la sécrétion de différentes enzymes protéolytiques.

En plus de provoquer une variation de la stabilité des enzymes, l’immobilisation chimique génère des modifications de la conformation de ces biocatalyseurs. Ces transformations peuvent, dans certains cas, provoquer des changements de conditions optimales d’utilisation des différents substrats de ces enzymes. Ainsi, elle peut résulter en une modification des proprieties cinétiques de l’enzyme (une variation des constantes cinétiques K_m et V_{max}) ou des conditions de pH et de températures optimales d’utilisation du substrat. Le Tableau 4.5 présente certaines modifications induites à des laccases immobilisées sur différents supports solides.

L'immobilisation d'enzyme sur des supports chargés mène souvent à un déplacement du profil de pH. Cette variation est due à la répartition non équivalente des concentrations en protons (H^+) et anions hydroxyles (OH^-) entre le microenvironnement environnant l'enzyme immobilisée et la solution. Ces profils s’établissent suite aux interactions électrostatiques entre ces ions et les charges de surface (D'Annibale et al., 1999).

Dans bien des cas, le pH optimal de la laccase tend à se déplacer vers un pH plus alcalin car les groupes négativement chargés de la matrice ont tendance à attirer une mince couche d'ions H^+ créant ainsi un microenvironnement plus acide pour l'enzyme liée que le pH de la solution que l'on mesure (Kennedy and Melo, 1990). Cependant, dans certains cas, le pH optimal se déplace vers un pH plus acide (Al-Adhami et al., 2002; Rogalski et al., 1999).

TABLEAU 3.4. VARIATION DE LA STABILITÉ DE DIFFÉRENTES LACCASES IMMOBILISÉES PAR DES MÉTHODES CHIMIQUES SUR DIFFÉRENTS SUPPORTS PAR RAPPORT À LA STABILITÉ DE L'ENZYME LIBRE[1]

Souche de WRF	Support	Immobilisation	Stabilité température	Stabilité pH	Stabilité à divers composés	Référence
Cerrena unicolor	Cellulose (DEAE) / support acrylique	Covalente	↑ 30 - 55°C [b]	↑ pH 2.5 -5 [b]		Al-Adhami et al., 2002
Cerrena unicolor	Billes de verre à porosité contrôlée	Covalente APTES-GLU	↑ 10 - 70°C [a]		Solvants organiques	Luterek et al., 1998
Cerrena unicolor	Billes de verre à porosité contrôlée	Covalente APTES-GLU	↑ 10 et 50°C [b]		NaN_3 Solvants organiques	Rogalski et al., 1999
Lentinula edodes	Chitosan	Adsorption et réticulation GLU	↑ 55 et 65°C [a]	↑ 2.5 - 5 [c]		D'Annibale et al., 1999
Lentinula edodes	Eupergit ® C	Covalente	↑ 55 et 65 °C [a]	↑ 2.5 - 4	Pronase	D'Annibale et al., 2000
Pyricularia oryzae	Membrane de polyesther-sulfone	Adsorption	↑ 30 et 40°C [b]	↑ 6.5 - 7.5		Lante et al., 2000
Pleurotus ostreatus	Eupergit ® C	Covalente	↑ 25°C [a]			Hublik and Schinner, 2000
Trametes versicolor	Kaolinite	Covalente APTES-GLU	↑ 20 - 80 °C [a]		NaN_3	Dodor et al., 2004
Trametes versicolor		CLEC $(NH_4)_2 SO_4$ + GLU	↑ 40 - 70°C [a]		Solvants organiques	Roy et al., 2006

[1] Augmentation de la stabilité (↑).

[a] Enzyme immobilisée plus stable à cette température ou dans cette gamme de température par rapport à l'enzyme libre. Pour les autres températures, pas de données mentionnées.

[b] Enzyme immobilisée plus stable à cette température ou dans cette gamme de température par rapport à l'enzyme libre. Pour les autres températures, l'enzyme immobilisée est moins stable que l'enzyme libre.

[c] Enzyme immobilisée plus stable à ce pH ou dans cette gamme de pH par rapport à l'enzyme libre.

TABLEAU 3.5. VARIATION DES CARACTÉRISTIQUES OPÉRATIONNELLES DE DIFFÉRENTES LACCASES IMMOBILISÉES PAR RAPPORT À L'ENZYME LIBRE[1]

Souche de WRF	Support	Immobilisation	pH optimal	Température optimale	Constantes cinétiques	Référence
Cerrena unicolor	Cellulose (DEAE) / support acrylique	Covalent	↓			Al-Adhami et al., 2002
Cerrena unicolor	Billes de verre poreux	Covalent APTES-GLU	↑	=		Luterek et al., 1998
Cerrena unicolor	Billes de verre à porosité contrôlée	Covalent APTES-GLU	↓	=	K_m ↑ V_{max} ↓	Rogalski et al., 1999 D'Annibale et al., 1999
Lentinula edodes	Chitosan	Adsorption et réticulation GLU	=	↑	K_m ↑	D'Annibale et al., 2000
Lentinula edodes	Eupergit® C	Liaisons covalentes			V_{max} ↓ K_m ↑	Rogalski et al., 1999
Panus conchatus	Alcool polyvinyliq ue carboxylé	Covalent par activation avec du N-HSI	↑			Yinghui et al., 2002
Phlebia radiata	Billes de verre à porosité contrôlée	Covalent APTES-GLU	=			Rogalski et al., 1995
Trametes versicolor	Kaolinite	Covalent APTES-GLU	=	↑		Dodor et al., 2004
Trametes versicolor		CLEC $(NH_4)_2$ SO_4 + GLU	↑		K_m ↑ V_{max} ↓	Roy and Abraham, 2006

[1]Augmentation du paramètre (↑), diminution du paramètre (↓) et paramètre inchangé (=).

Les changements dans les paramètres cinétiques et opérationnels sont attribués à différents facteurs, à savoir (i) changements dans la conformation de la protéine induits par les interactions entre l'enzyme et le support, (ii) l'encombrement stérique ou l'effet de diffusion et (iii) les interactions entre le support activé et le substrat.

De plus, la température optimale de l'enzyme est augmentée suite à l'immobilisation de la protéine. Cette température optimale est fonction de deux phénomènes compétitifs: le taux de réaction et la stabilité de l'enzyme. La vitesse de réaction est fonction de la température suivant l'expression d'Arrhenius. Plus la température est élevée, plus cette vitesse est élevé. En contrepartie, cette augmentation de la température est également impliquée dans la modification de la structure tertiaire de la protéine résultant en une perte d'activité enzymatique. L'augmentation de la température optimale observée sur les enzymes immobilisées peut s'expliquer par une augmentation de la constant cinétique (selon le principe d'Arrhenius) due à l'augmentation de la température et par l'effet protecteur de l'immobilisation qui, via ses liaisons covalentes, diminue la distorsion et les dommages induits par la chaleur sur l'enzyme (Wu et al., 2005).

Compte tenu de la modification du microenvironnement entourant l'enzyme et des modifications de sa structure tridimensionnelle, les propriétés cinétiques (K_m et k_{cat}) apparentes de l'enzyme peuvent varier. Elles peuvent diminuer (diminution du k_{cat} et/ou augmentation du K_m), par exemple, quand il y a répulsion électrostatique entre le substrat et le support. De plus, un changement de la structure tridimensionnelle peut engendrer des modifications du site catalytique de la protéine et ainsi modifier ses propriétés catalytiques. Aussi, l'encombrement stérique de l'enzyme par la proximité du support solide peut induire des limitations de transfert de masse du substrat (ou du produit) entre la solution et le site catalytique de l'enzyme. Ces limitations sont aussi responsables de la diminution des capacités cinétiques apparentes de l'enzyme immobilisée.

3.3.4 Avantages et inconvénients de l'immobilisation enzymatique via des liaisons covalentes

Les enzymes immobilisées par des liaisons covalentes présentent les avantages suivants :

- Augmentation de la stabilité du biocatalyseur formé puisque l'énergie impliquée dans les liaisons covalentes permet un maintien de l'enzyme sur le support ;

- Diminution des problèmes reliés au transfert de masse par rapport à d'autres méthodes d'immobilisation puisque les enzymes sont localisées sur la surface du support ;
- Augmentation de la stabilité thermique, au pH et vis-à-vis différents dénaturants chimiques et biologiques.
- Réalisation facile en laboratoire ;
- Manipulation du support aisée ;
- Amélioration potentielle des propriétés biocatalytiques de l'enzyme suite à un changement potentiel de la conformation de l'enzyme ;
- Nombreux supports possibles.

Toutefois, ce type d'immobilisation présente également certains inconvénients pouvant limiter son utilisation pour la formation d'un biocatalyseur solide, à savoir :

- La connaissance limitée des groupes fonctionnels présents sur les acides aminés de la protéine de façon à optimiser l'immobilisation et éviter l'inactivation de l'enzyme ;
- La nécessité de conditionner le support (e.g. silanisation) ;
- Une faible efficacité de liaison et de récupération de l'activité enzymatique;
- Une diminution de l'activité enzymatique ;
- Une répulsion stérique entre le substrat et le support ;
- Une modification de la structure tridimensionnelle de l'enzyme générant :
    - Une variation des propriétés cinétiques résultant en une potentielle diminution de l'affinité pour les substrats ;
    - Une diminution de l'activité enzymatique ;
- Une difficulté d'identification des conditions optimales d'immobilisation ;
- Une faible activité massique lorsque l'immobilisation se fait sur un support solide.

3.4 Conclusion

Finalement, l'objectif global de cette thèse de démontrer l'efficacité de la laccase de C. polyzona comme biocatalyseur pour éliminer les perturbateurs endocriniens NP, BPA et

TCS il est impératif de s'attarder à ces procédés d'immobilisation de façon à démontrer la grande versatilité de cette enzyme. Ces derniers permettront un traitement en continu des substances perturbatrices du système endocrinien à l'aide de cette enzyme ligninolytique et une stabilisation de cette dernière. Ces éléments permettront de développer un procédé biologique capable de rivaliser économiquement avec les procédés physico-chimiques traditionnels. De ce point de vue, l'utilisation de laccases immobilisées sur un support solide et via la réticulation des agrégats formés par précipitation s'avèrent être des alternatives attrayantes dans le développement de cette biotechnologie environnementale.

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CHAPITRE 4

ÉLIMINATION DES SUBSTANCES PERTURBATRICES DU SYSTÈME ENDOCRINIEN, LE NONYLPHÉNOL ET LE BISPHÉNOL A, ET UN INGRÉDIENT UTILISÉ DANS LA FORMULATION DE PRODUITS D'HYGIÈNE PERSONNELLE, LE TRICLOSAN, À L'AIDE DE LA PRÉPARATION ENZYMATIQUE OBTENUE DE LA SOUCHE FONGIQUE *CORIOLOPSIS POLYZONA*

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*

4.1 Avant-propos

Ce chapitre a préalablement été publié dans la revue *Chemosphere*. La référence exacte de ce texte est :
H. Cabana, J. L. Habid Jiwan, R. Rozenberg, V. Elisashvili, M. Penninckx, S. N. Agathos, J. P. Jones, 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*, *Chemosphere,* 2007, 67, 770-778.

4.2 Abstract / Résumé

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 utilization of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) 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.

L'élimination biocatalytique des substances perturbatrices du système endocrinien, le nonylphénol (NP) et le bisphénol A (BPA), ainsi qu'un ingrédient utilisé dans la formulation de produits hygiéniques, le triclosan (TCS), par la préparation enzymatique obtenue suite à la fermentation de la souche fongique *Coriolopsis polyzona* a été étudiée. L'analyse de la variance (ANOVA) démontre que le pH et la température de la solution sont des paramètres ayant des impacts statistiquement significatifs sur l'élimination du NP, BPA et TCS. La meilleure conversion du NP et du TCS a été obtenue à une température de 50°C tandis que la transformation du BPA a été optimale à 40°C. Le pH permettant la meilleure élimination de ces micropolluants a été de 5. Suite à un traitement enzymatique de 4 h, l'ensemble du NP et du BPA, présents initialement en solution à une concentration de 5 mg l^{-1}, ont été éliminé. Pour ce qui est du TCS, la conversion maximale atteinte a été de 65% et ce, après des temps de contact de 4 et 8 h. L'utilisation du 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) comme médiateur a significativement augmenté l'efficacité du système biocatalytique utilisé. Il a été démontré que l'élimination du NP et du BPA est directement lié à l'élimination de leur activité estrogénique respective. Des analyses par spectrométrie de masse ont démontrés que le traitement enzymatique produit des composés de haut poids moléculaire et ce, via un mécanisme de polymérisation radicalaire du NP, BPA et TCS. Ces oligomères sont formés via des liens covalents C-C ou C-O. L'oligomérisation du NP a produit des dimères, trimères, tétramères et pentamères ayant respectivement des masses moléculaires de 438, 656, 874 et

1092 uma. La polymérisation du BPA a formée des dimères, trimères et tétramères ayant des masses moléculaires de 454, 680 et 906 uma. Finalement, la polymérisation du TCS par la laccase a produit des dimères, trimères et tétramères dont les masses moléculaires sont respectivement de 574, 859 et 1146 uma.

4.3 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 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.

4.4 Experimental section

4.4.1 Organism and cultivation conditions

The WRF strain *C. polyzona* (MUCL 38443) was provided by the Mycothèque de l'Université Catholique de Louvain, partner of 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 ^{o}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^{o}C and milled) was carried out on a rotary shaker at 150 rpm and 27 ^{o}C in 250-m1 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^{o}C, and the supernatant was used as the source of crude enzyme.

4.4.2 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 (ε_{max} = 3.6 x 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.

4.4.3 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), 1 mg l^{-1} catalase from *Aspergillus niger,* crude enzyme preparation from *C. polyzona*, citric acid (0.5 M)/di-sodium hydrogen phosphate (0.5 M) 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. As a control, heat-inactivated (105°C, 4 h) enzyme solution was used under the same conditions. 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.

4.4.4 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 (37% w:w), 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%).

4.4.5 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 X 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.

4.4.6 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, U.K.). 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

$$A_{corrected} = (A_{540\ sample} - A_{540\ blank}) - (A_{620\ sample} - A_{620\ blank}). \quad (4.1)$$

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

4.4.7 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), 1 mg 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 by using HCl (37% w:w). 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.

4.4.8 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 units and collision gas pressure, 0.2 Pa.

4.5 Results

4.5.1 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 4.1. This statistical analysis is based on the model presented in Eq. 4.2.

$$y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \varepsilon_{ijk} \quad (4.2)$$

where y_{ijk} represents the observations of the experiment at the *i*th level of factor A (pH), *j*th level of factor B (temperature) and *k*th replication, μ is the overall mean effect, τ_i is the effect of the *i*th level of the factor A, β_j is the effect of the *j*th level B, $(\tau\beta)_{ij}$ is the effect of the interaction of τ_i and β_j and ε_{ijk} is a random error component. The aim of this analysis was to test the hypothesis presented in equations 4.3, 4.4 and 4.5.

$$H_{0pH}: \tau_{pH\,3} = \tau_{pH\,4} = \tau_{pH\,5} = 0 \quad (4.3)$$

$$H_{1pH}: \text{at least one } \tau_{pHi} \neq 0$$

$$H_{0\,T}: \beta_{T=20°C} = \beta_{T=40°C} = \beta_{T=50°C} = 0 \quad (4.4)$$

$$H_{1T}: \text{at least one } \beta_{Tj} \neq 0$$

$$H_{0\,pH/T}: (\tau\beta)_{ij} = 0 \text{ for all } i, j \quad (4.5)$$

$$H_{1pH/T}: \text{at least one } (\tau\beta)_{ij} \neq 0$$

If the null hypothesis (H_0) is rejected, then the corresponding factor has a significant effect on the observations of the factorial design.

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.

TABLE 4.1. 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

4.5.2 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. Figure 4.1 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. After half an hour, less than 15% of the TCS was removed from the solution. A one-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. After a 8-h treatment, less than 20 % of the EDCs present in solution were removed by using inactivated enzyme solutions (results not shown).

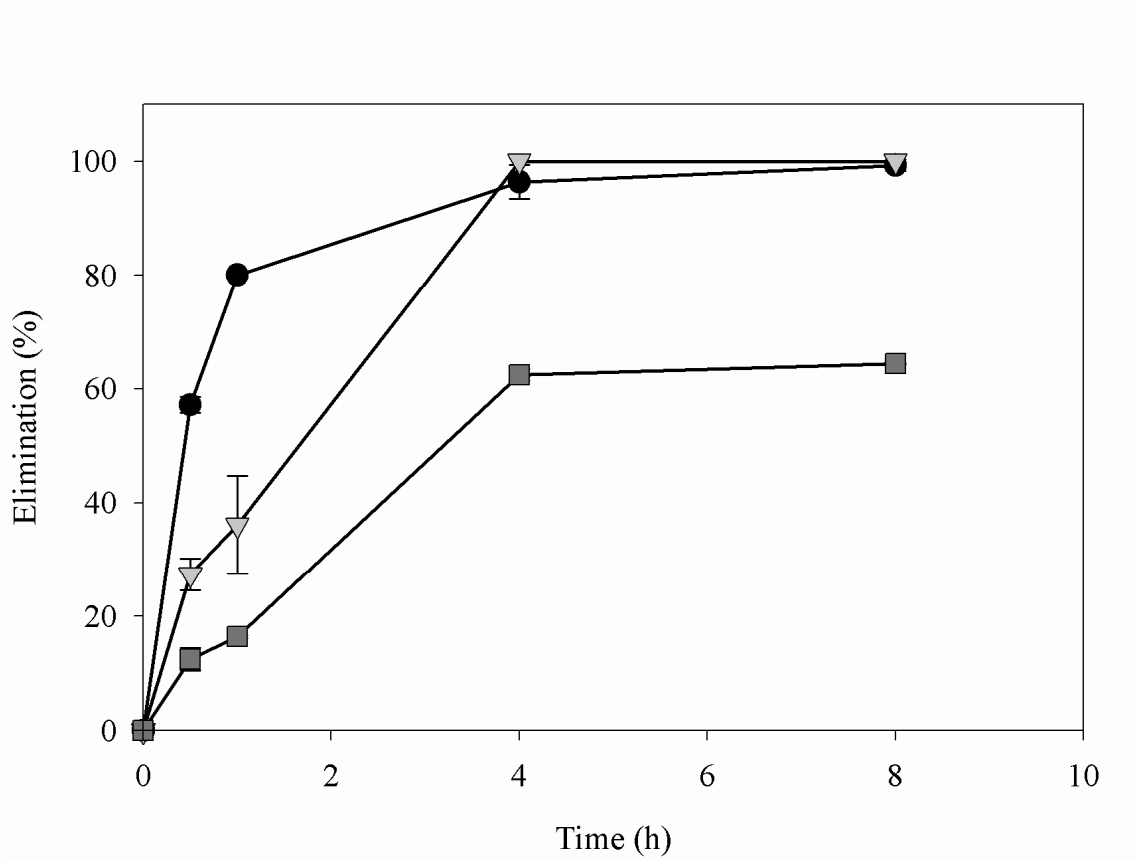


Figure 4.1. Elimination of (●) NP with 1 U l^{-1}, (▼) BPA with 10 U l^{-1} and (■) TCS with 100 U l^{-1} of laccase activity from *C. polyzona* at the previously established pH of 5 and temperature of 50 °C

3.5.3 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 one-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. 4.2. 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 affect the concentration of NP, BPA or TCS (results not shown).

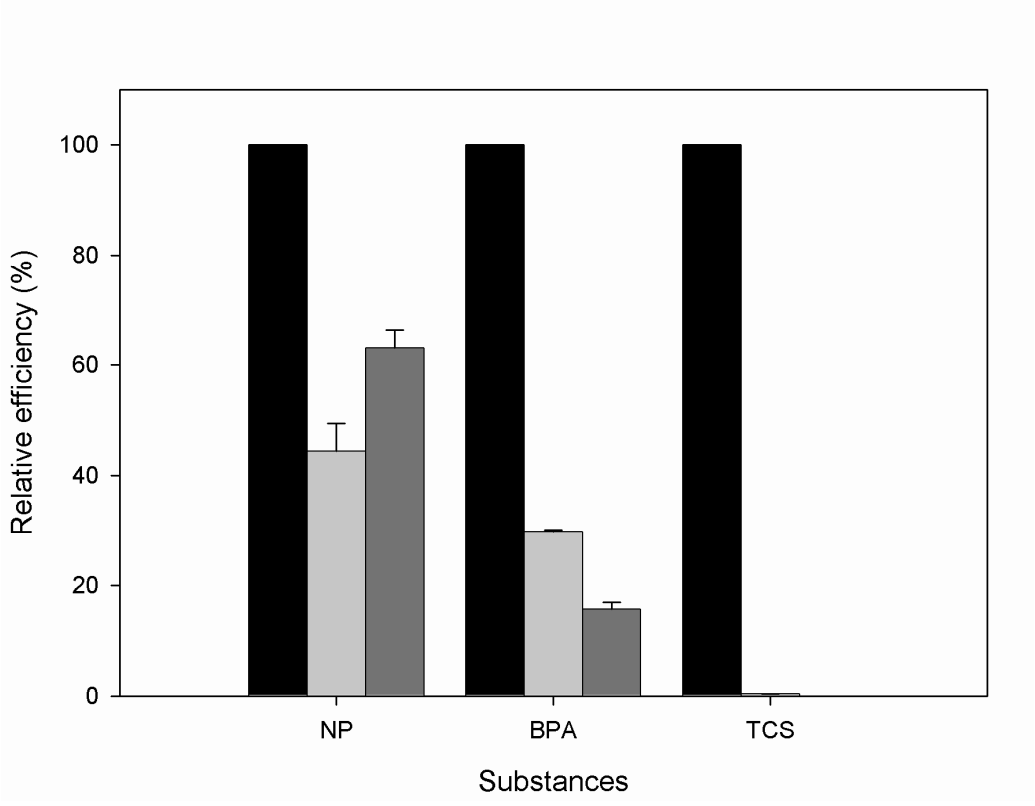


Figure 4.2. Effect of 10 µM of (■) ABTS, (■) 1-HBT or (■) absence of mediator on the removal of NP, BPA and TCS after a 1-h treatment at pH 4 and a temperature of 40 °C with the enzyme preparation containing 10 U l^{-1} of laccase from *C. polyzona*

4.5.4 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. Figure 4.3 shows that BPA and NP could bind to the hER at concentrations higher than 25 x 10^{-1} and 50 x 10^{-1} mg l^{-1} respectively, i.e., BPA had a higher tendency to bind to the hER 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.

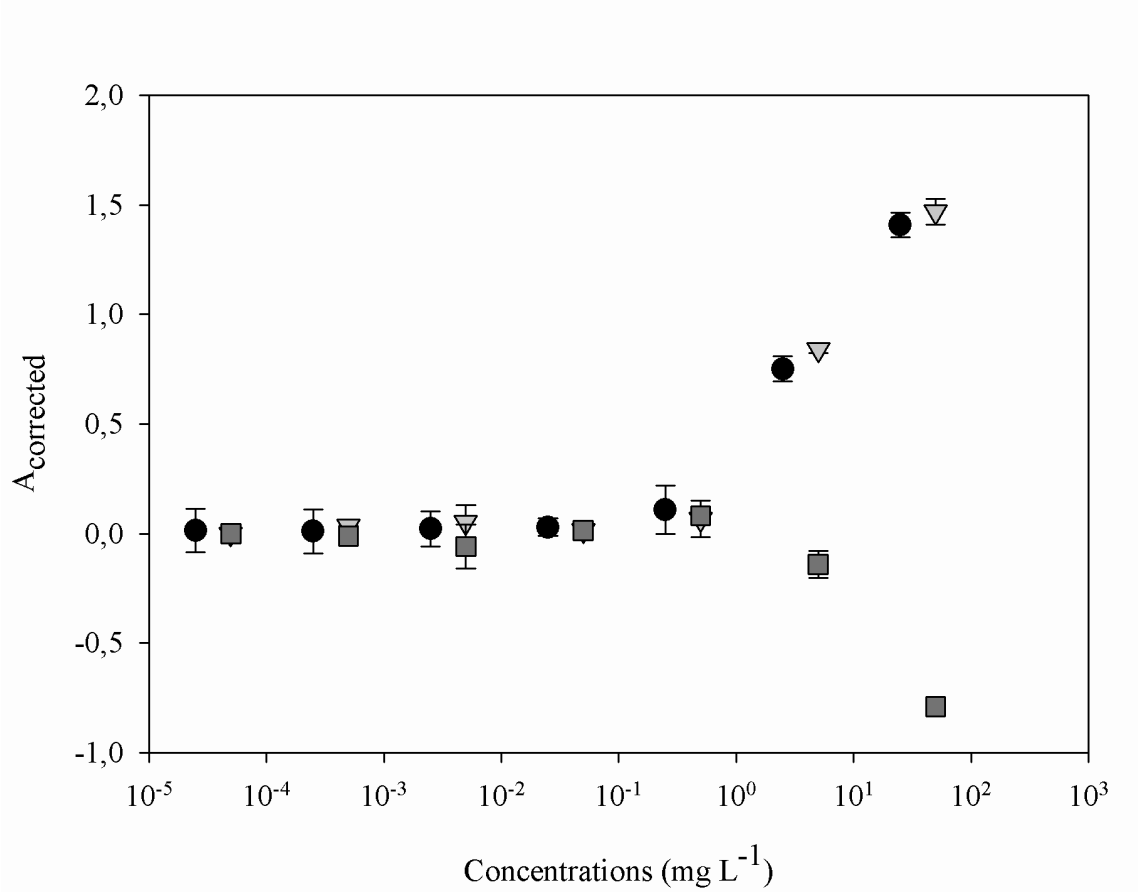


Figure 4.3. Responses of the Yeast Estrogenic Screen assay to (▼) NP, (●) BPA and (■) TCS

Figure 4.4 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.

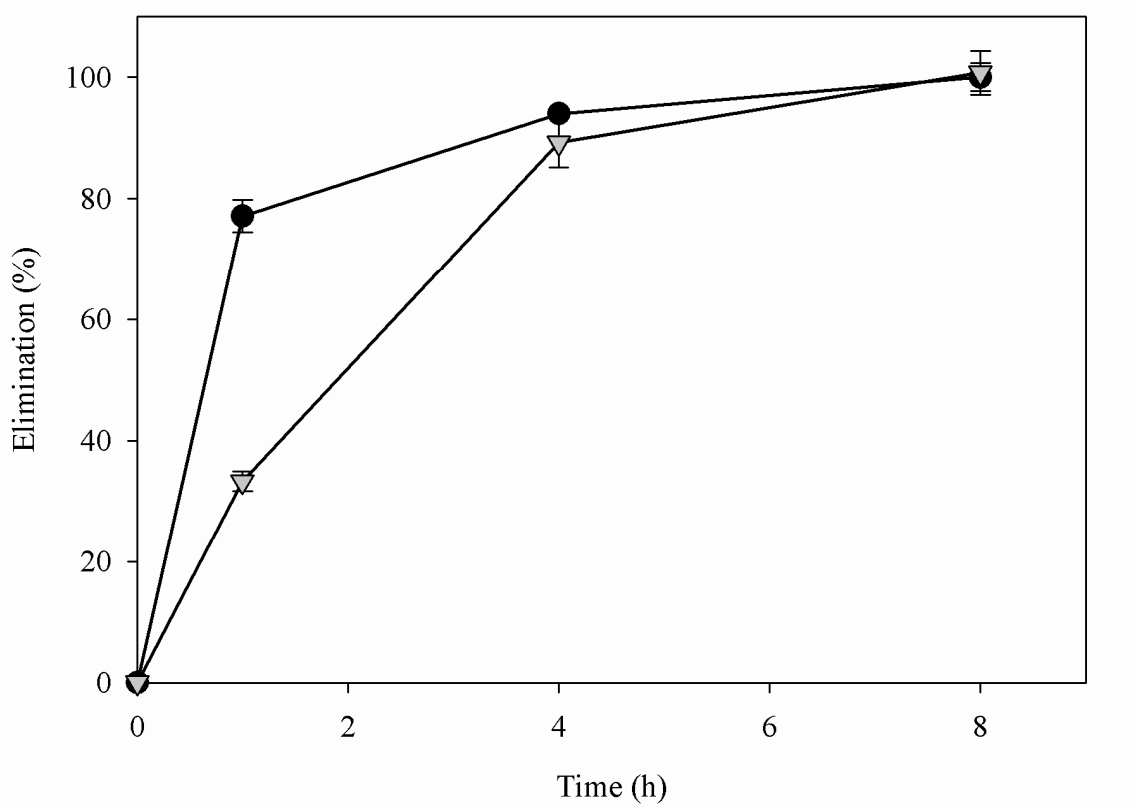


Figure 4.4. Elimination of the estrogenic activity of (●) NP and (▼) BPA with the enzyme preparation containing respectively 1 U l^{-1} and 10 U l^{-1} of laccase from *C. polyzona* at a pH of 5 and a temperature of 50 °C

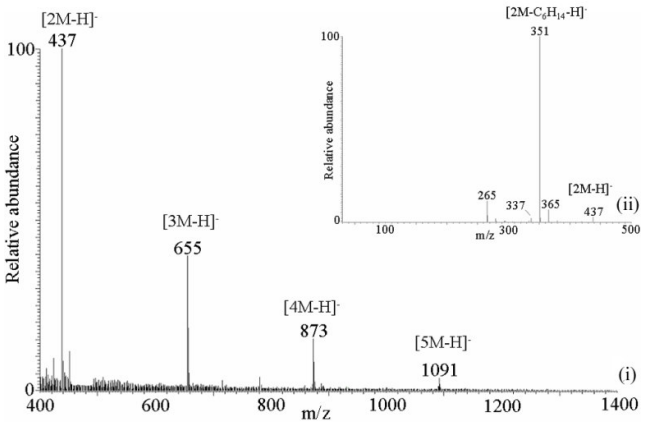
4.5.5 Identification of high molecular weight metabolites produced by the enzyme preparation treatment

Figure 4.5a shows the ESI(-) MS spectra of chloroform soluble metabolites of NP. On the full ESI(-) MS spectrum, *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.

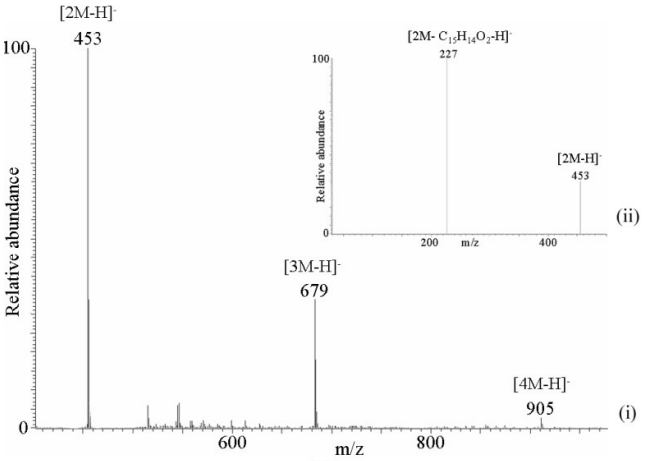
Figure 4.5b shows the ESI(-) MS spectra of chloroform-soluble metabolites of BPA. On the full ESI(-) MS spectrum, *m/z* values of 453, 679 and 905 represent respectively $[2M-H]^-$, $[3M-H]^-$ and $[4M-H]^-$of BPA. The molecular weights of the BPA dimer, trimer and

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.

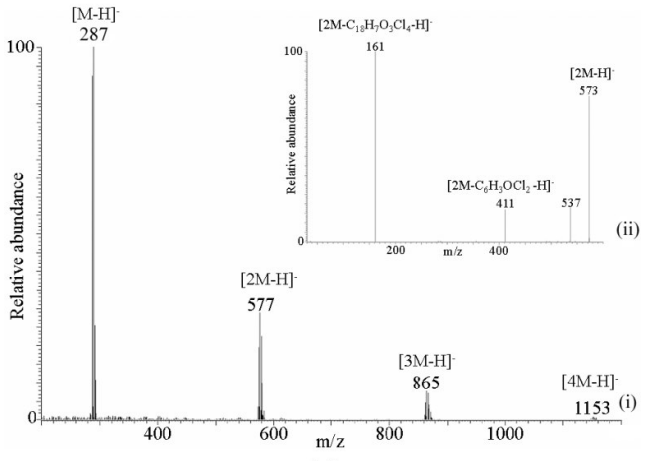
Figure 4.5c shows the ESI(-) MS spectra of chloroform-soluble metabolites of TCS. On the full ESI(-) MS spectrum, *m/z* values of 287, 573, 859 and 1145 represent respectively $[M-H]^-$, $[2M-H]^-$, $[3M-H]^-$ and $[4M-H]^-$ of TCS. The molecular weights of these compounds are respectively 288, 574, 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.



(a)



(b)



(c)

Figure 4.5. 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

4.6 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., 2006). 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 active 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 reached a plateau value 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. 4.3).

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 polymerisation 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 polymerisation could occur at the level of C-C or C-O bond formation. This type of bond could be between phenol moieties of NP, BPA or TCS. This way of polymerisation 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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CHAPITRE 5

IMMOBILISATION DE LA LACCASE DU CHAMPIGNON FILAMENTEUX *CORIOLOPSIS POLYZONA* ET UTILISATION DU BIOCATALYSEUR FORMÉ POUR L'ÉLIMINATION EN CONTINU DE SUBSTANCES PERTURBATRICES DU SYSTÈME ENDOCRINIEN

IMMOBILIZATION OF LACCASE FROM THE WHITE ROT FUNGUS *CORIOLOPSIS POLYZONA* AND USE OF THE IMMOBILIZED BIOCATALYST FOR THE CONTINUOUS ELIMINATION OF ENDOCRINE DISRUPTING CHEMICALS

5.1 Abstract / Résumé

Laccase from the white rot fungus strain *Coriolopsis polyzona* was immobilized covalently on the diatomaceous earth support Celite® R-633 using different strategies. A first methodology involved the sequential activation of the support surface with γ-aminopropyltriethoxysilane followed by the reaction of the functionalized surface with glutaraldehyde (GLU) or glyoxal (GLY) and the the immobilization of laccase on the activated surface. Another strategy tested the simultaneous internal cross-linking of the protein with GLU or GLY and the immobilization of the laccase on the silanized surface. Finally, these two strategies were modified to test the impact of the concomitant addition of bovine serum albumin (BSA) as a stabilizing agent during immobilization steps. The highest laccase activity and the greatest degree of activity recovery (while using 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the substrate) were achieved by the sequential immobilization procedure using GLU as the cross-linking agent.The solid catalysts involving the internal cross-linking of the protein showed significantly higher stability against several denaturants. The Michaelis-Menten kinetic parameters with respect to ABTS revealed a higher affinity for this substrate in the case of the sequential procedure compared to the simultaneous approach. The biocatalyst formed using GLU in the sequential procedure was used in a packed bed reactor for the continuous treatment of 5

mg l^{-1} solutions of the endocrine disrupting chemicals (EDCs) nonylphenol (NP), bisphenol A (BPA) and triclosan (TCS) by using repeated batch treatments. All of these EDCs could be eliminated at a contact time of less than 200 min by using respectively 3.75 units of laccase activity for BPA and TCS and 1.88 for NP. These performances of elimination were maintained over 5 consecutive treatment cycles using the same biocatalyst. This system could also remove these EDCs from 100 mg l^{-1} solutions. The Michaelis-Menten kinetic parameters with respect to these chemicals showed a decreasing affinity of the solid biocatalyst for NP, TCS and BPA in that order.

La laccase sécrétée par la souche fongique *Coriolopsis polyzona* a été immobilisée par la formation de liaisons covalentes sur de la terre de diatomées (Celite® R-633). Différentes stratégies d'immobilisation ont été testées. En premier lieu, une méthodologie séquentielle impliquant l'activation de la surface du support avec de γ-aminopropyltriéthoxysilane puis la réaction de cette surface fonctionnalisée avec les agents réticulants glutaraldéhyde (GLU) ou glyoxal (GLY). Une seconde stratégie implique la réticulation concomitante intra-protéique de la laccase et l'immobilisation sur la surface activée à l'aide de GLU ou de GLY. Finalement, l'ajout de sérum-albumine bovin lors de la réalisation de ces méthodologies d'immobilisation a également été testé. Le biocatalyseur solide formé présentant la plus grande activité laccase et la plus grande récupération de cette activité a été réalisé en utilisant la procédure séquentielle et le GLU comme agent réticulant et ce, vis-à-vis 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonique acide) (ABTS) comme substrat. Toutefois, les catalyseurs présentant une réticulation interne de la laccase présente une plus grande stabilité contre l'effet dénaturant de différents composés chimiques. Les paramètres cinétiques de type Michaelis-Menten, déterminés en utilisant l'ABTS comme substrat, indiquent une plus grande affinité pour ce substrat du biocatalyseur formé par la procédure séquentielle. Le catalyseur formé via la méthodologie séquentielle en utilisant le GLU comme agent réticulant a été utilisé dans un réacteur garni pour l'élimination en continue de solutions contenant 5 mg l^{-1} des perturbateurs endocriniens nonylphénol (NP), bisphénol A (BPA) et triclosan (TCS). Ce traitement continu a été simulé en répétant des traitements en cuvée. Ces substances ont été entièrement éliminées par le système et ce, pour des temps de contact de moins de 200 minutes. De plus, les performances d'élimination du système ont été maintenues sur 5 cycles consécutifs. Ce système a également utilisé avec succès pour l'élimination de solutions contenant 100 mg l^{-1} des

composés ciblés. Finalement, les paramètres cinétiques de Michaelis-Menten démontrent que ce catalyseur solide a plus d'affinité pour le NP, TCS et BPA respectivement.

5.2 Introduction

Over the last decades, progress in the understanding of the environmental impacts of persistent pollutants has prompted the development of new processes for their treatment. In an attempt to overcome some of the deficiencies of traditional chemical and biological treatment processes, research has focused on the use of enzymes for the elimination of a wide range of xenobiotics (Karam and Nicell, 1997). The use of ligninolytic enzymes from white rot fungi (laccase, lignin, manganese and versatile peroxidase) shows considerable promise due to their relative lack of specificity (Wesenberg et al., 2003) and their ability to catalyse the transformation of a wide range of pollutants. Laccase (polyphenoloxidase, E.C. 1.10.3.2) is a multicopper oxidase catalysing the oxidation of various aromatic and inorganic compounds (particularly phenols) with the concomitant reduction of oxygen to water (Duran et al., 2002). They are used, among others, in industrial applications such as pulp bleaching in the pulp and paper industry (Call and Mucke, 1997; Crestini and Argyropoulos, 1998) and decolourization or desizing in the textile industry (Wesenberg et al., 2003).

Compounds used in personal care products (PCPs) and substances which are either known or suspected endocrine disrupting chemicals (EDCs) can be transformed by laccases. Nonylphenol (4-nonylphenol), bisphenol A (2,2-bis(4-hydroxyphenol)propane), and triclosan (5-chloro-2(2,4-dichloro-phenoxy)phenol) are frequently detected in receiving waters downstream of intense urbanization (Boyd et al., 2004; Kolpin et al., 2002). These chemicals can 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 (Ishibashi et al., 2004; Jobling et al., 2003; Soto et al., 1991).

The promise of laccase for the elimination of PCPs and EDCs from both aqueous solutions and polluted soils has been established over the last few years (Cabana et al., 2007a; Kim and Nicell, 2006; Tanaka et al., 2003; Tanaka et al., 2001; Hundt et al., 2000) and recently reviewed (Cabana et al., 2007b). The resulting chemicals do not have any estrogenic

activity (Cabana et al., 2007a; Tanaka et al., 2003). These results have been extended with the use of the laccase from the WRF strain *Coriolopsis polyona* in the insolubilized yet fully active form of cross-linked enzyme aggregates (CLEAs) (Cabana et al., 2007c).

Efforts have been made to immobilize laccase on solid supports (Duran et al., 2002) in order to enhance the industrial utility of these enzymes in wastewater treatment processes, including the possibility to reuse them and to improve their stability. Several techniques may be applied to immobilize enzymes on solid supports. They are mainly based on chemical and physical mechanisms (Duran et al., 2002). Protocols for covalent enzyme immobilization often begin with a surface modification or an activation step. Silanization, the coating of the surface with organic functional groups using an organofunctional silane reagent, is a widely used strategy for initial surface modification of supports. Such added or native surface amino groups can be derivatized to aldehyde groups using a bi-functional compound such as glutaraldehyde or glyoxal (Duran et al., 2002). The large number of amine groups present in proteins, usually located on the surface, form covalent bonds with most of the reactive groups on the supports (Mateo et al., 2000). Furthermore the concurrent addition of free enzyme together with a cross-linking agent could result in internal cross-linking of the enzyme during the covalent binding step (Cao et al., 2003).

The first objective of this study was to immobilize the laccase from the WRF *C. polyzona* on a solid support and to determine the effect of the immobilization conditions on the properties of the biocatalyst. The factors examined were the nature of the cross-linking agent used, the order in which the cross-linking agent is added and the supplementation of the laccase with bovine serum albumin (BSA) during the contacting phase. The stability of free and immobilized laccase was assessed in the presence of chemical denaturants and at different pH and temperature. The second objective of the present study was to evaluate the performance of immobilized laccase for the continuous elimination of the endocrine disrupting chemicals p353NP (a branched isomer of NP), BPA and TCS in a packed-bed reactor (PBR), including the effects of pH and temperature on their removal.

5.3 Experimental section

5.3.1 Chemical reagents

All chemicals were of analytical grade (or of the highest purity available). 3,5-Dimethyl-3-heptanol was from Alfa Aesar (Karlsruhe, Germany). Celite® R-633 was kindly supplied by World Minerals (Santa Barbara, CA). The specific surface area of this support was 1.3 $m^2\ g^{-1}$ as determined by the Brunauer–Emmett–Teller (BET) method on a Micromeritics Flow Sorb II 2300 sorptometer (Micromeritics Instrument Corporation, Norcross, GA). All other chemicals were from Sigma-Aldrich (Saint-Louis, MO). All solvents were of HPLC grade.

5.3.2 Organism and Cultivation Conditions

The WRF strain *C. polyzona* (MUCL 38443) was provided by the Mycothèque de l'Université Catholique de Louvain, partner of the Belgian Coordinated Collection of Microorganisms (BCCM™/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 standard 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. The medium was adjusted to pH 6.0 with 2 M NaOH prior to autoclaving. After 10 days of cultivation the fungal biomass was filtered and the solids were separated by centrifugation at 3000 g for 15 min at 4°C. Enzymes were precipitated using 600 g l^{-1} ammonium sulfate. This preparation was dissolved in distilled water and dialyzed using a 13 kDa membrane. After freezing at -18°C to precipitate polymers, the solution was filtered and used as the source of laccase. This precipitation step results in less than 10% laccase activity lost.

5.3.3 Enzyme Assays

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

(ε_{max} = 3.6 X 10 ^{4} M^{-1} cm^{-1}) (Bourbonnais and Paice, 1990). The assay mixture contained 0.5 mM ABTS. The pH was adjusted to 3 using citric acid (0.5 M)/disodium hydrogen phosphate (0.5) buffer and the temperature was set at 20°C. One unit (U) of laccase activity was defined as the amount of enzyme forming 1 µmol of $ABTS^{\cdot+}$ per min.

Immobilized laccase activity was assayed by incubating approximately 50 mg of support in 5 ml of buffer at pH 3, after addition of 5 ml of a 1 mM solution of ABTS and incubation at 20°C under continuous orbital stirring at 150 rpm. The absorbance was monitored every minute by withdrawing 1 ml of the solution. After measurement, the solution was remixed with the immobilized laccase. After 10 minutes, the solution was filtered and the support dried overnight at 105°C.

The activity recovery was defined as the apparent laccase activity of the solid biocatalyst formed divided by the laccase activity theorically binded to the support. The laccase activity theorically binded to the support is defined as the difference between the initial laccase activity and those remaining in the supernatant at the end of the immobilization procedures after the washing step. The binding yield is defined as the activity theorically binded to the support divided to the initial activity and the effective binding yield represent the effective activity of the biocatalyst formed divided by the initial laccase activity.

5.3.4 Immobilization procedures

The immobilization of laccase involved the formation of covalent bonds between amino acids not involved in the activity of the enzyme and the diatomaceous earth support Celite® R-633. Two different approaches were used, one sequential and the second simultaneous (Figure 5.1).

In the standard sequential immobilization procedure (CR633-GLU) 1 g of support was silanized with 12.5 ml of a 4% (v:v) solution of γ-aminopropyltriethoxysilane (APTES) in acetone at 45°C for 24 hours. The organic phase was then discarded and the carriers were washed off with distilled water and dried overnight at 45°C. The support was then put in contact with 12.5 ml of a 5% (v:v) solution of glutaraldehyde (GLU) in a 0.5 M phosphate buffer (pH = 7) for 2 h. The support was then washed with distilled water, filtered and

dried for 48 h at 50°C. Finally, 10 ml of a 1 U ml^{-1} laccase solution in distilled water were added to the support. The suspension was placed in a 50 ml erlenmeyer an orbitally shaked at 100 rpm for 24 h at room temperature. The pellets were subsequently washed several times with distilled water until no laccase activity was detected in the supernatant and finally stored in water at 4°C. A slight modification of the above standard immobilization procedure involved the addition of BSA to the laccase solution during the phase of contact of laccase with the solid support. The following mass ratios of BSA to laccase activity were used: 0.01 mg U^{-1} (preparation designated as CR633-GLU-BSA-0.01), 0.1 mg U^{-1} (CR633-GLU-BSA-0.1) and 1 mg U^{-1} (CR633-GLU-BSA-1).

In the simultaneous immobilization procedure the internal cross-linking of the laccase was undertaken during the covalent binding step (biocatalyst designated as CR633-SIM-GLU). The GLU activation step was carried out at the same time as the laccase-to-support contacting phase. This immobilization procedure was performed until no more laccase activity was detected in the supernatant. The same procedure was also modified with the addition of BSA using different mass ratios of BSA to U (e.g. for a BSA:enzyme ratio of 1 mg U^{-1}, CR633-SIM-GLU-BSA-1).

All of these procedures were also performed using the same molar concentration of glyoxal (GLY) instead of GLU as the cross-linking agent.

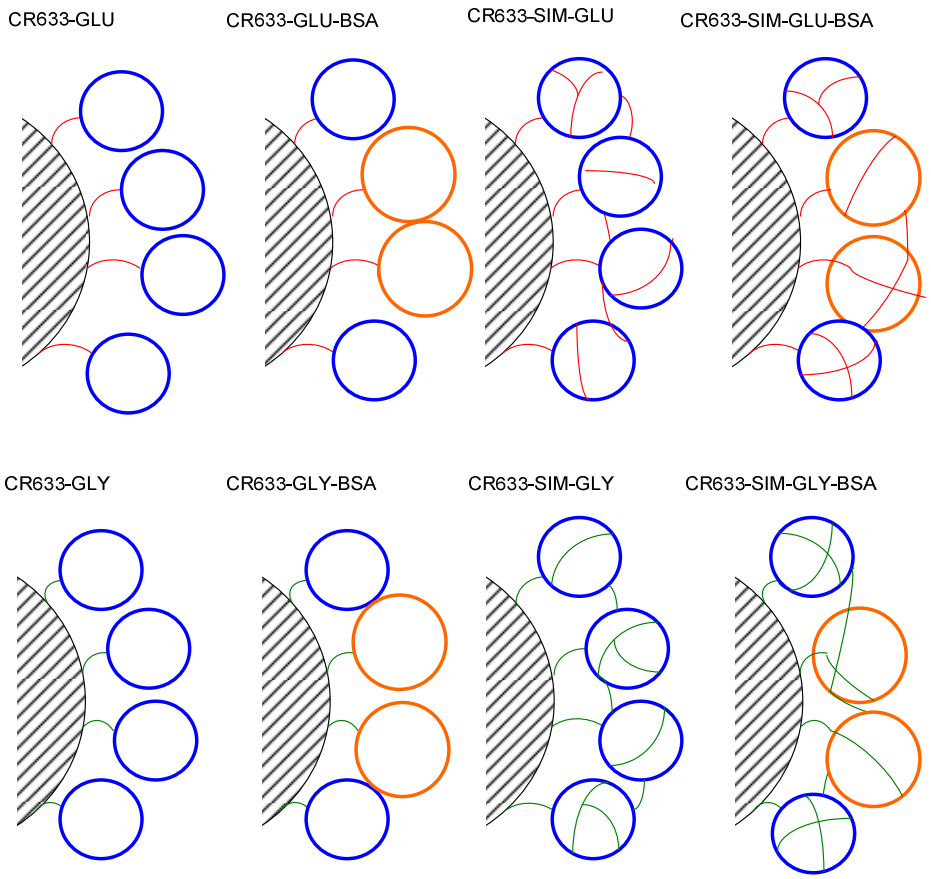

Figure 5.1. Schematic of the biocatalyst expected using different procedures. Proteins: (○) laccase; (○) BSA. Cross-linking agents: (—) GLU; (—) GLY

5.3.5 Stability against thermal and chemical denaturation

The thermal inactivation of the solid immobilized laccase (and of the soluble laccase as a control) was investigated at 40 °C and pH 3 in citric acid (0.5 M)/disodium hydrogen phosphate (0.5 M) buffer. Solid catalysts (0.1 g) or free laccase (0.1 U) were incubated in 500 µL of buffer solution containing 100 µM of phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity. The initial activities were compared with the residual activities after a 24-h incubation.

The stability against different denaturants was tested using 0.1 g of support. The denaturing solutions consisted of 10 µM $CaCl_2$, 10 µM ethylenediamine-tetraacetic acid (EDTA), 25% (v/v) methanol and 25% (v/v) acetone in citric acid (0.5 M)/disodium

hydrogen phosphate (0.5 M) buffer at a pH of 3 and a temperature of 20°C, and the incubations lasted 30 min.

5.3.6 Determination of kinetic parameters

The Michaelis–Menten kinetic parameters K_m and k_{cat} of free and immobilized laccase were determined by measuring the laccase activity using ABTS as substrate over a 25 to 1500 μM range of initial concentrations. Furthermore, the same kinetic parameters were determined using the selected EDC as substrate using initial concentrations varying from 5 to 150 mg l^{-1}. The parameter values were obtained by curve fitting of the plot of reaction rate versus substrate concentrations using the Sigma Plot 7.0 software (SPSS Inc., Chicago, IL).

5.3.7 Synthesis of 4(3',5'-dimethyl-3'-heptyl)-phenol (p353NP)

The p353NP isomers of NP were synthesized by Friedel-Crafts alkylation from phenol and 3,5-dimethyl-3-heptanol, and consisted of two diastereomers. The reaction medium contained 1.88 g phenol, 4.33 g 3,5-dimethyl-3-heptanol, 35 ml BF_3-ether complex and 200 ml anhydrous petroleum ether placed in a 500 ml two-necked flask equipped with a reflux condenser. The reaction was allowed to run for 15 min at 50°C with stirring, and then stopped by adding 200 ml of water. After intensive stirring for a further 15 min, the aqueous phase was removed and the organic phase was washed 5 times with water in order to remove unreacted phenol, and then dried over Na_2SO_4 (Corvini et al., 2004). After removing the petroleum ether under vacuum, the yield of p353NP was 1.72 g with a purity of 98.0% (GC).

5.3.8 Elimination of endocrine disrupting chemicals

A packed bed reactor (PBR), operated in a repeated batches mode, was used for the continuous elimination of each EDC from two series of aqueous solutions, one of 5 mg l^{-1} and another one of 100 mg l^{-1} of p353NP, BPA or TCS. The reactor consisted of a 1.6 cm diameter and 20 cm height glass column. The PBR was fed with the EDC solution at a

flow rate of 1.0 ml min^{-1}. The reactor was packed with 5 g of immobilized laccase (0.75 U g^{-1}) for the elimination of BPA and TCS and 2.5 g for the elimination of p353NP. The solution pH was maintained at 5 using a citric acid (0.5 M)/disodium hydrogen phosphate (0.5 M) buffer except when otherwise specified. Catalase (1 mg l^{-1}) from *Aspergillus niger* was added to the solution to eliminate hydrogen peroxide. Each EDC solution was air saturated prior to feeding in the PBR. The elimination of the EDCs was tested at room temperature. After the elimination was complete, the liquid phase was drained and the reactor supplemented with fresh aliquots of the EDC solution.

To quantify the potential adsorption of the EDCs on the support with immobilized laccase, the same amount of support with previously inactivated laccase was used under the conditions applied to the elimination in the PBR. The laccase on the support was inactivated by incubating it at 105°C for 4h.

The impact of the operational conditions pH and temperature was determined by using 0.5 g of support with immobilized laccase incubated batchwise with 5 ml of a 5 mg l^{-1} solution of EDC at pH 3, 4 or 5 and temperature 30, 40 or 50°C for 3 h respectively.

5.3.9 Extraction of EDCs prior to quantitative analysis

The p353NP isomers were 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 p353NP isomers were eluted from the cartridge using 3 ml of ethyl acetate followed by 6 ml of methylene chloride. The organic phase was then evaporated under a gentle stream of nitrogen gas.

BPA and TCS were extracted using methylene chloride in a 1:1 (v:v) ratio (methylene chloride:treated solution). The solutions were acidified to a pH of 2 with HCl and shaken for 20 min. The organic phase was separated and evaporated under a gentle stream of nitrogen gas. Each sample was dissolved in 100 µL of methylene chloride prior to quantitative chemical analysis.

5.3.10 GC-MS analysis

The gas chromatography-mass spectrometry analysis was performed on a G1800A gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with an electron ionization detector and an HP-5 MS fused-silica column (30 m X 0.25 mm i.d., 0.25 mm film thickness) (Hewlett-Packard, Palo Alto, CA). Helium was used as a carrier gas at a flow rate of 0.70 ml min^{-1}. The column was held at 70°C for 3 min, then heated up to 160°C at a rate of 20°C min^{-1}, and finally up to 300°C at a rate of 10°C min^{-1} and held for 7 min (Diaz et al., 2002). The injector temperature was set at 250°C. Analyses were conducted in single ion monitoring mode (SIM). For each target compound three unique identifier ions were chosen for SIM. The chosen ions for SIM were 121, 149 and 220 for p353NP, 119, 213 and 228 for BPA and 146, 218 and 288 for TCS.

5.4 Results

5.4.1 Activity of the solid biocatalyst

The different immobilization procedures used influenced the laccase activity of the solid biocatalysts formed and the percentage of enzyme activity recovered. Figure 5.2 and Table 5.1 present the laccase activity, the degree of activity recovery, the binding yield and the effective binding yield using the different immobilization procedures. The highest laccase activity on the solid support was obtained using the sequential immobilization procedures compared to the simultaneous immobilization and cross-linking of the enzyme. Using the sequential procedures, higher laccase activity was achieved with GLU than with GLY. The highest laccase activity was approximately 0.8 U g^{-1} and was reached using the CR633-GLU biocatalyst. In contrast, it was the adoption of GLY that improved the activity of the biocatalyst when using the simultaneous procedure. The activity of the CR633-SIM-GLY support was 0.3 U g^{-1}. Although the co-immobilization of BSA and laccase decreased the activity of the catalyst formed with the sequential procedure, the addition of BSA did not influence the laccase activity of the catalyst formed using the simultaneous approach.

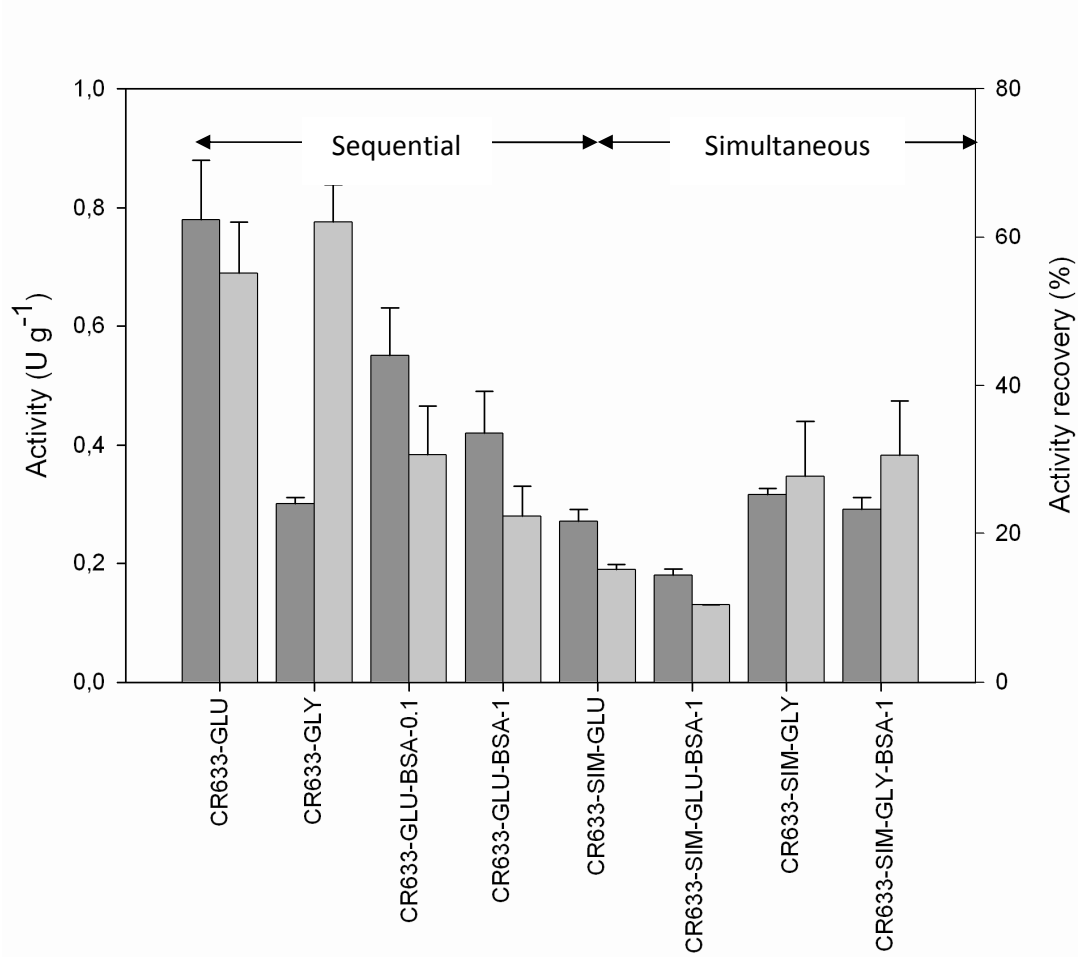

Figure 5.2. Laccase activity (■) and percent activity recovery (■) using different immobilization procedures (means of triplicates ± standard deviation)

TABLE 5.1. IMMOBILIZATION YIELDS USING THE DIFFERENT IMMOBILIZATION STRATEGIES PROPOSED[a]

Immobilization procedure	Activity recovery (%)	Binding yield (%)	Effective binding yield (%)
CR633-GLU	55 ± 8	45 ± 9	27 ± 7
CR633-GLY	62 ± 7	23 ± 3	14 ± 3
CR633-GLU-BSA-0.1	31 ± 6	86 ± 8	17 ± 2
CR633-GLU-BSA-1	22 ± 4	45 ± 2	9 ± 2
CR633-SIM-GLU	15 ± 1	100 ± 3	14 ± 1
CR633-SIM-GLU-BSA-1	10 ± 1	100 ± 8	11 ± 2
CR633-SIM-GLY	29 ± 7	35 ± 3	9 ± 2
CR633-SIM-GLY-BSA-1	31 ± 7	30 ± 4	9 ± 3

[a] The values represent means of triplicates ± standard deviation

Laccase activity recovery was maximized when using the sequential procedure. A 60 % recovery was obtained with the CR633-GLY biocatalyst. The highest laccase activity recovery using the simultaneous immobilization methodology was approximately 30 % (CR633-SIM-GLY-BSA-1). The addition of BSA decreased the laccase activity recovery with the sequential procedure and slightly improved it when using the simultaneous approach.

5.4.2 Stability of the immobilized enzyme

Figure 5.3 presents the residual activity for different forms of laccase after a 24-h incubation period under denaturing conditions at a pH of 3 and a temperature of 40°C. The activity of the various immobilized forms of laccase, assayed with ABTS as a substrate, was better conserved than the free form under the conditions tested. By far the best stability was displayed by the laccase immobilized by the simultaneous procedures: The CR633-SIM-GLY and CR633-SIM-GLY-BSA-1 retained 90% of the initial activity on the support while the corresponding biocatalysts with GLU resulted in 90% activity retention. The residual activity of the laccase immobilized by the sequential procedures was considerably lower (about 30% of the initial activity) but still approximately 3 times higher than that of the free form, which, in turn, represented only 10% of the initial activity.

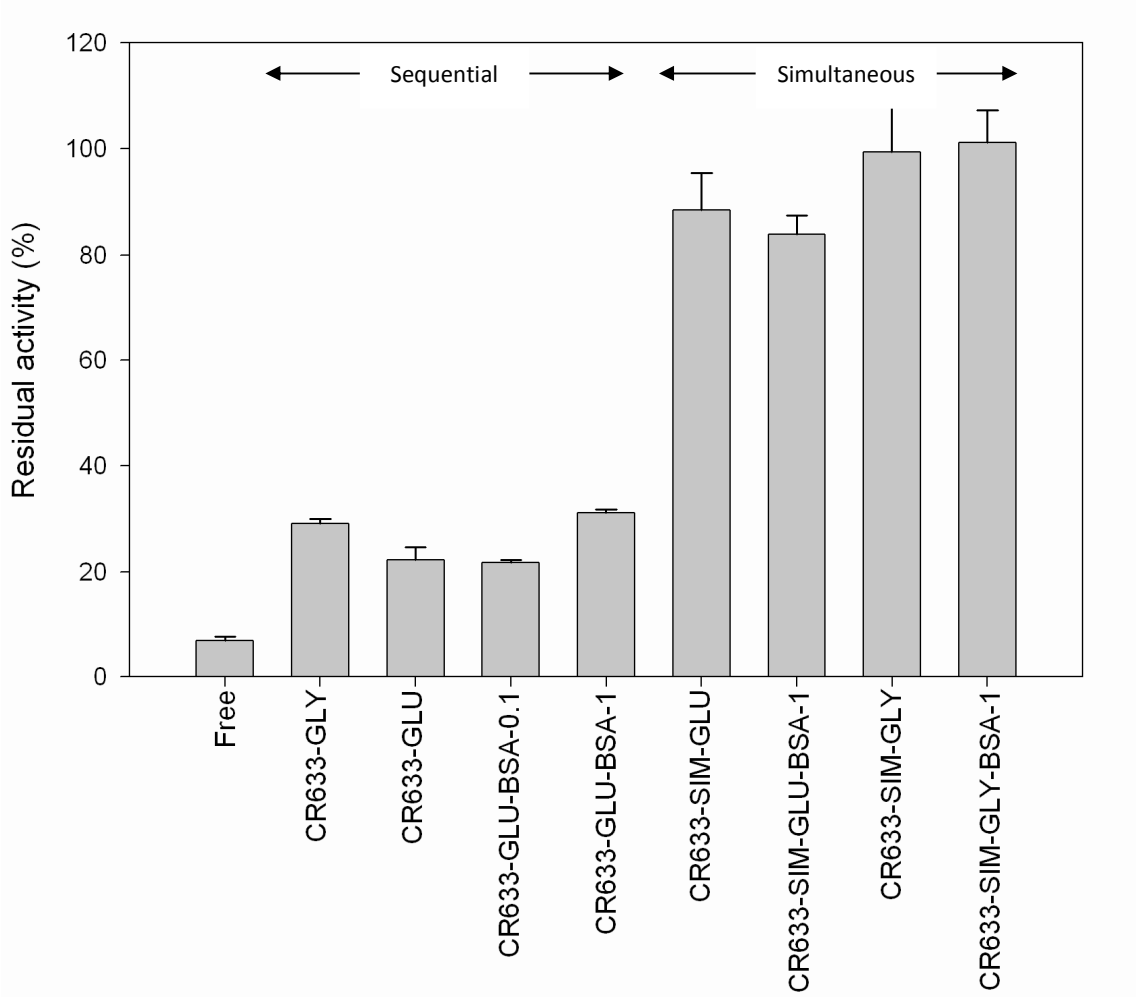

Figure 5.3. Residual laccase activity after a 24-h incubation period at a temperature of 40°C and a pH of 3 (means of triplicates ± standard deviation)

Various forms of laccase (free, CR633-GLU and CR633-SIM-GLU) were exposed to different denaturants in the activity assay mixture (substrate ABTS) for 30 min to determine the influence of the internal cross-linking of the laccase on its resistance to these agents (Figure 5.4). The biocatalyst CR633-GLU had no higher laccase stability than the free enzyme. These two forms of laccase had residual activities of 50%, 55%, 45% and 50%, respectively, in the presence of a 25% (v/v) methanol, 25% (v/v) acetone, 10 μM solution of EDTA or 10 μM $CaCl_2$. The internally cross-linked laccases showed significantly higher residual activities, which reached levels of 120%, 85%, 120% and 110%, respectively, for methanol, acetone, EDTA and $CaCl_2$ solutions (Figure 5.4).

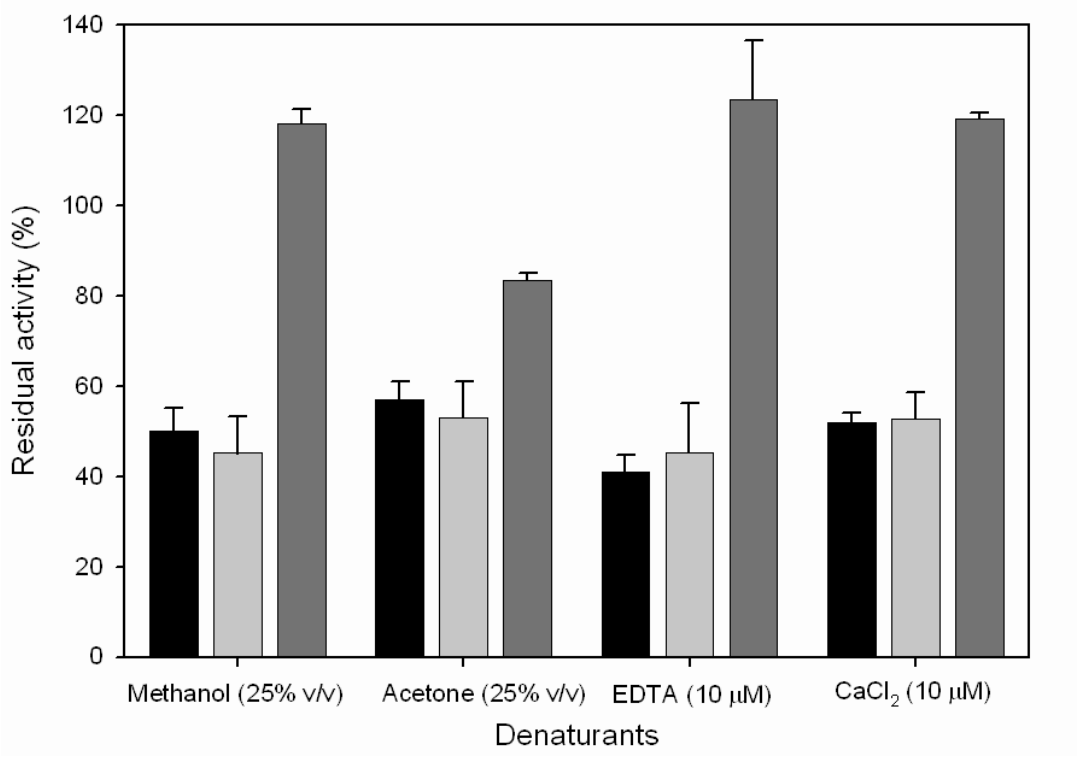

Figure 5.4. Residual laccase activity of (■) free laccase, (■) CR633-GLU and (■) CR633-SIM-GLU supports after a 30-min incubation with different denaturants at room temperature and a pH of 3 (means of triplicates ± standard deviation)

5.4.3 Kinetics of immobilized laccase

The Michaelis-Menten kinetic constants for the oxidation of ABTS are presented in Table 5.2 for the biocatalysts resulting from different immobilization procedures using GLU as the cross-linking agent (for reaction as a function of substrate concentrations, see Annexe 3). The CR633-SIM-GLU biocatalyst had a higher maximum rate of transformation of ABTS compared to its counterpart originating from the sequential procedure. The Michaelis-Menten constant, K_m, of the free laccase was 7 times lower than the K_m of the CR633-GLU laccase and 20 times lower than the constant for the CR633-SIM-GLU biocatalyst. Finally, the efficiency of the biocatalytic process constant, $k_{cat}\ K_m^{-1}$, of the CR633-GLU was approximately 3 times higher than the efficiency constant of the CR633-SIM-GLU for the oxidation of the ABTS.

Table 5.3 presents the Michaelis-Menten kinetic parameters for the elimination of the EDCs p353NP, BPA and TCS using the CR633-GLU biocatalyst. The K_m values are of the same order of magnitude for all three target substrates. However, the k_{cat} value for p353NP

is approximately 5 times higher than for the other EDCs tested. Based on the efficiency of the biocatalytic process constant of the CR633-GLU for the various EDCs, these solid biocatalysts have a greater capacity of elimination for p353NP, followed by TCS and by BPA.

TABLE 5.2. MICHAELIS-MENTEN KINETIC CONSTANTS OF LACCASE IMMOBILIZED ON CELITE® R-633 FOR THE OXIDATION OF ABTS AT pH 3 AND A TEMPERATURE OF 20°C[a]

Support	k_{cat} (µmol min^{-1} g^{-1})	K_m (µM)	k_{cat} K_m^{-1} (l min^{-1} g^{-1})
Free	11.2 ± 0.6	31.0 ± 2.0	3.6 X 10^{-1}
CR633-GLU	0.7 ± 0.02	192.0 ± 11.1	3.6 X 10^{-3}
CR633-SIM-GLU	0.9 ± 0.05	711.0 ± 15.1	1.3 X 10^{-3}

[a] The values represent means of triplicates ± standard deviation

TABLE 5.3. MICHAELIS-MENTEN KINETIC CONSTANTS FOR THE ELIMINATION OF p353NP, BPA AND TCS WITH CR633-GLU CATALYST AT A pH OF 5 AND A TEMPERATURE OF 20°C[a]

Substrate	K_m (mM)	k_{cat} (µmol min^{-1} g^{-1})	k_{cat} K_m^{-1} (l g^{-1} min^{-1})
p353NP	0.219 ± 0.022	4.3 ± 0.1	0.02
BPA	0.180 ± 0.017	0.89 ± 0.07	0.005
TCS	0.128 ± 0.01	0.92 ± 0.1	0.007

[a] The values represent means of triplicates ± standard deviation

5.4.4 Elimination of EDCs using the CR633-GLU biocatalyst

The continuous elimination of EDCs was tested in a PBR using a repeated batch mode. Figure 5.5 presents the enzymatic and adsorption-based removal of p353NP, BPA and TCS from 5 mg l^{-1} solutions over 5 consecutive cycles of a nominal contact time of 200 min each, using, respectively, 5 g of catalyst CR633-GLU for BPA and TCS and 2.5 g of the same catalyst for p353NP. The solid biocatalyst had an activity of 0.75 Ug^{-1}. Between cycles the catalyst beads were washed with 100 ml buffer at a flow rate of 1 ml min^{-1}. The degrees of removal due to adsorption to the support of CR633-GLU whose laccase had been thermally inactivated varied from 60% to 40% for p353NP, 50% to 40% for BPA and

60% to 40% for TCS between the first and the fifth cycle. The degree of adsorption of the EDCs correlated to the hydrophobicity of each chemical.

The complete enzymatic elimination of p353NP, BPA and TCS was achieved over 5 consecutive cycles representing contact times of approximately 200 min in the immobilized laccase bed. The extents of enzymatic removal were well conserved over these sequential treatment cycles. In an effort to extend the performance of the immobilized laccase, these biocatalysts were used for the continuous elimination of each of the three EDCs from 100 mg l^{-1} solutions (Figure 5.6). Considerable removal extents were achieved for all of these chemicals. At a contact time of 20 min, 60% elimination for p353NP, 40% for BPA and 55% for TCS were achieved. The degrees of removal reached 85% for p353NP, 70% for BPA and 80% for TCS after the residence time was adjusted to 200 min in the PBR for the same 100 mg l^{-1} solutions. The percent elimination attributed to the adsorption of these substances on the support was less than 40% for p353NP, 15% for BPA and 30% for TCS.

Table 5.4 presents the analysis of variance (ANOVA) of the data on the elimination of EDCs under different conditions of temperature and pH using the CR633-GLU laccase. This statistical analysis can determine objectively the impact of each condition on the reduction of the concentration of each substance in solution. The temperatures studied were 30, 40 and 50°C and the pHs were 3, 4 and 5. 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 value of 0.992 for the elimination of p353NP, 0.998 for that of BPA and 0.994 for that of TCS suggest that the fitted linear-plus-interactions models could explain 99.2%, 99.8% and 99.4% respectively of the total variation (Montgomery, 2005). This implies satisfactory representations of the elimination processes by such mathematical representations. The F-values of 140.4 for p353NP elimination, 515.0 for BPA and 200.2 for TCS elimination and a p value <0.001 for all eliminations indicate that the assumed linear-plus-interactions models are statistically significant and can predict well the experimental results.

TABLE 5.4. ANALYSIS OF VARIANCE (ANOVA) FOR THE SELECTED LINEAR PLUS INTERACTIONS MODEL FOR ELIMINATION OF p353NP. BPA AND TCS WITH CR633-GLU CATALYST

Substance	Source	Sum of squares	Degrees of freedom	Mean square	F-value	Prob > F	Coefficient of determination (R^2)
p353NP	Model	18939.4	8	2367.4	140.4	< 0.0001	0.992
	Temperature	509.3	2	254.6	15.1	0.0013	-
	pH	15696.3	2	7848.1	465.5	< 0.0001	-
	Interaction temperature/pH	2733.8	4	683.5	40.5	< 0.0001	-
	Residual error	151.7	9	16.9	-	-	-
	Total	19091.1	17	-	-	-	-
BPA	Model	16310.3	8	2038.8	515.0	< 0.0001	0.998
	Temperature	473.3	2	236.6	59.8	< 0.0001	-
	pH	14222.9	2	7111.4	1796.5	< 0.0001	-
	Interaction temperature/pH	1614.2	4	403.5	101.9	< 0.0001	-
	Residual error	35.6	9	4.0	-	-	-
	Total	16345.9	17	-	-	-	-
TCS	Model	19460.1	8	2432.5	200.2	< 0.0001	0.994
	Temperature	151.3	2	75.6	6.2	0.0201	-
	pH	17745.2	2	8872.6	730.3	< 0.0001	-
	Interaction temperature/pH	1563.6	4	390.9	32.2	< 0.0001	-
	Residual error	109.3	9	12.1	-	-	-
	Total	19569.5	17	-	-	-	-

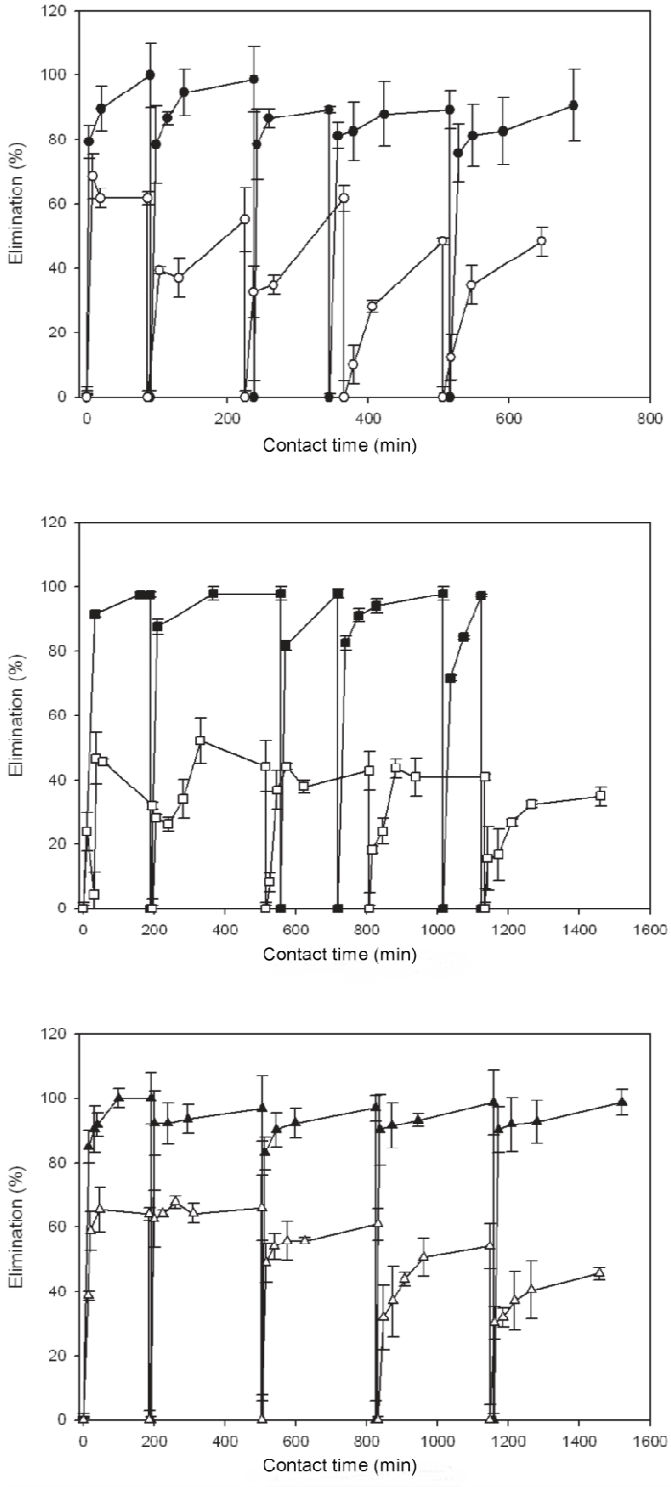

Figure 5.5. Elimination of (●) p353NP, (■) BPA and (▲) TCS from a 5 mg l^{-1} solution of each EDC using immobilized laccase (CR633-GLU) in a PBR at a temperature of 20°C and a pH of 5. The open figures represent the removal accounted for by the adsorption of the EDCs on the support (means of triplicates ± standard deviation)

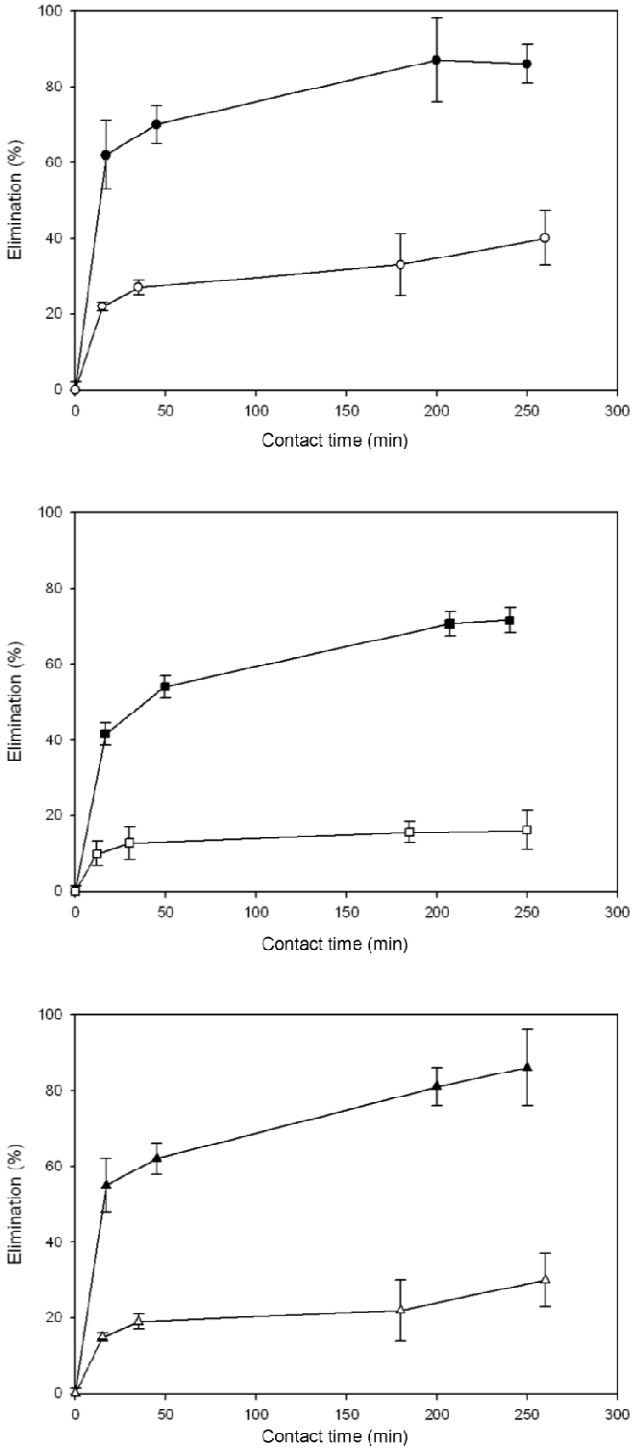

Figure 5.6. Elimination of (●) p353NP, (■) BPA and (▲) TCS from a 100 mg l^{-1} solution of each EDC using immobilized laccase (CR633-GLU) in a PBR at a temperature of 20°C and a pH of 5. The open figures represent the removal accounted for by the adsorption of the EDCs on the support (means of triplicates ± standard deviation)

5.5 Discussion

The bifunctional aldehyde activation of silanized supports is one of the most widely used techniques to immobilize laccase (Duran et al., 2002). These immobilization procedures involve the formation of covalent bounds between the aldehyde moiety of the cross-linking agent and the protein. Furthermore, the APTES amino groups confer ionic exchanger properties (Betancor et al., 2006; Mateo et al., 2000).

Most of the studies dealing with the immobilization of laccase have used sequential-type immobilization procedures and GLU as the cross-linking agent. It has been demonstrated that the enzymatic activity of a solid biocatalyst depends of the activation, immobilization and post-immobilization procedures used (Lopez-Gallego et al., 2007; Betancor et al., 2006). Our own results point out the necessity to determine the impact of the immobilization procedures (order of steps, use and nature of cross-linking agent, addition of neutral protein as a stabiliser) on the biocatalysts formed in terms of enzymatic activity and stability. To do so, we used either GLU or GLY as cross-linking agents, we tested a sequential and a simultaneous immobilization procedure and we determine the impact of the co-immobilization of BSA and laccase on the diatomaceous earth support using.

In this study, we investigated the influence of the length of the cross-linking agent's carbon chain on the robustness of the catalyst formed by testing the 2-carbon atom GLY and the 5-carbon atom GLU as alternative cross-linking agents. The highest biocatalyst activity (see Figure 5.2), using the sequential procedure, was obtained using the GLU as the cross-linking agent. These higher activities could result from the lower steric hindrance for ABTS due to the longer linker (5-carbon atoms GLU) between the support and the protein. Based on the activity balances, the utilization of GLU resulted in more laccase activity transferred from the soluble phase to the immobilized one compared to the GLY activated support (see Table 5.1). That mass transfer phenomenon during the immobilization procedure, possibly due to differences in the kinetics of bond formation between the protein and the GLY- and GLU-activated support, could explain the higher activity of the CR633-GLU catalyst. In addition, the co-immobilization of BSA with laccase resulted in decreased activity of the solid catalyst formed. This could be due to the competition between the binding sites. BSA binds to sites where normally the laccase could have been

bound. This would result in a decrease of laccase bound to the support. Finally, the addition of BSA could enhance steric hindrance effects for the substrate in the vicinity of the laccase and resulting in lower apparent laccase activity.

By using simultaneous immobilization procedures (CR633-SIM forms of immobilized laccase) the impact of the internal cross-linking of the laccase was investigated. This internal rigidification of the enzymatic structure has been used recently for the formation of cross-linked laccase aggregates and resulted in highly stabilizing laccase activity (Cabana et al., 2007c). These simultaneous immobilization procedures result in a lower activity of the biocatalyst. The reduction of the laccase activity on the support may be the result of rigidification of the enzyme due to internal cross-linkage. These internal chemical bonds could reduce the laccase activity by decreasing the mobility of the catalytic site.

The stability of immobilized and free laccase against different denaturants was also tested in an effort to anticipate their behavior in applications where such conditions may be present. Globally, the immobilization procedures used result in higher laccase stability of the biocatalysts compared to the free form of the enzyme. It appears that the internal cross-linking of the laccase by both GLU and GLY results in a significantly higher residual enzyme activity after having been incubated at 40°C and pH 3 for 24 h. Furthermore, the higher stability of that form of solid catalyst was also observed against the chemical denaturants methanol, acetone, EDTA and $CaCl_2$. The rigidification of the protein, due to the presence of internal cross-linking, would explain this significant difference between the free and immobilized catalysts tested.

The kinetic study of the different catalysts formed using GLU as cross-linking agent (CR633-GLU and CR633-SIM-GLU) showed that the CR633-GLU was a better catalysts for the oxidation of the substrate ABTS than CR633-SIM-GLU when the efficiency of the biocatalytic process constant k_{cat} K_m^{-1} is used for comparison. This reduction in catalytic efficiency could be explained by the internal network of covalent bonds that decrease the mobility of the catalytic site and increase the steric hindrance *vis-à-vis* the substrate. However, Eisenthal et al. (2007) have demonstrated that this measure of the catalytic efficiency could lead to misleading conclusions when comparing the ability of different enzymes to transform the same substrate. These authors demonstrated that enzyme having

higher catalytic efficiency (based on k_{cat} K_m^{-1} values) can catalyze lower reaction rate of substrate transformation than one having a lower k_{cat} K_m^{-1} value (Eisenthal et al., 2007).

The different immobilization procedures used resulted in various biocatalytic properties. The sequential procedure resulted in higher laccase activity and the simultaneous approach led to higher stabilization of the enzyme. These specificities resulting from the immobilization methodologies make both types potentially attractive for biotechnology applications. Our results are consistent with previous studies indicating that the immobilization of laccase from different WRF strains improved the enzymatic properties such as the reusability and thermal and chemical stability compared to the free form of laccase (Hu et al., 2007; Dodor et al., 2004; D'Annibale et al., 2000; Shuttleworth and Bollag, 1986).

The low laccase activity obtained on the support used could be due to the Celite® R-633 specific surface area. The specific surface area of this particular support is 1.3 m^2 g^{-1} as measured by the BET method. This specific surface area is lower than that of other supports used for the immobilization of laccase that exhibit higher enzyme activity. In comparison, γ-Al_2O_3 immobilized laccase from *Trametes modesta* had an activity of 13.26 U g^{-1} (Kandelbauer et al., 2004) while the support used has a specific surface area, given by the supplier, of approximately 31 m^2 g^{-1}(Lembke, 2007). However, when comparing the activity per surface of biocatalyst, the CR633-GLU biocatalyst exhibited a laccase activity of 0.6 U m^{-2} comparatively to 0.4 U m^{-2} for the one produced by Kandelbauer et al. (2004).

The adsorption of the p353NP, BPA and TCS present in solution on the inactivated laccase varied from 40 to 60% for the elimination of these EDCs from the 5 mg l^{-1} solutions and from 15 to 40% for the 100 mg l^{-1} solutions. This physical removal of the EDCs is linked to the solubility and hydrophobicity of the different compounds. The highest adsorption was seen in the case of p353NP which is the least water-soluble compound tested. The octanol-water partition coefficient (log K_{ow}) and water solubility of NP are, respectively, 4.48 (Ahel and Giger, 1993) and 4.9 mg l^{-1} (Brix et al., 2001), of BPA 3.32 (Latorre et al., 2003) and 110-320 mg l^{-1} (Shareef et al., 2006) and of TCS 4.76 (Yoon et al., 2006) and 10 mg l^{-1} (Mcavoy et al., 2002).

Even if part of the elimination observed is associated with the adsorption of these chemicals to the support, the enzymatic action is clearly at work in the removal of these EDCs. The immobilized laccase from *C. polyzona* used in our experiments eliminated the totality of each EDC present in the 5 mg l^{-1} solutions in less than 200 min of residence in the bioreactor bed (based on void volume). The elimination of the p353NP isomer was accomplished with half of the catalyst used for the elimination of BPA or TCS. The results of the elimination of the 100 mg l^{-1} solutions also underscore the fact that this insolubilized laccase has a decreasing ability to transform p353NP, TCS and BPA in that order. These results illustrate the ability of the solid catalysts used to oxidize a wide range of EDCs concentrations in aqueous solutions. That oxidative ability confers on the CR633-GLU immobilized laccase the possibility of being used to treat effluents in which the concentration of the chemicals could vary. The efficiency of the biocatalytic process constants determined from the kinetic study confirmed that the biocatalyst formed had a reducing ability to transform TCS, p353NP and BPA in that order and conform to our previously published data (Cabana et al., 2007c). The elimination of the EDCs by immobilized laccase involves different physical and biochemical steps. This process appears to involve the simultaneous adsorption of the EDC to the support and its enzymatic elimination. The possibility of reusability of the proposed catalysts is of considerable interest for the potential use of immobilized laccase in environmental applications. When testing the reusability of the CR633-GLU biocatalyst for the elimination of the EDCs, a significant stability was observed over the five consecutive treatment cycles executed for each substance.

The ANOVA analysis of the elimination of the EDCs using CR633-GLU biocatalyst point out the significant influences of temperature and pH on the enzymatic transformation of p353NP, BPA and TCS. This analysis allowed us to determine the best operational conditions for enzymatic transformation of p353NP, BPA and TCS among the range of temperatures and pH's studied. The same efficiency of elimination for all of the EDCs tested was obtained at pH 4 and 40°C as compared to pH 5 and 50°C. These results are reasonable in view of the expected combination of stability produced by a higher pH (closer to the enzyme optimum) and a higher catalytic activity resulting from a higher temperature. All other combinations of temperature/pH result in lower elimination of the target compounds.

Our results are consistent with those obtained with immobilized laccase using different immobilization procedures for the continuous elimination of environmental pollutants has been used for the elimination of dyes (Palmieri et al., 2005; Peralta-Zamora et al., 2003; Zille et al., 2003), pesticides (Jolivalt et al., 2000), PAHs (Dodor et al., 2004), phenolic compounds (Ahn et al., 2002; Hublik and Schinner, 2000; Lante et al., 2000; Shuttleworth and Bollag, 1986) and olive-mill wastewater treatment (D'Annibale et al., 1999; D'Annibale et al., 2000). The continuous elimination of EDCs by laccase immobilised on a solid support has been reported only for BPA using laccase from *Trametes* sp. immobilized on a nylon membrane in a bioreactor (Diano et al., 2007) and covalently immobilized onto alkylaminated controlled porosity glass (Iida et al., 2003; Iida et al., 2002). Our group also used insolubilized laccase, as cross-linked enzyme aggregates, in a fluidized bed reactor for the continuous elimination of p353NP, BPA and TCS (Cabana et al., 2007c).

5.6 Conclusion

These results demonstrate that the immobilization procedure has a significant impact on the biocatalysts formed in regard to the specific activity, stability under different conditions and kinetic parameters. This is the first time that a PBR filled with immobilized laccase on a solid support was used for the continuous elimination of the EDCs p353NP, BPA and TCS. The growing attention accorded to the elimination of these EDCs from environmental matrices makes immobilized laccase an attractive candidate in our bioremediation arsenal.

5.7 Acknowledgements

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CHAPITRE 6

PRÉPARATION ET CARACTÉRISATION DE LACCASE IMMOBILISÉE SOUS FORME D'AGRÉGATS D'ENZYMES RÉTICULÉS ET LEUR UTILISATION POUR L'ÉLIMINATION DE MOLÉCULES PERTURBATRICES DU SYSTÈME ENDOCRINIEN

PREPARATION AND CHARACTERIZATION OF CROSS-LINKED LACCASE AGGREGATES AND THEIR APPLICATION TO THE ELIMINATION OF ENDOCRINE DISRUPTING CHEMICALS

6.1 Avant-Propos

Ce chapitre a préalablement été publié dans la revue *Journal of Biotechnology*. La référence exacte de ce texte est :
H. Cabana, J. P. Jones, S. N. Agathos**,** Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals, *J. Biotechnol.* **2007**. 132, 23-31.

6.2 Abstract / Résumé

Laccase from the white rot fungus *Coriolopsis polyzona* was immobilized for the first time through the formation of cross-linked enzyme aggregates (CLEAs). Laccase CLEAs were produced by using 1000 g of polyethylene glycol per liter of enzyme solution as precipitant and 200 μM of glutaraldehyde as a cross-linking agent. These CLEAs had a laccase activity of 148 U g^{-1} and an activity recovery of 60.2% when using 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate. CLEAs formed by co-aggregation with bovine serum albumin (BSA) as a stabilizer showed lower laccase activity and affinity for ABTS than those without BSA. The thermoresistance was higher for CLEAs than for free laccase and also higher for CLEAs co-aggregated with BSA than for simple CLEAs when tested at a pH of 3 and a temperature of 40°C. The CLEAs co-aggregated with BSA showed higher residual activity after denaturing treatment with

protease, NaN_3, EDTA, methanol and acetone. Finally, laccase CLEAs were tested for their capacity to eliminate the known or suspected endocrine disrupting chemicals nonylphenol, bisphenol A and triclosan in a fluidized bed reactor. A 100-ml reactor with 0.5 mg of laccase CLEAs operated in repeated batch mode at a contact time of 150 min at room temperature and pH 5 could remove all three EDCs from a 5 mg l^{-1} solution.

La laccase sécrétée par la souche fongique *Coriolopsis polyzona* a été immobilisée pour la première fois via la formation d'aggrégats d'enzymes réticulés (CLEAs). Ces CLEAs ont été produits en utilisant comme précipitant 1000 g de polyéthylène glycol par litre de solution enzymatique et 200 µM de glutaraldéhyde comme agent réticulant. Les CLEAs ainsi formés présentent une activité laccase de 148 U g^{-1} et ont récupéré 60.2% de l'activité de cette enzyme en utilisant le 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) comme substrat. Les CLEAs formés par co-aggrégation avec du sérum-albumine bovin (SAB), utilisé comme stabilisant, démontrent une activité et une affinité pour l'ABTS moindre que les CLEAs formés sans SAB. Toutefois, ces CLEAs réalisés par co-agrégation présentent une plus grande stabilité à l'encontre d'une protéase, du NaN_3, de l'EDTA, du méthanol et de l'acétone. La thermorésistance des CLEAs est supérieure à celle des laccases libres tandis que cette propriété des CLEAs formées par co-agrégation avec de la SAB est supérieure à celle des CLEAs à un pH de 3 et une température de 40°C. Finalement, les CLEAs ont été testés pour leur capacité à éliminer les perturbateurs endocriniens nonylphénol, bisphénol A et triclosan dans un réacteur à lit fluidisé. Pour ce faire, un réacteur de 100 ml a été utilisé dans un mode de réacteur en cuvé répété avec 0.5 mg de CLEA, à température ambiante et à un pH de 5. Après un temps contact de 150 minutes, le système testé était en mesure d'éliminer l'ensemble de ces composés initialement présents en solution à une concentration de 5 mg l^{-1}.

6.3 Introduction

Laccase (polyphenoloxidase, EC 1.10.3.2) is a class of multicopper lignin-modifying enzymes catalyzing the oxidation of phenol-like compounds, aromatic amines and some inorganics. Over the last decades, the use of laccase has been explored for the biodegradation of xenobiotics (Durán and Esposito, 2000; Torres et al., 2003), for

bleaching in the pulp and paper industry (Call and Mucke, 1997; Crestini and Argyropoulos, 1998) and for decolorization in the textile industry (Wesenberg et al., 2003).

Compounds used in personal care products (PCPs) and substances which are either known or suspected endocrine disrupting chemicals (EDCs) can be transformed by laccases. Nonylphenol (4-nonylphenol), bisphenol A (2,2-bis(4-hydroxyphenol)propane), and triclosan (5-chloro-2(2,4-dichloro-phenoxy)phenol) are frequently detected in receiving waters downstream of intense urbanization (Boyd et al., 2004; Kolpin et al., 2002). These chemicals can 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 (Ishibashi et al., 2004; Jobling et al., 2003; Soto et al., 1991).

The promise of laccase for the elimination of PCPs and EDCs from both aqueous solutions and polluted soils has been established over the last few years (Cabana et al., 2007; Fukuda et al., 2001; Kim and Nicell, 2006; Tanaka et al., 2000; Tanaka et al., 2001). The resulting chemicals do not have any estrogenic activity (Cabana et al., 2007).

Efforts to immobilize laccase on solid supports aim at enhancing its industrial utility, including its repeated utilization (Duran et al., 2002). Immobilization generally results in laccase stabilization against thermal and chemical denaturation and in kinetic behavior modifications. A disadvantage of immobilization on a solid support is the low enzyme/support weight ratio. Cross-linked enzyme aggregates (CLEAs) have been recently proposed as an alternative to conventional immobilization on solid supports and to cross-linked enzyme crystals (Sheldon et al., 2007; Schoevaart et al., 2006; Cao, 2005; Mateo et al., 2004; Schoevaart et al., 2004). This immobilization involves the precipitation of the enzyme and the chemical cross-linking of the protein using bifunctional compounds. Cross-linking prevents the solubilisation and possible loss of the aggregates after removing the precipitating agent. Some additives have been proposed for the stabilization of CLEAs such as bovine serum albumin (BSA) and polyionic polymers (Shah et al., 2006; Wilson et al., 2004).

The first objective of this study was proving the concept of CLEAs with a crude laccase solution, including the production of laccase CLEAs, the formation of CLEAs stabilized with BSA and their biocatalytic characterization. Since none of the previous reports on

applying laccase for the removal of PCPs and EDCs used immobilized enzyme, the second objective of the present study was the utilization of stabilized CLEAs for the elimination of p353NP (a branched isomer of NP), BPA and TCS.

6.4 Experimental section

6.4.1 Chemical reagents

All chemicals were of analytical grade (or of the highest purity available). 3,5-dimethyl-3-heptanol and polyethylene glycol were from Alfa Aesar (Karlsruhe, Germany). All other chemicals were from Sigma-Aldrich (Saint-Louis, MO). All solvents were HPLC grade.

6.4.2 Organism and Cultivation Conditions

The WRF strain *C. polyzona* (MUCL 38443) was provided by the Mycothèque de l'Université Catholique de Louvain, partner of the Belgian Coordinated Collection of Microorganisms (BCCM^{TM}/MUCL). The inoculum was grown in a rotary shaker at 150 rpm and 27^{o}C in 250-ml flasks containing 100 ml of standard 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. The pH was adjusted to 6.0 with 2 M NaOH prior to autoclaving. After 10 days of cultivation the biomass was filtered and the solids were separated by centrifugation at 3000 g for 15 min at 4^{o}C. Enzymes were precipitated using 600 g l^{-1} ammonium sulfate. This preparation was dialyzed against distilled water using a 13 kDa membrane and then used as the source of laccase.

6.4.3 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 (ε_{max} = 3.6 X 10^{4} M^{-1} cm^{-1}) (Bourbonnais and Paice, 1990). The assay mixture contained 0.5 mM ABTS. The pH was adjusted to 3 using citric acid (0.5 M)/di-sodium hydrogen phosphate (0.5 M) buffer and the temperature was set at 30°C. One unit of activity was defined as the amount of enzyme forming 1 µmol of $ABTS^{\cdot+}$ per min.

The activity recovery was defined as the apparent laccase activity of the solid biocatalyst formed divided by the laccase activity theorically binded to the support. The laccase activity theorically binded to the support is defined as the difference between the initial laccase activity and those remaining in the supernatant at the end of the immobilization procedures after the washing step.

6.4.4 Aggregate activity

Ninety µL of precipitant with proper dilution was added to 10 µL of laccase solution (1U ml^{-1}). The enzyme was allowed to precipitate for 16 h at 20°C. Next, 900 µL of phosphate buffer (10 mM, pH = 7) was added. A 100 µL aliquot was then transferred into a cuvette to determine the residual laccase activity (Schoevaart et al., 2004). The precipitation step was optimized by testing polyethylene glycol (PEG) (average molecular weight of 1500), ammonium sulfate, ethanol or acetone as precipitants.

6.4.5 Preparation of CLEAs

CLEAs were prepared using 2 ml of a 1 U ml^{-1} laccase solution, the best precipitant, glutaraldehyde (GLU) as the cross-linking agent, and bovine serum albumin (BSA) as a stabilizer. Different concentrations of GLU and BSA were tested (see Results). The solution was stored at 4°C for 24 h. Subsequently, the solution was centrifuged for 15 min at 1000 g. The pellets were washed repeatedly with distilled water until no laccase activity was detected in the supernatant.

6.4.6 Determination of kinetic parameters

The Michaelis-Menten kinetic parameters K_m and k_{cat} of laccase CLEAs prepared with and without BSA were determined by measuring the laccase activity using ABTS as substrate over a 5 to 750 µM concentrations. Furthermore, the same kinetic parameters were determined using the selected EDCs as substrate using initial concentration of EDCs varying from 0.5 to 200 mg l^{-1}. The parameter values were obtained by curve fitting of the plot of reaction rate versus substrate concentrations using the Sigma Plot 7.0 software (SPSS Inc., Chicago, IL).

6.4.7 CLEAs stability against chemical denaturants

The stability against different denaturants was tested using CLEAs representing 10 μU of laccase activity. The denaturing solutions consisted of NaN_3, $ZnCl_2$, $CoCl_2$, $CaCl_2$, ethylenediamine-tetraacetic acid (EDTA), methanol or acetone in citric acid/di-sodium hydrogen phosphate buffer at pH 3. The tests lasted 30 min at 20°C. The denaturation with a serine protease from *Streptomyces griseus* was carried out at pH 7 and 30°C for 6 h.

6.4.8 Kinetics of thermal inactivation

The time course of thermal inactivation of CLEAs and of soluble laccase was investigated at 40 °C and pH 3. One hundred μU of CLEAs or free laccase were incubated in 500 μL of buffer solution containing 100 μM of phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity. Residual activity was determined every 30 min. The inactivation of free laccase and CLEAs was modeled using the 3-parameter model of Aymard and Belarbi (2000):

$$\frac{(A)_t}{(A)_0} = Ce^{-\alpha t} + (1-C)e^{-\beta t} \qquad (6.1)$$

The ratio $\frac{(A)_t}{(A)_0}$ represents the enzyme activity remaining after time *t*, C is a dimensionless constant and α and β have the dimension of first-order rate constants. The physical meaning of the parameters and their expressions as a function of individual rate constants differs according to the mechanism considered. This expression could be used irrespective of the thermal inactivation mechanism involved (Aymard and Belarbi, 2000). The values of the different parameters of this model were obtained by curve-fitting of the plot of the residual enzyme activity versus time using the Sigma Plot 7.0 software.

6.4.9 Scanning electron microscopy of CLEAs

Scanning electron micrographs (SEMs) of CLEAs were obtained on Hitachi S-4700 FESEM and S-3000N VPSEM (Tokyo, Japan) electron microscopes. The samples were previously dried at ambient temperature and then coated with platinum using an Emitech K550 (Ashford, U.K.) sputter coater.

6.4.10 Synthesis of 4(3',5'-dimethyl-3'-heptyl)-phenol (p353NP)

The p353NP isomers of NP were synthesized by Friedel-Crafts alkylation from phenol and 3,5-dimethyl-3-heptanol (nonanol). The reaction medium contained 1.88 g phenol, 4.33 g 3,5-dimethyl-3-heptanol, 35 ml BF_3-ether complex and 200 ml anhydrous petroleum ether in a 500 ml two-necked flask equipped with a reflux condenser. The reaction was run for 15 min at 50°C with stirring, and then stopped by adding 200 ml of water. After intensive stirring for a further 15 min, the aqueous phase was removed and the organic phase was washed 5 times with water in order to remove non-reacted phenol, and then dried over Na_2SO_4 (Corvini et al., 2004). After removing the petroleum ether under vacuum, the yield of p353NP was 1.72 g with a purity of 98.0% (GC).

6.4.11 Elimination of endocrine disrupting chemicals by CLEAs

A fluidized bed reactor (FBR) was used for the transformation of each EDC. The glass column (1.6 cm diameter, 20 cm height) FBR was fed with the EDC solution (air saturated) at a flow rate of 1.5 ml min^{-1}. The reactor was packed with 0.5 mg of laccase CLEAs and operated in a repeated batch mode at room temperature and at pH 5. The EDCs were eliminated from an aqueous solution of 5 mg l^{-1} whose pH was kept at 5 with citric acid (0.5 M)/di-sodium hydrogen phosphate buffer (0.5 M). Catalase (1 mg l^{-1}) from *Aspergillus niger* was added to the solution to eliminate hydrogen peroxide. The reactor was fed with 100 ml of the EDC solution until complete elimination of EDC. After the elimination was complete, the liquid phase was drained and the reactor supplemented with fresh aliquots of the EDC solution.

6.4.12 Extraction of EDCs

The p353NP isomers were extracted with a C_{18} solid phase extraction cartridge 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 about 2 ml min^{-1} under vacuum. The p353NP isomers were eluted from the cartridge using 3 ml of ethyl acetate and 6 ml of methylene chloride. BPA and TCS were extracted using methylene chloride in a 1:1 (v/v) ratio with the treated solution. The solutions were acidified to pH 2 with HCl and shaken for 20 min. The organic phase was then separated and evaporated under a gentle stream of nitrogen gas.

Each sample was dissolved in 100 µL of methylene chloride prior to quantitative chemical analysis.

6.4.13 GC-MS analysis

The gas chromatography-mass spectrometry analysis was performed on a G1800A gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with an electron ionization detector and an HP-5 MS fused-silica column (30 m X 0.25 mm i.d., 0.25 mm film thickness). Analysis was conducted in full-scan mode over a m/z range of 50 to 450 amu. Helium was used as carrier gas at a flow rate of 0.70 ml min^{-1}. The column was held at 70°C for 3 min, then temperature increased to 160°C at a rate of 20°C min^{-1}, and it finally increased to 300°C at a rate of 10°C min^{-1} and held for 7 min (Diaz et al., 2002). The injector was set at 250°C.

6.5 Results

6.5.1 Preparation of laccase CLEAs

The impact of the formation procedures on the activity of laccase CLEAs was studied in two steps: (1) determining the best precipitant and its concentration and (2) determining the impact of cross-linker concentration (Schoevaart et al., 2004). Figure 6.1 shows the maximum activity recovery after 16 h of precipitation using PEG, ammonium sulfate, methanol or acetone. The best results (recovery of about 130% of the initial laccase activity) were obtained using 100% (w/v) of PEG (1000 g l^{-1}) as precipitant. Next, in a step combining aggregation and cross-linking, we used a 1000 g l^{-1} solution of PEG and a range of GLU concentrations to produce laccase CLEAs (López-Serrano et al., 2002). GLU at 200 µM led to both higher laccase activity and higher overall recovery (results not shown).

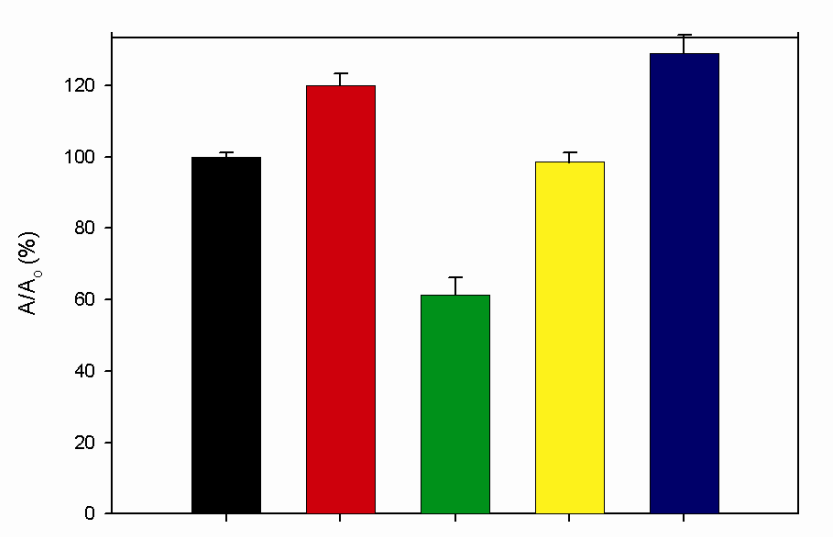

Figure 6.1. Laccase activity recovery after a 16-h precipitation at pH 7 and a temperature of 20°C using (■) buffer, (■) 55% (w/volume of enzyme solution) ammonium sulfate, (■) 79% (w/volume of enzyme solution) ethanol, (■) 67% (w/volume of enzyme solution) acetone or (■) 100% (w/volume of enzyme solution) PEG as precipitant (mean of triplicates ± standard deviation)

6.5.2 Formation of laccase CLEAs with the addition of BSA as a stabilizer

Given that the co-aggregation of lipase and penicillin acylase with BSA improved the stability, % recovery and activity of these two enzymes (Shah et al., 2006), CLEAs were made by co-aggregating laccase with different amounts of BSA per unit of activity and using the best conditions found above. The absolute activities initially obtained and the percent activities recovered for the different quantities of BSA added are shown in Table 6.1. This co-aggregation resulted in a decrease of the laccase activity of the CLEAs formed and a drop in the percentage of activity recovered.

TABLE 6.1. ACTIVITY OF LACCASE CLEAs AND ACTIVITY RECOVERY USING DIFFERENT AMOUNTS OF BSA[a]

BSA (mg U^{-1})	Activity (U g^{-1})	Activity recovery (%)
0	148 ± 12	60.2 ± 4.1
0.01	78 ± 10	32.7 ± 2.1
0.1	40 ± 3	28.7 ± 2.1
1	44 ± 4	30.3 ± 3.1

[a] The values represent means of triplicate experiments ± standard deviation

6.5.3 Kinetics of laccase CLEAs

Table 6.2 presents the different Michaelis-Menten kinetic constants of CLEAs with and without BSA for the oxidation of ABTS (for reaction rate as a function of initial substrate concentration, see Annexe 3). CLEAs had a higher maximum rate of ABTS transformation compared to free laccase. The k_{cat} value of CLEAs not supplemented with BSA was found to be 6 times higher than that of free laccase and 15 times higher than the k_{cat} of CLEAs supplemented with BSA at 0.01 mg U^{-1} (CLEA-BSA-0.01) with ABTS as substrate. When 0.1 and 1 mg U^{-1} of BSA were used, the maximum rates of ABTS transformation by the respective CLEAs decreased significantly. The Michaelis-Menten constants, K_m, of the free and insolubilized forms were about the same. The biocatalytic efficiency of the unsupplemented CLEAs, based on $k_{cat}\ K_m^{-1}$ value, was 7 times higher than that of the free laccase for the oxidation of ABTS. CLEA-BSA-0.1 and CLEA-BSA-1 were far less effective catalysts than the other forms of laccase as reflected by their $k_{cat}\ K_m^{-1}$ value which were one and two orders of magnitude lower.

TABLE 6.2. MICHAELIS-MENTEN KINETIC CONSTANTS OF LACCASE CLEAs FOR THE OXIDATION OF ABTS[a]

	K_m (μM)	k_{cat} (μmol s^{-1} mg^{-1})	k_{cat} K_m^{-1} (l mg^{-1} s^{-1})	R^2
Free	32.0 ± 2.5	10.2 ± 0.5	0.300	0.987
CLEA	28.5 ± 3.8	63.9 ± 2.2	2.20	0.9839
CLEA-BSA-0.01	29.8 ± 6.4	4.3 ± 0.9	0.10	0.9975
CLEA-BSA-0.1	28.5 ± 3.4	0.3 ± 0.01	0.01	0.9831
CLEA-BSA-1	31.9 ± 4.2	0.02 ± 0.02	0.006	0.9806

[a] The values represent means of triplicate experiments ± standard deviation

Table 6.3 presents the Michaelis-Menten kinetic parameters for the elimination of the EDCs p353NP, BPA and TCS using laccase CLEAs unsupplemented with BSA. The kinetic constants obtained are of similar value for all three target substrates. Based on the affinity and kinetic constants of the CLEAs for the various EDC, these insolubilized biocatalysts have a greater capacity of elimination for TCS, followed by p353NP and by BPA.

TABLE 6.3. MICHAELIS-MENTEN KINETIC CONSTANTS FOR THE ELIMINATION OF P353NP, BPA AND TCS WITH LACCASE CLEAs UNSUPPLEMENTED WITH BSA AT A pH OF 5 AND A TEMPERATURE OF 20°C[a]

Substrate	K_m (mM)	k_{cat} (μmol s^{-1} mg^{-1})	k_{cat} K_m^{-1} (l mg^{-1} s^{-1})	R^2
p353NP	0.45 ± 0.002	0.8 ± 0.01	0.02	0.943
BPA	0.23 ± 0.017	0.16 ± 0.01	0.0069	0.944
TCS	0.12 ± 0.01	0.24 ± 0.03	0.020	0.961

[a] The values represent means of triplicate experiments ± standard deviation

6.5.4 Stability against chemical denaturants

Figure 6.2 presents the residual activity of the free and immobilized laccases incubated with chemical denaturants and a protease. Unsupplemented CLEAs had higher stability than free laccase in the presence of solutions of 10 μM of EDTA, 30 and 150 μM of NaN_3 and 25% v/v methanol and acetone. The BSA-supplemented CLEAs had improved stability only against EDTA. Finally, laccase in the form of CLEAs was not more stable against a 10 μM solution of $ZnCl_2$, $CoCl_2$ or $CaCl_2$ than free laccase. The laccase CLEAs

displayed a residual activity twice as high as the free laccase against the proteolytic action of a 2 g l^{-1} solution of the serine protease from *S. griseus*. The addition of BSA improved the stability of the immobilized laccase against the proteolytic action. For example, the stability of the CLEA-BSA-1 was 50% higher than that of the CLEAs without BSA.

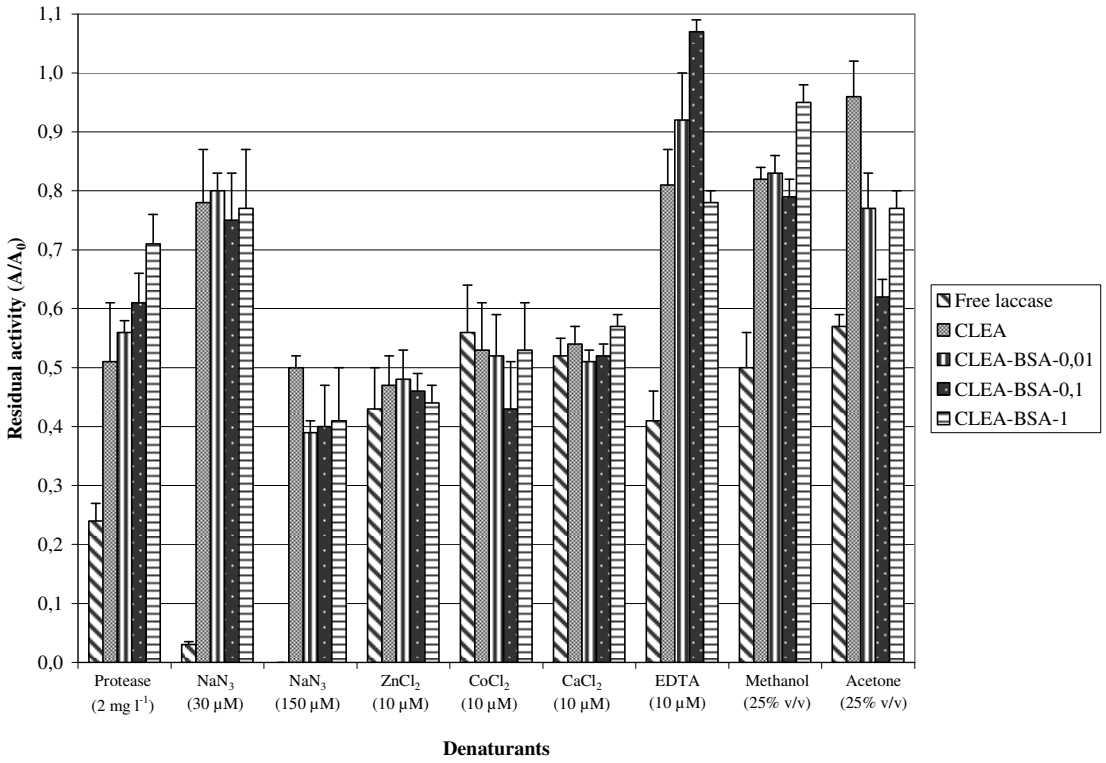

Figure 6.2. Residual activity (A/A_0) of laccase CLEAs after incubation with denaturants (mean of triplicates ± standard deviation)

In a parallel experiment, BSA-free CLEAs incubated with the different EDCs before their transformation had a stabilizing effect on the enzyme. At the end of the incubation period, the residual activity of the CLEAs in the absence of EDC was 50% of the initial activity versus 85% in their presence for all three substances tested (results not shown).

6.5.5 Kinetics of thermal inactivation

Figure 6.3 presents the temporal variation of activity for the different forms of laccase over a 2-day period. The activity of the immobilized forms of laccase was better conserved than the free form at pH 3 and 40°C. After a 24-h incubation period, the free laccase had no

residual activity while CLEA, CLEA-BSA-0.01 and CLEA-BSA-0.1 still showed 20% of their initial laccase activity *versus* 40% for the CLEA-BSA-1.

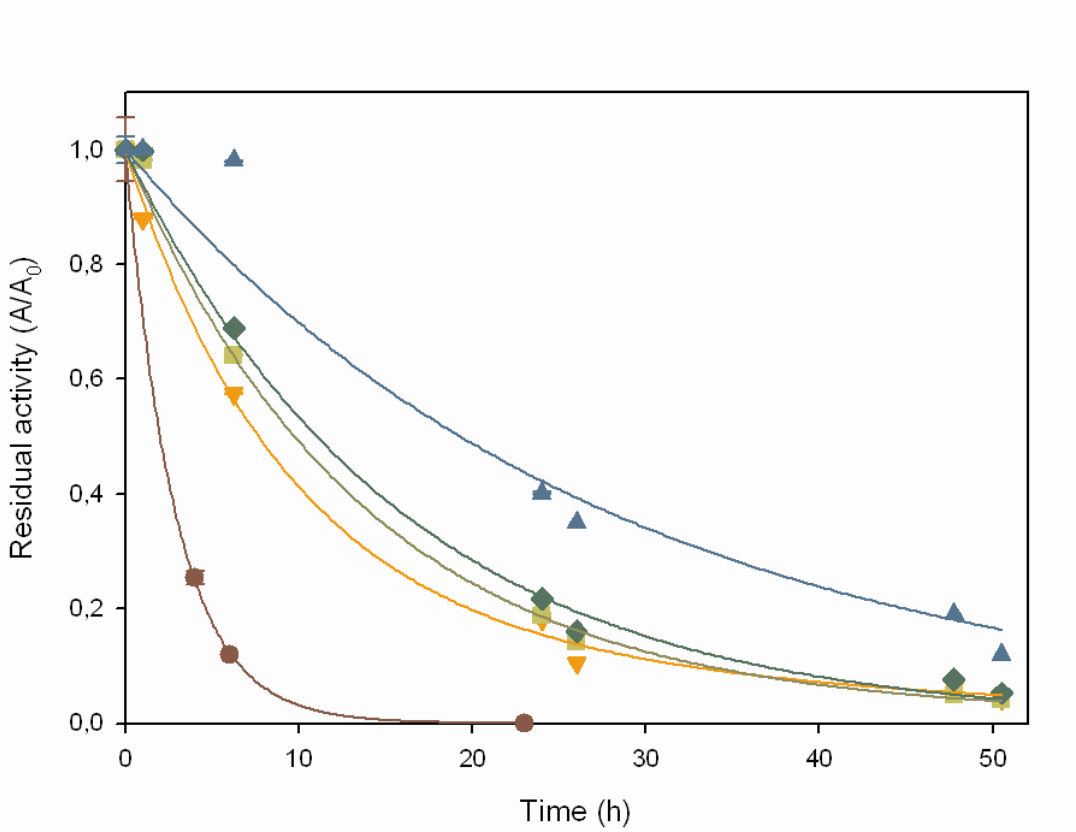

Figure 6.3. Thermal inactivation of free laccase (●, —), CLEA (▼, —), CLEA-BSA-0.01 (■, —), CLEA-BSA-0.1 (♦, —) and CLEA-BSA-1 (▲, —) subjected to a temperature of 40°C and a pH of 3. The solid lines represent the inactivation kinetics modelled with the biexponential model proposed by Aymard and Belarbi (2000)

Table 6.4 presents the kinetic inactivation parameters for free and immobilized laccase. Using these parameters, the half-life of the free laccase, CLEA, CLEA-BSA-0.01, CLEA-BSA-0.1 and CLEA-BSA-1 was found to increase in this order, from 2.0 to 19.3 h.

TABLE 6.4. KINETIC INACTIVATION PARAMETERS FOR LACCASE $CLEA_S$ AND $CLEA_S$ FORMED WITH THE ADDITION OF BSA, SUBJECTED TO A TEMPERATURE OF 40 °C AND A PH OF 3[a]

	C	α (h^{-1})	β (h^{-1})	R^2	half-life (h)
Free laccase	0.53	0.35	0.35	0.989	2.0
CLEA	0.80	0.11	0.03	0.977	7.7
CLEA-BSA-0.01	0.99	0.07	0	0.997	9.7
CLEA-BSA-0.1	0.49	0.06	0.06	0.994	11.0
CLEA-BSA-1	0.51	0.04	0.04	0.960	19.3

[a] The values represent means of triplicate experiments

6.5.6 SEMs of CLEAs

Figure 6.4 shows the SEMs of CLEAs of laccase prepared without BSA and with 1 mg of BSA per unit of laccase activity. The BSA-free CLEAs were smaller than those made with the protein feeder, their length being between 1 and 5 µm although some bigger ones up to 50 µm were also observed (Figures 6.4A and 6.4B). The CLEAs of laccase made with 1 mg U^{-1} of BSA were bigger (100 - 200 µm) (Figures 6.4C and 6.4D). The SEMs of both types of laccase CLEAs showed a uniform surface structure.

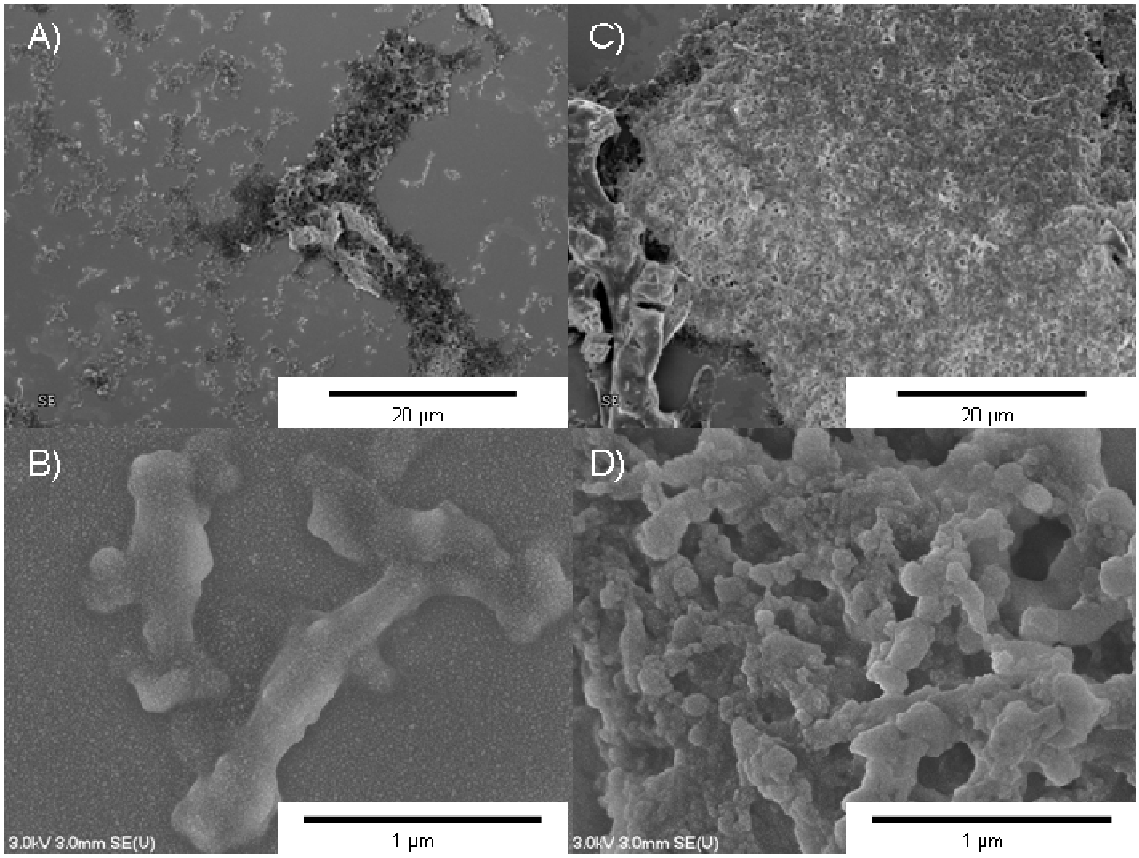

Figure 6.4. SEMs of laccase CLEAs and CLEAs formed with 1 mg BSA U^{-1}A) CLEA (working distance = 11.4 mm, acceleration voltage = 5 kV) B) CLEA (working distance = 3.0 mm, acceleration voltage = 3 kV) C) CLEA-BSA 1 mg U^{-1} (working distance = 11.4mm, acceleration voltage = 5 kV) and D) CLEA-BSA 1 mg U^{-1} (working distance = 3.0 mm, acceleration voltage = 3 kV)

6.5.7 Elimination of EDCs

The elimination of EDCs from a 5 mg l^{-1} solution using 0.5 mg of laccase CLEAs at pH 5 and room temperature in a FBR is shown in Figure 6.5. A contact time of 50 min in the FBR allowed a 90% elimination of p353NP and TCS and a 30% removal of the BPA. At a 150-min residence time, more than 95% of the p353NP, BPA and TCS were transformed.

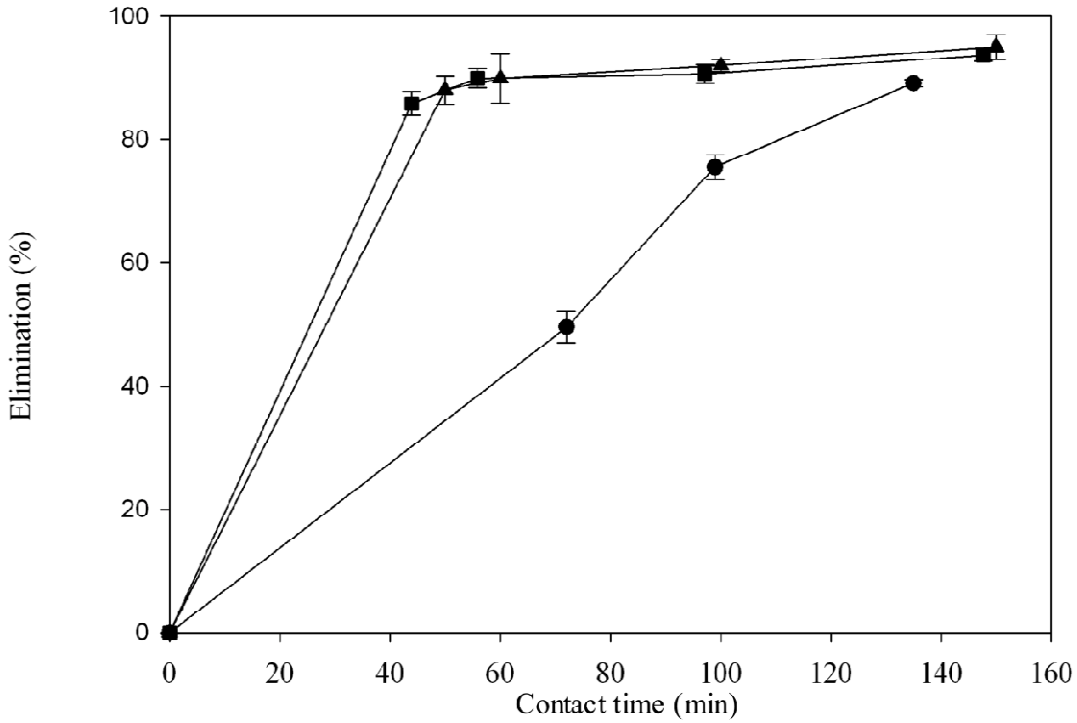

Figure 6.5. Elimination of a 5 mg l^{-1} solution of (▲) p353NP, (●) BPA and (■) TCS at a pH of 5 and at room temperature using 0.5 mg of CLEAs of laccase in a fluidized bed reactor as a function contact time

6.6 Discussion

The results from the precipitation tests indicated that quenching the laccase solution with high precipitant concentrations led to a high recovery of enzyme activity. In the case of other enzymes this fast precipitation procedure has shown higher activity recovery than the slow addition of precipitants (Schoevaart et al., 2004). The higher remaining activity of the laccase precipitated with PEG compared to free laccase could be explained by the hypothesis that this polymer tends to fold on itself thereby allowing to bind more water molecules in its three-dimensional structure (Donato et al., 1996). Denaturation upon precipitation with organic solvents results from the hydrophobic interactions between the solvent and the nonpolar groups of the laccase which shifts the equilibrium towards the denatured state (Makarov et al., 1995). For thermodynamic reasons ethanol has a higher preferential solvation for enzymes than acetone at the same activity in solution, hence

ethanol leads to greater denaturation of laccase compared to acetone (Kovrigin and Potekhin, 1997).

Determining the impact of the cross-linking agent's concentration is necessary due to the potential inactivation of enzymes by this type of chemical. The use of 200 µM GLU as the cross-linking agent gave, among the concentrations tested, the best laccase CLEAs activity and recovery compared to higher concentrations. The reduction of the CLEAs activity at GLU concentrations higher than 200 µM could be explained by a rigidification of the enzyme's three-dimensional structure that hinders the conformational change needed for the oxidation of substrates at the active site. This trend was also observed recently in the production of laccase crossed-linked enzyme crystals (Roy and Abraham, 2006). In a study of lipase and alcohol dehydrogenase CLEAs, Schoevaart et al. (2004) showed that concentrations of GLU higher than 8 µM inactivated the alcohol dehydrogenase CLEAs but concentrations between 5 and 100 µM did not significantly change the activity of lipase CLEAs.

Recently, the formation of CLEAs by co-aggregation of lipase or penicillin acylase with BSA (Shah et al., 2006) and glutaryl acylase or penicillin acylase with polymers (Lopez-Gallego et al., 2005; Wilson et al., 2004) was proposed. These protocols improve the recovery, stability, activity, enantio-selectivity and specificity of the enzyme. In our case, the addition of BSA at levels from 0.01 mg to 1 mg per unit of laccase activity led to lower CLEAs activity, possibly due to inadequate exposure of the laccases catalytic center to ABTS because of shielding by the BSA. In addition, this activity drop could reflect mass transfer limitations from the bulk substrate solution to the laccase active site. This hypothesis is consistent with the visual evidence from the SEMs of laccase CLEAs without BSA and with 1 mg BSA U^{-1}: the surface/volume ratio of CLEAs decreased when increasing the proportion of BSA. The addition of BSA may disturb the internal structure of the aggregates resulting in narrower channels that limit the diffusion of the substrate ABTS and the co-substrate oxygen in the CLEAs. Furthermore, these channels could trapped the oxidized ABTS and reduced the ability (by limiting the mass transfer's driving force) of the substrate to migrate up to the catalytic site of the laccase present in the amorphous structure of the CLEAs. Those limitations could result in a lower apparent laccase activity.

The kinetic study showed that the different forms of laccase studied have almost the same affinity, based on K_m values, for the substrate ABTS. CLEA is a better catalyst for the oxidation of ABTS than free laccase when the rate of transformation, k_{cat}, and the efficiency of the process, $k_{cat}\ K_m^{-1}$, are used for comparison. The CLEAs supplemented with BSA had a ten-fold lower efficiency than free laccase. This drop in catalytic activity could also be explained by mass transfer limitations as discussed above.

Testing the stability of CLEAs and free laccase against different denaturants, it seems that CLEAs formation diminished the structural alteration of the catalytic site of laccase induced by azide binding (Battistuzzi et al., 2003). The aggregation of laccase as CLEAs improved enzyme stability against the chelator EDTA. This stabilization could be due to hindering mass transfer of EDTA into the CLEAs' amorphous structure. The stabilities against the chaotropic salts $ZnCl_2$, $CoCl_2$ and $CaCl_2$ were the same for free and insolubilized laccase, likely due to the small dimension of the ions that allowed them to easily migrate into the structure of the CLEAs and inactivate the laccase to an equal extent. Nonetheless, the low concentrations used could explain the salts' low impact. Laccase CLEAs showed higher stability to serine proteases from *S. griseus* than free laccase, possibly because proteolytic enzymes could not efficiently diffuse into the aggregates to hydrolyse the laccase. Furthermore, the addition of BSA as a protective agent significantly enhanced that stability. This could be due to the higher affinity or access of the protease to BSA compared to the laccase present in CLEAs. Finally, the stability against the organic solvents acetone and methanol was higher for the laccase CLEAs than for free laccase. As seen before, the formation of CLEAs stabilizes the enzyme in the presence of organic cosolvents (Tyagi et al., 1999; Wilson et al., 2004) due to the rigidity of the biocatalyst formed.

Laccase CLEAs showed higher stability against thermal denaturation compared to free laccase because the cross-linking (presence of multiple links on the protein surface) prevented the unfolding of laccase under thermal stress conditions (Fernandez-Lafuente et al., 1995). These results differ from those obtained by López-Gallego et al. (2005) who observed the same residual activity for free glutaryl-7-aminocephalosporanic acid acylase compared to the enzyme immobilized as CLEAs. The addition of BSA as a protective agent led to a higher half-life of the CLEAs formed. These findings are in agreement with those of Shah et al. (2006) who obtained more thermally stable CLEAs of penicillin

acylase when they added BSA. This higher stability of laccase CLEAs with BSA could be explained by the microenvironment induced by the presence of large amounts of BSA in the immediate surroundings of laccase that enhances the cross-linking and decreases the entropy of the enzyme's denatured state. It is likely that a favourable interaction is established between laccase and BSA and that the cross-linking may be reinforcing it.

The structures of laccase CLEAs formed without BSA and with addition of 1 mg of BSA per unit laccase activity were similar. The laccase CLEAs formed with the addition of BSA show a structure analogous to CLEAs of lipase from *Pseudomonas cepacia* and from *Candida rugosa* (Schoevaart et al., 2004; Shah et al., 2006). It appears that the aggregates formed with the addition of BSA consisted of the laccase particles without BSA assembled in a macro structure 100-200 µm long compared to BSA-free CLEAs which were only 1-5 µm. This size difference is consistent with a variation in the CLEAs' surface/volume ratio resulting in mass transfer limitations which are greater in CLEAs formed with BSA than for those without.

In this study we investigated the elimination of three known or suspected EDC: NP, BPA and TCS. Due to the complex mixture of isomers present in technical NP (Wheeler et al., 1997) which gives rise to a complex metabolic pattern, we studied the elimination of p353NP, a branched isomer of NP. In a FBR we could reach more than 90% elimination of p353NP and TCS with a 40-min contact time while 130 min was required for 90% removal of BPA. These findings are in agreement with the kinetic measurements obtained with batch eliminations of EDC using laccase CLEAs. The kinetic constants obtained confirmed that the insolubilized biocatalyst formed had decreasing efficiency towards TCS, p353NP and BPA in that order. We have previously demonstrated that free laccases from *C. polyzona* had decreasing elimination efficiency for NP, BPA and TCS in that order (Cabana et al., 2007). This difference could be due to a modification of the catalytic properties of the enzyme upon insolubilization (Shah et al., 2006).

6.7 Conclusion

For the first time, CLEAs of laccase from the WRF *C. polyzona* have been described in and used for the elimination of EDCs. The immobilization process was investigated with

respect to the precipitation step and the concentration of the cross-linking agent GLU. Furthermore, laccase CLEAs with the addition of BSA as a stabilizer were formed and their performance and stability examined. Finally, the laccase CLEAs were used for the successful elimination of the EDC p353NP, BPA and TCS. Overall, our results show that laccase immobilization via the formation of CLEAs is a promising tool to increase the applicability and reusability of laccase in biotechnology.

6.8 Acknowledgement

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CHAPITRE 7

UTILISATION D'AGRÉGATS DE LACCASE RÉTICULÉS DANS UN RÉACTEUR À PERFUSION POUR L'ÉLIMINATION EN CONTINUE DE SUBSTANCES PERTURBATRICES DU SYSTÈME ENDOCRINIEN

UTILIZATION OF CROSS-LINKED LACCASE AGGREGATES IN A PERFUSION BASKET REACTOR FOR THE CONTINUOUS ELIMINATION OF ENDOCRINE DISRUPTING CHEMICALS

7.1 Abstract / Résumé

A perfusion basket reactor (BR) was developed for the first time for the continuous utilization of insolubilized laccase as cross-linked enzyme aggregates (CLEAs). The BR consisted of an unbaffled basket made of a metallic filtration module filled with CLEAs and continuously agitated by a 3-blade marine impeller. The agitation conditions influenced both the apparent laccase activity in the reactor and the stability of the biocatalyst. The optimal laccase activity was obtained for a rotational speed of 12.5 rps, while the highest stability was obtained at speeds of 1.7 rps or lower. The activity and stability of the biocatalyst drastically varied after the appearance of vortices in the reaction medium. This reactor was used for the continuous elimination of the endocrine disrupting chemicals (EDCs) nonylphenol (NP), bisphenol A (BPA) and triclosan (TCS). Optimization of EDC elimination by laccase CLEAs as a function of temperature and pH was achieved by response surface methodology using a central composite factorial design. The best conditions were a pH of 4.8, 4.7 and 4.9 and a temperature of 40.3, 48.0 and 41.2 °C for the elimination of p353NP (a branched isomer of NP), BPA and TCS respectively. Finally, BR was used for the continuous elimination of these EDCs from a 5 mg l^{-1} aqueous solution using 1 mg of CLEAs at a pH of 5 and room temperature. Our results showed that at least 85% of these EDCs could be eliminated with a hydraulic retention time of 325 min. The performances of the BR were quite stable over a 7-day period of continuous treatment. Furthermore, this system could eliminate these EDCs from a 100 mg l^{-1} solution. Finally, a suitable kinetic model was developped to predict the elimination of

these xenobiotics in the BR using laccase CLEAs. This model combines the Michaelis-Menten kinetics of the CLEAs and the continuous stirred tank reactor behaviour of the BR.

Pour la première fois, un réacteur à perfusion (BR) a été développé pour l'utilisation en continu de laccases insolubilisées par réticulation d'agrégats d'enzymes (CLEAs). Ce BR consiste en un panier sans chicane fabriqué à l'aide de média filtrant métallique contenant et retenant les CLEAs. La solution aqueuse contenue dans ce panier est agitée à l'aide d'une hélice marine à trois palles. Ce réacteur est alimenté dans le panier et l'effluent traité est évacué par gravité à travers du média filtrant. Les conditions d'agitation du BR ont un impact sur l'activité laccase et la stabilité des CLEAs présents dans ce réacteur. L'activité maximale a été obtenue pour une agitation de 12.5 rps, tandis que la stabilité maximale a été obtenue pour une agitation variant entre 0 et 1.7 rps. L'apparition de tourbillons dans le milieu réactionnel a un impact significatif sur l'activité et la stabilité des CLEAs. Ce réacteur a été utilisé pour l'élimination en continu des perturbateurs endocriniens nonylphénol (NP), bisphénol A (BPA) et triclosan (TCS). L'optimisation de l'élimination de ces composés en fonction du pH et de la température a été effectuée à l'aide d'une approche statistique permettant d'obtenir une surface de réponse. Pour ce faire, un design factoriel construit en étoile. Les conditions optimales sont un pH de 4.8, 4.7 et 4.9 et une température de 40.3, 48.0 et 41.2°C pour l'élimination du p353NP (un isomère du NP), du BPA et du TCS respectivement. Finalement, ce BR a été utilisé pour l'élimination en continu de solution contenant 5 mg l^{-1} de ces composés en utilisant 1 mg de CLEAs et ce, à un pH de 5 et à température ambiante. Nos résultats démontrent qu'une élimination minimale de 85% de ces composés a été obtenue pour un temps de rétention hydraulique de 325 minutes. Les performances d'élimination du BR se sont avérées relativement stables sur une période de 7 jours. De plus, ce système a été utilisé avec succès pour l'élimination de solutions contenant 100 mg l^{-1} de ces composés. Finalement, un modèle cinétique a été développé de façon à prédire l'efficacité d'élimination de ces polluants à l'aide du système. Ce modèle combine la cinétique de type Michaelis-Menten des laccases immobilisées sous forme de CLEAs et le comportement continu du réacteur (de type CSTR).

7.2 Introduction

Laccase (polyphenoloxidase, EC 1.10.3.2) is a class of multicopper lignin-modifying enzymes catalyzing the oxidation of phenol-like compounds, aromatic amines and some inorganics. Over the last decades, the use of laccase has been explored for the bioremediation of xenobiotics (Durán and Esposito, 2000; Torres et al., 2003), for bleaching in the pulp and paper industry (Call and Mucke, 1997; Crestini and Argyropoulos, 1998) and for decolourization in the textile industry (Wesenberg et al., 2003).

Phenolic compounds used in personal care products and substances which are either known or suspected endocrine disrupting chemicals (EDCs) can be transformed by laccases (Cabana et al., 2007b; Kim and Nicell, 2006a; Tanaka et al., 2000). Nonylphenol (4-nonylphenol) (NP), bisphenol A (2,2-bis(4-hydroxyphenol)propane) (BPA), and triclosan (5-chloro-2(2,4-dichloro-phenoxy)phenol) (TCS) are known or suspected EDCs frequently detected in receiving waters downstream of intense urbanization (Boyd et al., 2004; Kolpin et al., 2002). These chemicals have been reported to interact with estrogen, androgen and thyroid hormone receptors (Veldhoen et al., 2006; Ishibashi et al., 2004; Jobling et al., 2003; Soto et al., 1991).

The application of lignin modifying enzymes (LMEs) including laccase in free form (in solution) for the treatment of EDCs is beset by significant operational barriers: reusability, cost and denaturation of the enzyme (Cabana et al., 2007b). Laccase immobilization has been proposed as a strategy to overcome these limitations (Duran et al., 2002). Generally, the immobilization of such LMEs on solid supports significantly enhances 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 (Cao et al., 2003). The general approach involves the precipitation of soluble enzyme with an appropriate aggregation additive and its subsequent cross-linking with a bifunctional agent leading to the formation of so-called cross-linked enzyme

aggregates (CLEAs) (Cao et al., 2003). The resulting solid biocatalysts exhibit high activity/volume ratio (Cao et al., 2003).

The formation of CLEAs of laccase from the white rot fungi *Coriolopsis polyzona* has been recently described and used for the continuous elimination of NP, BPA and TCS in a fluidized bed reactor (FBR) (Cabana et al., 2007c). However, the fluidized laccase particles were difficult to maintain in the bioreactor, resulting in a loss of enzyme activity. In order to overcome this operational difficulty and, by extension, increase the applicability and reusability of laccase CLEAs in bioprocesses we developed an innovative perfusion type basket reactor (BR) that allows the continuous use of CLEAs (see Figure 1). Our newly-developed BR consists of a metallic filtration membrane-like module that allows the retention of the solid biocatalyst in the reactor. The filtration module separates the laccase CLEA containing environment from the EDC-free effluent. This separation allows retention of the CLEAs in the reaction media. The content of the BR is continuously agitated with an impeller that maximizes the homogenization of the CLEAs in the reaction medium and ensures the presence of sufficient oxygen (the laccase co-substrate) to efficiently remove the xenobiotics by the enzymatic action. Finally, the treated effluent flows through the porous module by gravity.

Specifically, this paper presents the concept of continuous utilization of laccase CLEAs in a BR under realistic conditions, characterizes the biocatalytic activity as a function of the agitation conditions, quantifies and models the elimination of p353NP (a branched isomer of NP), BPA and TCS in such a system. To our knowledge, this is the first time that a BR is used to segregate an insolubilized biocatalyst in substrate-containing solution and is applied for continuous elimination of xenobiotics from aqueous solutions.

7.3 Experimental section

7.3.1 Chemical reagents

All chemicals were of analytical grade (or of the highest purity available). 3,5-dimethyl-3-heptanol and polyethylene glycol (mean molecular weight of 1 500 amu) were from Alfa

Aesar (Karlsruhe, Germany). All other chemicals were from Sigma-Aldrich (MO, USA). All solvents were HPLC grade.

7.3.2 Organism and Cultivation Conditions

The WRF strain *C. polyzona* (MUCL 38443) was provided by the Mycothèque de l'Université Catholique de Louvain, partner of the Belgian Coordinated Collection of Microorganisms (BCCM^{TM}/MUCL). The inoculum was grown in a rotary shaker at 150 rpm and 27^{o}C in 250-ml flasks containing 100 ml of standard 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. The medium was adjusted to pH 6.0 with 2 M NaOH prior to autoclaving. After 10 days of cultivation the fungal biomass was filtered and the solids were separated by centrifugation at 3000 g for 15 min at 4^{o}C. Enzymes were precipitated using 600 g l^{-1} ammonium sulfate. The precipitated enzyme preparation was dissolved in distilled water and dialyzed against distilled water using a 13 kDa membrane. This solution was used as the source of laccase.

7.3.3 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 (ε_{max} = 3.6 X 10^{4} M^{-1} cm^{-1}) (Bourbonnais and Paice, 1990). 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.

7.3.4 Preparation of CLEAs

CLEAs were prepared using 2 ml of a 1 U ml^{-1} laccase solution to which were added 2 g of polyethylene glycol as precipitant and 0.16 µl of a 25% (w:w) solution of glutaraldehyde as the cross-linking agent. The solution was stored at 4°C for 24 h. Subsequently, the solution was centrifuged for 15 min at 1000 g. The pellets were washed repeatedly with

distilled water until no laccase activity was detected in the supernatant. The CLEAs used had a laccase activity of 148 U g^{-1} (Cabana et al., 2007c).

7.3.5 Perfusion basket reactor

Figure 7.1 shows the experimental set-up used as a perfusion basket reactor (BR). This BR was made of 316L stainless steel porous medium having a particle removal efficiency of 99% for 2.2 µm particles (Mott Corporation, CT, USA). The diameter of the unbaffled reactor and the height of the liquid were of 0.075 m each. The solution inside of the BR was agitated by a 0.025 m diameter 3 blade marine propeller (pitch of 1.5:1) situated at 0.025 m from the reactor bottom. The working volume of the BR was of 33.1 X 10^{-5} m^3. The BR was continuously fed by a programmable HPLC pump (Model 590, Waters Corporation, MA, USA) over a range of flowrates of 0-20 ml min^{-1}. This reactor overflow was transferred into a glass beaker by gravity. The agitation speed was varied from 0-25 rps. The BR was filled with 1 mg CLEAs.

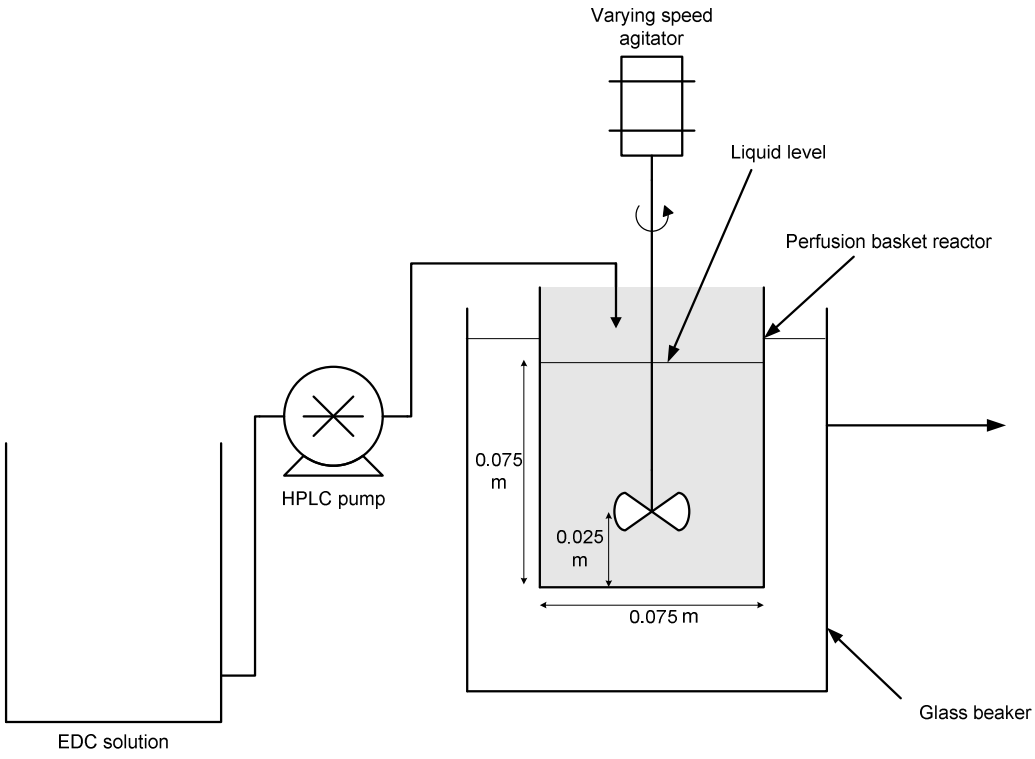

Figure 7.1. Experimental set-up used as perfusion basket reactor

7.3.6 Mixing conditions

The power dissipated in the liquid by the marine propeller was estimated by determining the power number (N_p) of the system as proposed by McCabe et al. (2001). The N_p is defined by equations 1 and 2. In an unbaffled tank, at Reynold's number (Re) greater than about 10 000, the flow is fully turbulent and the N_p is dependant of the Froude number (Fr) and Re as expressed by equation 7.1.

$$N_p = K_t Fr^{\frac{a-\log \mathrm{Re}}{b}} \qquad (7.1)$$

Where K_t, a and b are constants depending of the type of impeller and the geometry of the system (K_t = 0.87, a = 1.7 and b = 18 for a 3 blade marine impeller with a 1.5 pitch) (McCabe et al., 1993).

The N_p is related to the power dissipated into the mixed solution according to equation 7.2.

$$N_p = \frac{P}{n^3 D^5 \rho} \qquad (7.2)$$

Where P is the power (W) dissipated in the solution, n is the rotational speed (rps) of the agitator, D is the diameter of the agitator (m) and ρ the density (kg m^{-3}) of the reaction media.

The intensity of the mixing was also correlated to the average shear rate by using the G factor which was derived from the fact that, at a particular point in the bulk solution, the product of shear stress and shear rate represents power per unit volume and is equal to the product of viscosity by the square of shear rate (Nagata, 1975). In this way, the square root of the power input per unit volume divided by the viscosity of the solution is directly linked with the shear rate at that point. Using the effective power input to the system, as determined with the N_p value, equation 7.3 was used to determine the average shear rate of the system.

$$G = \left(P \mu^{-1} V^{-1}\right)^{1/2} \qquad (7.3)$$

Where G represents the rate of shear (s^{-1}), P the power input (kg m^2 s^{-3}), μ the viscosity of the solution (kg s^{-1} m^{-1}) and V the volume of the reactor (m^3).

7.3.7 Impact of agitation speed on CLEAs activity and stability

The impact of the agitation speed on the CLEA activity was assayed by agitating 331 ml of a solution containing 0.5 mM of ABTS at a pH of 3 and at room temperature in a beaker having the same dimensions and dispositions as the BR and containing 1 mg of CLEAs. The rotational speed of the propeller was varied over the range of 0.83 to 25 rps. The absorbance of the reaction medium was monitored spectrophotometrically every minute at 420 nm over a 10 min period.

This system, without ABTS in solution, was also used to determine the residual activity of the CLEAs as a function of agitation characteristics. The biocatalytic stability was monitored over a 24 h period by sampling 100 µl of the solution and determining the residual laccase activity in solution.

7.3.8 Synthesis of 4(3',5'-dimethyl-3'-heptyl)-phenol (p353NP)

The p353NP isomers of NP were synthesized by Friedel-Crafts alkylation from phenol and 3,5-dimethyl-3-heptanol. The reaction medium contained 1.88 g phenol, 4.33 g 3,5-dimethyl-3-heptanol, 35 ml BF_3-ether complex and 200 ml anhydrous petroleum ether in a 500 ml two-necked flask equipped with a reflux condenser. The reaction was run for 15 min at 50°C with stirring, and then stopped by adding 200 ml of water. After intensive stirring for a further 15 min, the aqueous phase was removed and the organic phase was washed 5 times with water in order to remove non-reacted phenol, and then dried over Na_2SO_4 (Corvini et al., 2004). After removing the petroleum ether under vacuum, the yield of p353NP was 1.72 g with a purity of 98.0% (GC).

7.3.9 Impact of temperature and pH on the elimination of EDCs by laccase CLEAs

A two-factor central composite factorial design comprising 4-axial points and 4-factor points was designed to find the optimal conditions of pH and temperature for the transformation of the EDCs. For this, a 1-h batch treatment of the different EDCs was performed under the conditions of pH and temperature presented in Table 7.1 using 0.1 mg of laccase CLEAs in a 10-ml reaction volume. The reaction mixture consisted of 5 mg l^{-1}

of each of p353NP (0.023 mM), BPA (0.022 mM) or TCS (0.018 mM), 1 mg l^{-1} catalase from *Aspergillus niger,* citric acid (0.5 M)/di-sodium hydrogen phosphate (0.5 M) buffer and 1% v/v methanol. The statistical analysis was performed using the Design Expert 6.0.11 software (Stat-Ease inc., MN, USA).

TABLE 7.1. VARIABLES UNDER INVESTIGATION IN THE CENTRAL COMPOSITE FACTORIAL EXPERIMENTAL DESIGN

Independent variables	Coded symbols	Levels				
		-1.41	-1	0	1	1.41
pH	X_1	2.4	3.0	4.5	6.0	6.6
Temperature (°C)	X_2	13.8	20.0	35.0	50.0	56.2

7.3.10 Continuous elimination of endocrine disrupting chemicals by CLEAs

The BR was used for the continuous transformation of each EDC at 5 or 100 mg l^{-1} in an aqueous solution whose pH was maintained at 5 using a citric acid/di-sodium hydrogen phosphate buffer. Catalase (1 mg l^{-1}) from *A. niger* was added to the solution to eliminate hydrogen peroxide and contained 1% v/v methanol to ensure a good solubility of the EDCs. The BR was fed with 5 mg l^{-1} and 100 mg l^{-1} EDC solutions (air saturated) at a flow rate depending on the hydraulic residence time chosen, packed with 1 mg laccase CLEAs and operated at room temperature. The agitation speed was set at 1.7 rps in order to preserve both the stability and the activity of the enzyme (see results). Prior to continuous elimination, the system was saturated with EDC by operating for 16 h without laccase CLEAs. After this start-up period, the EDC concentrations were the same at the inlet and outlet of the reactor. By sampling 250 µl of solution in the BR the residual laccase activity was spectrophotometrically determined during the treatment process.
The elimination of these EDCs in the BR was performed under steady-state conditions. To do this, at least 4 BR volumes flowed through the reactor before sampling.

7.3.11 Modelling EDC elimination in the BR

A continuous stirred tank reactor (CSTR) model of the EDC elimination process in the BR was developed. The following assumptions were made: the reaction occurred in a continuous, ideally and intentaneously mixed reactor, it followed Michaelis-Menten type

reaction kinetics and it was not oxygen limited. The governing CSTR model equations may be described as followed. Equation 7.4 represents the mass balance of the EDCs under steady-state conditions:

$$F_{in}C_{EDC,in} - F_{out}C_{EDC} = -Vr_{EDC} \quad (7.4)$$

where F_{in}, F_{out} represent the volumetric flowrates, C_{EDC} the molar concentrations of each EDC, V the volume of the reactor and r_{EDC} the reaction rate. This reaction rate was represented by the Michaelis-Menten kinetic expression shown in equation 7.5:

$$-r_{EDC} = -\frac{k_{cat}m_{CLEA} * C_{EDC}}{K_m + C_{EDC}} \quad (7.5)$$

with k_{cat} representing the rate constant, m_{CLEA} the mass of the laccase CLEAs in the BR and K_m the affinity constant. Combining equations 7.4 and 7.5 the hydraulic residence time (τ) could be determined as a function of the concentration of the EDCs in the BR:

$$\tau = -\frac{\left(C_{EDC,in} - C_{EDC}\right) * \left(K_m + C_{EDC}\right)}{k_{cat}m_{CLEA} * C_{EDC}} \quad (7.6)$$

The kinetic constants, k_{cat} and K_m used were published by our group elsewhere (Cabana et al., 2007c).

7.3.12 Extraction of EDCs prior to quantitative analysis

The p353NP isomers were extracted using a C_{18} solid phase extraction cartridge (Alltech, IL, USA). 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 p353NP isomers were eluted from the cartridge using 3 ml of ethyl acetate and 6 ml of methylene chloride. BPA and TCS were extracted using methylene chloride in a 1:1 (v/v) ratio (methylene chloride:treated solution). The solutions were acidified to pH 2 with HCl and shaken for 20 min. The organic phase was then separated and evaporated under a gentle stream of nitrogen gas. Each sample was dissolved in 100 µl of methylene chloride prior to quantitative chemical analysis.

7.3.13 GC-MS analysis

The gas chromatography-mass spectrometry (GC-MS) analysis was performed on a G1800A gas chromatograph (Hewlett-Packard, CA, USA) equipped with an electron ionization detector and an HP-5 MS fused-silica column (30 m X 0.25 mm i.d., 0.25 mm film thickness) (Hewlett-Packard, CA, USA). Analysis was conducted in full-scan mode over an m/z range of 50 to 450 amu. Helium was used as carrier gas at a flow rate of 0.70 ml min^{-1}. The column was held at 70°C for 3 min, then the temperature increased to 160°C at a rate of 20°C min^{-1}, and it was finally increased to 300°C at a rate of 10°C min^{-1} and held for 7 min (Diaz et al., 2002). The injector temperature was set at 250°C.

7.4 Results

7.4.1 Effect of agitation conditions on the activity and stability of laccase CLEAs

The impact of the agitation conditions on the apparent laccase activity was determined by changing the rotational speed of the marine impeller and using ABTS as substrate. Table 7.2 presents the experimental design used and the impact of the mixing intensity on the laccase apparent activity and on the stability of the CLEAs used as a function of rotational speed, mixing energy and the rate of shear. Over the range of conditions tested, the maximum laccase activity was obtained for a rotational speed of 12.5 rps. Above this speed, the laccase activity did not change up to a rotational speed of 25 rps. For speeds lower than 12.5 rps, the laccase activity significantly decreased. The relative activity was of 87%, 63%, 53% and 46% for rotational speed of 8.3, 4.2, 2.5 and 0.8 rps respectively.

The stability of the laccase CLEAs, as a function of the agitation conditions, was monitored over a 24-h period. Table 7.2 shows the residual laccase activity after that incubation period for the agitation conditions tested. The residual activity of the CLEAs subjected to different mixing conditions decreased from 97% when the system was not agitated to 42% when the agitation speed was set at 25 rps. Specifically, the CLEAs were not denaturated over the range of agitation from 0 to 1.7 rps but the residual activity

dropped drastically between 1.7 and 4.2 rps from 98% down to 54%. For rotational speeds between 4.2 and 25 rps, the residual activity, after the 24-h incubation period, decreased linearly from 54% to 42%.

TABLE 7.2. IMPACT OF AGITATION CONDITIONS ON THE ACTIVITY AND STABILITY OF LACCASE CLEAS IN THE BASKET REACTOR USING ABTS AS SUBSTRATE AT A PH OF 5 AND A TEMPERATURE OF 20°C[a]

Agitation speed (rps)	Reynolds number	Froude number	Mixing energy ($W\ m^{-3}$)	G factor (s^{-1})	Relative activity ($A\ A_{max}^{-1}$) (± std dev.)	Residual activity ($A_{24}\ A_0^{-1}$) (± std dev.)
0	0	0.00	0.00	0.0	ND.	0.97 (0.06)
0.8	51823	0.0018	0.04	6.5	0.46 (0.07)	0.96 (0.06)
1.7	103647	0.007	0.3	17.2	0.49 (0.10)	0.98 (0.03)
2.5	155471	0.016	0.9	29.8	0.53 (0.09)	0.82 (0.09)
3.3	207294	0.028	1.9	44.0	0.64 (0.10)	0.72 (0.05)
4.2	259118	0.044	3.5	59.3	0.63 (0.03)	0.54 (0.10)
8.3	518236	0.18	21.8	147.5	0.87 (0.06)	0.66 (0.02)
12.5	777355	0.40	62.0	248.6	1.00 (0.09)	0.51 (0.06)
16.7	1036473	0.70	128.8	358.4	0.92 (0.06)	0.47 (0.05)
20.8	1295592	1.11	226.0	474.7	0.96 (0.03)	0.46 (0.03)
25.0	1554710	1.59	356.0	596.1	0.96 (0.10)	0.42 (0.06)

[a] The values represent means of triplicate experiments

ND. Not determined

7.4.2 Effect of temperature and pH on elimination of p353NP, BPA and TCS by laccase CLEAs

Preliminary results indicated that the elimination of these EDCs by free laccase from *C. polyzona* were temperature and pH dependant (Cabana et al., 2007a). Further results highlighted the ability of CLEAs formed with laccase from the same WRF to remove these xenobiotics without indicating the impact of the wastewater conditions on the elimination performances of the CLEAs (Cabana et al., 2007c). In order to investigate the effect of pH and temperature on the removal of p353NP, BPA and TCS, a Central Composite factorial design was used. This design can determine statistically the impact of each condition on the reduction of the concentration of each substance in solution and could be used to develop a second order model that could determine the optimal conditions necessary for

the conversion of these xenobiotics. The class of model that best fitted the experimental observations based on the limited factorial design used turned out to be of the linear-plus-interactions form presented by equation 7.7 as seen from data of Tables 7.3 and 7.4:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum_{i<j} \sum \beta_{ij} x_i x_j \qquad (7.7)$$

where Y represents the relative efficiency of elimination, β regression coefficients and Xi the coded symbol representing the independent variables pH and temperature.

Table 7.3 shows the results obtained for each set of operational conditions and Table 7.4 presents the corresponding analysis of variance (ANOVA). 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 0.976 for the elimination of p353NP, 0.929 for that of BPA and 0.978 for that of TCS suggested that the fitted linear-plus-interactions models could explain 97.6, 92.9 and 97.8% respectively of the total variation. This implies satisfactory representations of the processes by the above models. The F-values of 56.5 for p353NP elimination, 26.2 for BPA elimination and 61.1 for TCS elimination and *p* values < 0.001 for p353NP and TCS eliminations and of 0.001 for BPA elimination indicate that these linear-plus-interaction models are statistically significant and can predict the experimental results well. The ANOVA analysis highlighted the statistical significance of the independent variables on the elimination of these EDCs by laccase CLEAs. The Central Composite design can determine the quadratic response surface over the range of parameters tested. The second order models for the relative efficiency of elimination (Y_i) of p353NP, BPA and TCS respectively are presented through equations 7.8-7.10 considering only the significant terms determined by the ANOVA analysis.

$$Y_{p353NP} = -259.3 + 106.4 \times X_1 + 4.76 \times X_2 - 12.87 \times X_1^2 - 0.086 \times X_2^2 + 0.45 \times X_1 \times X_2 \qquad (7.8)$$

$$Y_{BPA} = -311.65 + 124.52 \times X_1 + 4.7 \times X_2 - 13.25 \times X_1^2 - 0.049 \times X_2^2 \qquad (7.9)$$

$$Y_{TCS} = -266.65 + 107.43 \times X_1 + 4.99 \times X_2 - 13.04 \times X_1^2 - 0.09 \times X_2^2 + 0.49 \times X_1 \times X_2 \qquad (7.10)$$

The 3D-surface responses obtained by these quadratic expressions are presented in Figure 7.2 to show the effects of the independent variables temperature and pH and the interactive

effects of each variable on the relative efficiency. The optimal conditions of pH and temperature for the removal of p353NP, BPA and TCS by laccase CLEAs could be determined by calculating the derivative of the regression models (equations 7-9) with regards to the independent variables X_1 and X_2. The optimal temperatures are 40.3, 48.0 and 41.2 °C for p353NP, BPA and TCS respectively. As regards the optimum pH values, they are 4.8, 4.7 and 4.9 respectively for the elimination of p353NP, BPA and TCS.

TABLE 7.3. OBSERVED RESPONSES IN CENTRAL COMPOSITE FACTORIAL DESIGN FOR THE ELIMINATION OF THE EDCs p353NP. BPA AND TCS BY LACCASE CLEAs

			p353NP		BPA		TCS	
Run	**pH**	**Temperature (°C)**	**Elimination (%)**	**Relative efficiency (%)**	**Elimination (%)**	**Relative efficiency (%)**	**Elimination (%)**	**Relative efficiency (%)**
1	4.5	35.0	67.5	86.4	60.3	92.5	76.3	91.7
2	2.4	35.0	14.2	18.2	14.6	22.5	16.1	19.4
3	6,0	50.0	56.8	72.7	47.2	72.5	62.4	75,0
4	3,0	50.0	30.2	38.6	24.5	37.5	30.0	36.1
5	6.6	35.0	42.6	54.5	27.7	42.5	48.5	58.3
6	4.5	56.2	55.0	70.5	44.5	68.3	62.4	75,0
7	4.5	35.0	74.5	95.5	65.2	100,0	83.2	100.0
8	6,0	20.0	19.5	25.0	11.4	17.5	20.8	25,0
9	4.5	35.0	69.2	88.6	53.8	82.5	76.3	91.7
10	4.5	35.0	71.0	90.9	58.7	90,0	74.0	88.9
11	4.5	13.8	32.0	40.9	24.5	37.5	32.4	38.9
12	3,0	20.0	24.9	31.8	11.4	17.5	25.5	30.6
13	4.5	35.0	78.1	100.0	52.2	80,0	83.2	100.0

TABLE 7.4. ANALYSIS OF VARIANCE (ANOVA) FOR THE SELECTED LINEAR PLUS INTERACTIONS MODEL FOR THE RELATIVE ELIMINATION OF p353NP, BPA AND TCS AFTER A 1-H TREATMENT WITH LACCASE CLEAS FROM *C. POLYZONA*

Substance	Source	Sum of squares	Degrees of freedom	Mean square	F-value	Prob > F	Coefficient of determination (R^2)	Coefficient of variation
p353NP	Model	9885.6	5	1977.1	56.5	< 0.0001	0.976	9.45
	pH	774.2	1	774.2	22.1	0.0022	-	-
	Temperature	1159.9	1	1159.9	33.2	0.0007	-	-
	pH^2	5829.8	1	5829.8	166.7	< 0.0001	-	-
	$Temperature^2$	2588.5	1	2588.5	74,0	< 0.0001	-	-
	Interaction temperature/pH	418.4	1	418.4	12.0	0.0106	-	-
	Residual error	244.8	7	35.0	-	-	-	-
	Total	10130.3	12	-	-	-	-	-
BPA	Model	10201.6	4	2550.4	26.2	0.0001	0.929	16.89
	pH	500.6	1	500.6	5.1	0.0432	-	-
	Temperature	1723.6	1	1723.6	17.7	0.0030	-	-
	pH^2	6182.9	1	6182.9	63.4	< 0.0001	-	-
	$Temperature^2$	2730.7	1	2730.7	28,0	0.0007	-	-
	Residual error	780.1	8	97.5	-	-	-	-
	Total	10981.7	12	-	-	-	-	-
TCS	Model	10802.1	5	2160.4	61.1	< 0.0001	0.978	9.3
	pH	975.3	1	975.3	27.6	0.0012	-	-
	Temperature	1421.1	1	1421.1	40.2	0.0004	-	-
	pH^2	5988.5	1	5988.5	169.5	< 0.0001	-	-
	$Temperature^2$	2870.2	1	2870.2	81.2	< 0.0001	-	-
	Interaction temperature/pH	493.8	1	493.8	14	0.0073	-	-
	Residual error	247.3	7	35.3	-	-	-	-
	Total	11049.4	12	-	-	-	-	-

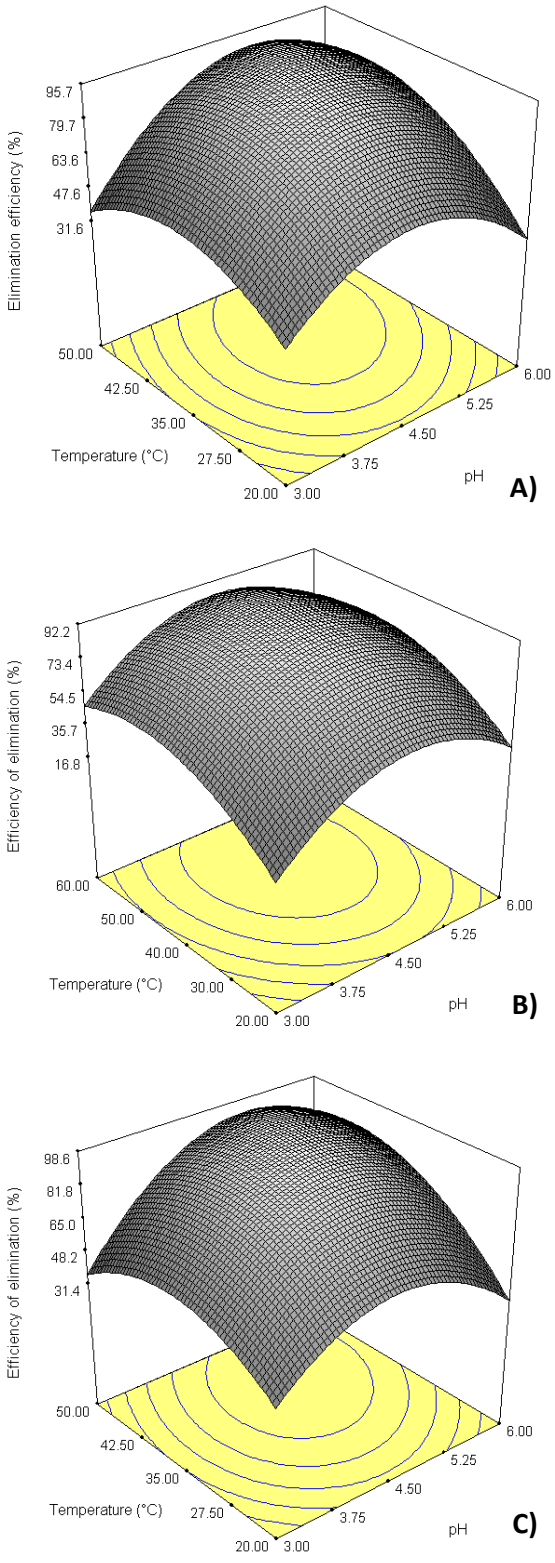

Figure 7.2. Response surface plots showing the effect of temperature and pH on the efficiency of elimination of A) p353NP, B) BPA and C) TCS from 5 mg l^{-1} solutions using 0.1 mg of laccase CLEAs after a 1-h-treatment

7.4.3 Continuous elimination of p353NP, BPA and TCS using laccase CLEAs in the basket reactor

The elimination of the EDCs was monitored as a function of the hydraulic residence time in the BR. Figure 7.3A shows the time course of the elimination of p353NP, BPA and TCS present in 5 mg l^{-1} solutions as a function of the hydraulic retention time at a pH of 5, a temperature of 20°C and an agitation of 1.7 rps using 1 mg of laccase CLEAs retained in the BR. Eliminations of p353NP, BPA and TCS of more than 85% were achieved at a hydraulic retention time of 325 min. The Figure 7.3B highlights the ability of the system to eliminate high concentrations of EDCs by using 1 mg of laccase CLEAs. At a residence time of 400 min, more than 95% of the p353NP and TCS present in aqueous solutions at a concentration of 100 mg l^{-1} was removed. For a residence time of 425 min, a BPA-elimination of 80% was achieved by the bioreactor.

Using the Michaelis-Menten kinetic constant obtained from data of batch elimination of these EDCs (Cabana et al., 2007c), the modeled elimination is presented in Figure 7.3. The models predicted adequately the behavior of the system in regard to its capacity to remove the target compounds.

Figure 7.4 presents the elimination of a these xenobiotics from 5 mg l^{-1} solutions and the variation of the laccase activity in the reactor as a function of the number of BR volumes that pass through the reactor. These treatments were performed over 30 BR volumes which represent 7 days of continuous operation at a hydraulic retention time of 331 min. For this hydraulic retention time, complete elimination of p353NP and TCS was initially obtained (for less than 10 BR volumes) while 90% of the BPA presents in solution was eliminated up to 5 BR volumes flow through the BR. After seven days of continuous operation, the elimination of p353NP, BPA and TCS decreased respectively by 20%, 10% and 15% compared to the initial BR performances. The laccase activity present in the BR was stable when incubated in presence of p353NP and decreased by 30% and 20% in the presence of BPA and TCS respectively after the 7-day operation of the bioreactor. Furthermore, these results demonstrated the ability of the BR to greatly retain the CLEAs within the reaction volume.

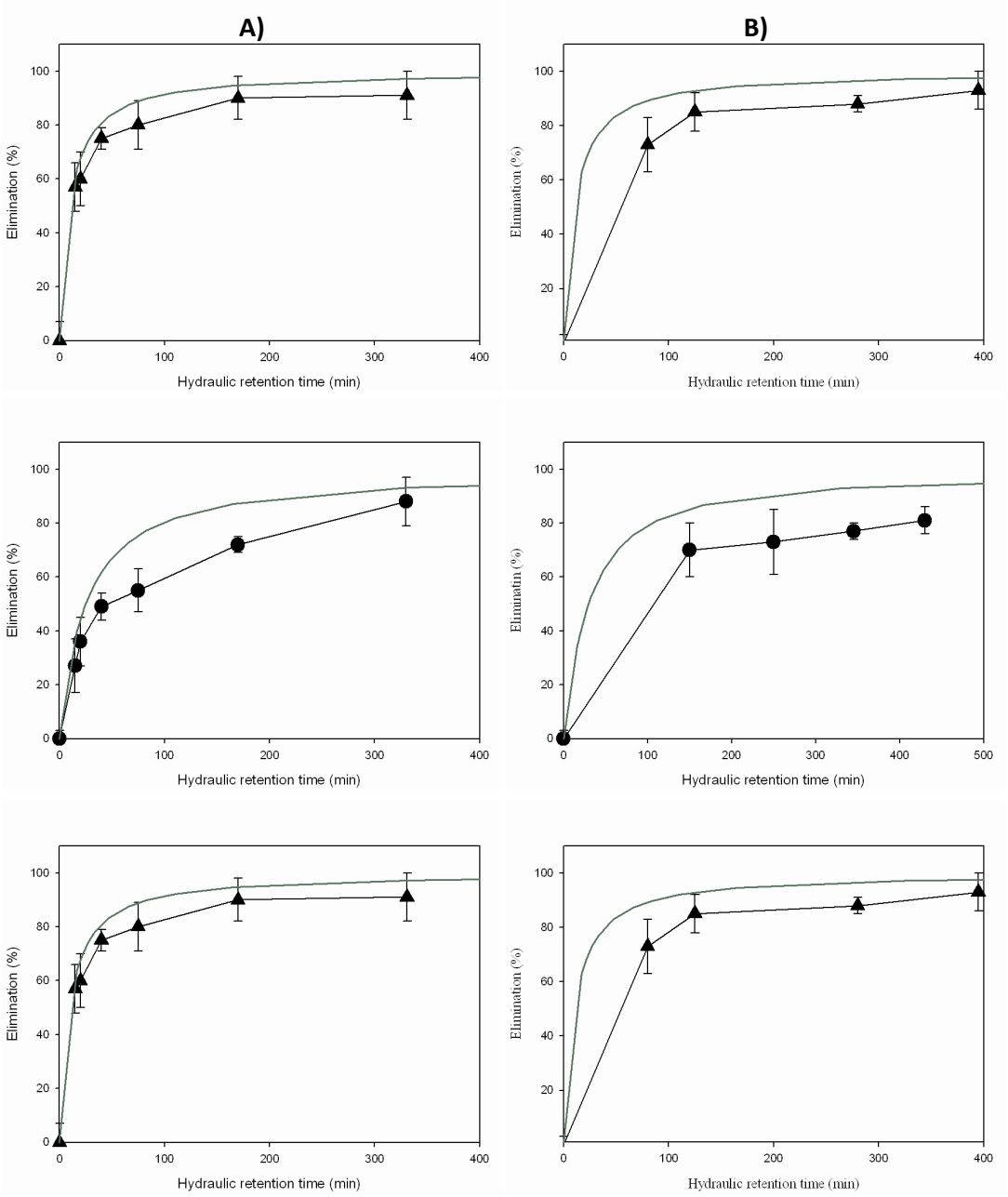

Figure 7.3. Continuous treatment of a (A) 5 mg l^{-1} and (B) a 100 mg l^{-1} solutions of (▲) p353NP, (●) BPA and (■) TCS at pH 5 and at room temperature using 1 mg of laccase CLEAs in the BR as a function of hydraulic residence time. The solid lines (—) represent the modelling of the processes

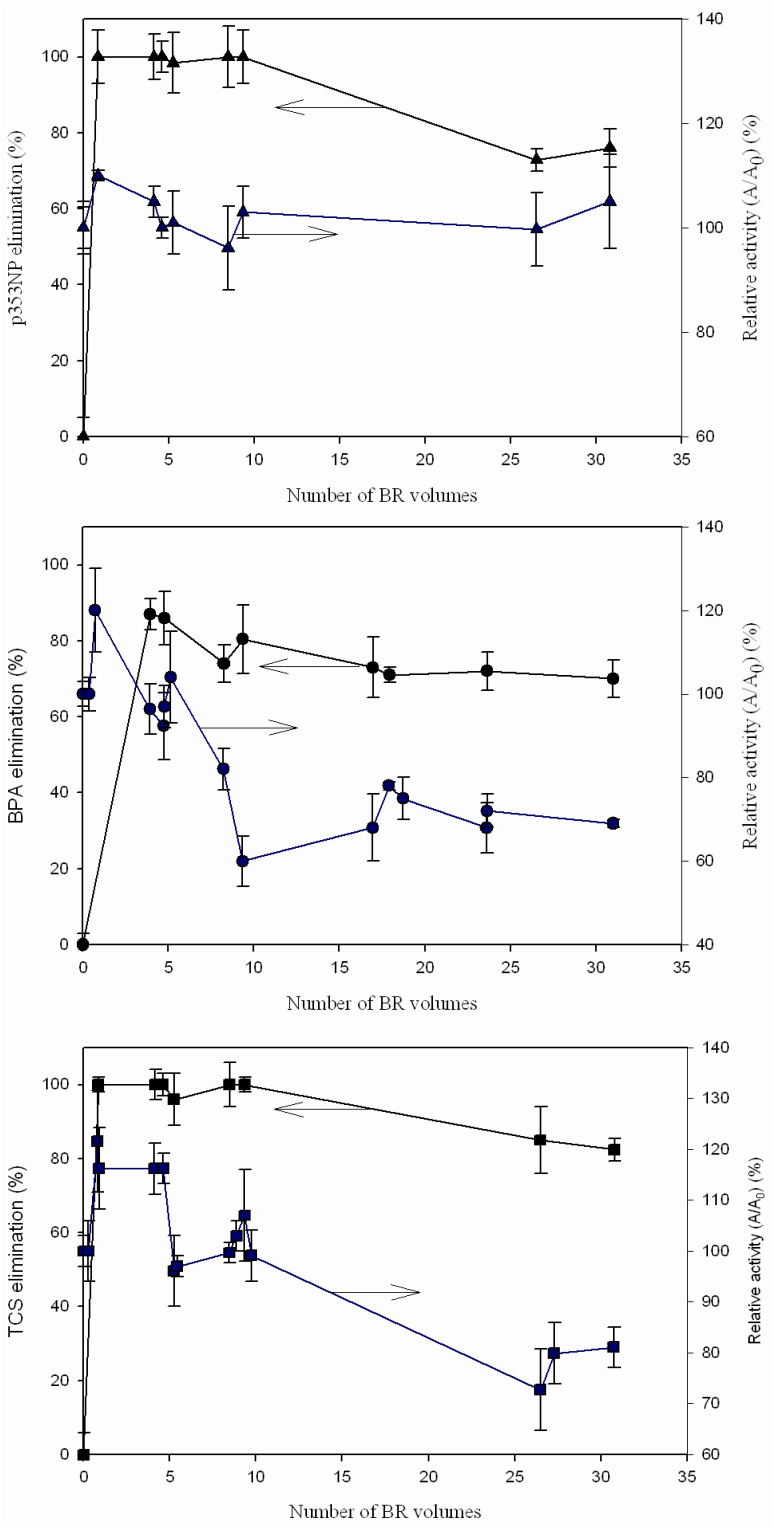

Figure 7.4. Elimination of (▲) p353NP, (●) BPA and (■) TCS from 5 mg l^{-1} solutions at pH 5 and at room temperature using 1 mg of laccase CLEAs in the BR and variation of the laccase activity in the BR as a function of the number of BR volumes that flow through the reactor. The hydraulic retention time was of 331 min

7.5 Discussion

Marine type impellers are generally considered to be suitable for agitating fragile biomaterials such as animal cell cultures because flow characteristics give full suspension of the biocatalysts and so reduce their damage at low speeds from possible shear/interface effects (Jan et al., 1993). This impeller ensured the good mixing of the reactant medium containing laccase CLEAs and EDCs and the mass transfer of the substrates

The use of an unbaffled BR allowed a high fluid-particle mass transfer rate for a given power consumption (Grisafi et al., 1998) and the existence of a central vortex plays an important role in enhancing the mass transfer process (Johnson and Huang, 1956). An increase of the agitation speed resulted in an increase of the energy provided to the system which translates to a rise of the fluid motion. This motion apparently increases the rate of substrate-enzyme collisions. These considerations could explain the variation of the relative activity of the laccase CLEAs as a function of the rotational speed. The laccase activity drastically increased when the agitation conditions above 4.2 rps formed a vortex ($Fr > 0.044$) in the BR.

The activity profile of the laccase CLEAs as a function of agitation conditions (see Table 7.2) showed that the inactivation is rotation-speed dependant. However, for low rotational speed range (0 - 1.7 rps) where there no visible vortices, the stability of the biocatalyst remained the same. These vortices enhanced the interfacial area and, by extension, the denaturation of the proteins. Reports on the inactivation of proteins by shear stress indicate that the protein structure disruption is linked to the acceleration of the turnover of the air-liquid interface and not by high shear stress or shear rate (Maa and Hsu, 1997; Oliva et al., 2003). This is consistent with observations done by Maa and Hsu (1996) on the denaturation of recombinant human growth hormone and recombinant human deoxyribonuclease showing that in the absence of air-liquid interface neither high shear nor high shear rate had a significant effect on protein inactivation (Maa and Hsu, 1996). Conversely, the presence of an air-liquid interface caused the loss of activity of recombinant human growth hormone (Maa and Hsu, 1997). The inactivation of the enzymes at the interface is due to the bipolar nature of the proteins that causes them to concentrate at the interface. Protein denaturation at hydrophobic interfaces is due to the

unfolding of the molecule and exposure of the hydrophobic regions to this interface. Finally, the macromolecules undergo aggregation over their hydrophobic regions (Maa and Hsu, 1997). Furthermore, in unstirred reactors, the air-liquid interface is tipically saturated with denaturated enzymes, thus limiting the inactivation of moer protein. Agitation of the reaction medium causes the movements of the enzymes present at the interface and is responsible for the renewal of the species at this interface causing the denaturation of more enzymes (Lencki et al., 1993).

Overall, our agitation conditions appear to have had two opposite effects: an increase in the agitation speed enhanced the laccase activity in the BR while the stability of the enzyme decreased. Since it is preferable to choose conditions in which the long-term activity of the biocatalyst is ensured compared to merely a higher initial activity (Duran et al., 2002), the agitation of the BR was set to 1.7 rps for the continuous elimination of the EDCs.

The Central Composite factorial design used and the ANOVA analysis highlighted the statistically significant influence of temperature and pH on the enzymatic transformation of p353NP, BPA and TCS with laccase CLEAs. The statistical models and response surface plots obtained enabled the determination of the optimal operational of temperature and pH. As for the case of free laccase from *C. polyzona*, these values represent a compromise between the stability produced by a higher pH and catalytic activity resulting from a higher temperature and are consistent with our previous finding (Cabana et al., 2007a). 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* (Okazaki et al., 2002) and an optimal pH of 5 for commercial laccase from *T. versicolor* (Kim and Nicell, 2006a) were shown previously. The latter laccase showed the same optimal pH for TCS as for BPA (Kim and Nicell, 2006b) whereas its optimal temperature for the bioconversion of BPA abd TCS lay between 45 and 50°C (Kim and Nicell, 2006a and 2006b).

The immobilized laccase from *C. polyzona* used in the BR for the continuous elimination of the EDCs under conditions ensuring good biocatalyst stability enabled the system to completely remove 90% of p353NP and TCS and eliminate 80% of BPA present in solution at concentrations of 5 and 100 mg l^{-1} for hydraulic residence time of less than 500 min using 1 mg of laccase CLEAs. These results point out the flexibility of this system as

to its ability to react to a shock load of phenolic pollutants. Comparison of the elimination predicted by the CSTR numerical model and the experimental data showed that the model developed is suitable for prediction of the behavior of the biocatalyst in the BR for the elimination of the phenolic EDCs tested.

The robustness of the system was tested by continuously feeding with EDC solutions at a concentration of 5 mg l^{-1} and at an hydraulic retention time of 331 min over a 7-day period (representing 30 BR volumes). The performance of the system decreased somewhat over this whole period. This decrease in efficiency was linked to the reduction of the laccase activity in the BR. Nonetheless, this represented a reasonable level of durability in the reactor performance attributable to the high stability of the immobilized laccase (Cabana et al., 2007c; Duran et al., 2002; Palmieri et al., 2005).

7.6 Conclusion

For the first time, a perfusion basket reactor was used for the continuous utilization of a solid biocatalyst formed by the cross-linking of an enzyme aggregate. An evaluation of the impact of the operation conditions of this reactor on the activity and stability of the laccase CLEAs used showed the robustness of the system in addition to its usefulness in the successful elimination of the EDCs p353NP, BPA and TCS. The development of this reactor for the continuous elimination contaminants exemplifies the value of designing a cost-competitive and easily operable treatment scheme to render the enzymatic processes more widely applicable to the environmental area. From this point of view, this project offers a basis for the development of laccase based bioprocesses for EDC elimination. The system used an easy to produce biocatalyst with high mass/activity ratio that can efficiently remove EDCs over a wide range of concentrations. The reactor developed represents a promising tool for the use of immobilized laccase in industrial and environmental biotechnologies.

7.7 Acknowledgement

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CONCLUSION

L'ensemble de ce travail démontre clairement que la laccase sécrétée par la souche fongique *C. polyzona* est un biocatalyseur adéquat et performant pour l'élimination des perturbateurs endocriniens NP, BPA et TCS présents en solution aqueuse à des concentration de 5 et 100 mg l^{-1} et ce, dans des conditions de laboratoire. L'élimination de ces polluants a été effectuée en utilisant ce biocatalyseur sous forme libre (soluble) et immobilisée. Ces travaux ont permis d'obtenir des informations fondamentales et appliquées qui pourront être utilisées dans le développement des bioprocédés industriels dévoués à l'élimination de ces substances, mais aussi, de façon générale, dans le développement de procédés utilisant cette enzyme.

L'élimination de ces composés en utilisant la laccase libre a permis de démontrer que cette enzyme est apte à éliminer efficacement ces composés et ce, sous différentes conditions de pH et de température. Les résultats obtenus, soutenus par une analyse statistique de type ANOVA, démontre que l'élimination optimale de ces composés réside en une combinaison de stabilité et d'activité catalytique générée par ces paramètres opérationnels. Il apparaît que ce biocatalyseur peut éliminer ces composés en utilisant de faibles concentrations d'activité enzymatique. L'élimination de ces composés est grandement améliorée en utilisant l'ABTS comme médiateur dans le système laccase/médiateur. L'action des laccases de *C. polyzona* sur ces substances génère des oligomères (dimères à pentamères) suite à l'interaction des radicaux phénoxy produits par l'oxydation enzymatique de ces composés phénoliques. Comme ces composés initialement présents induisent des changements hormonaux dans divers organismes, il était impératif de déterminer si les substances produites présentent des similarités structurelles avec des hormones endogènes. En ce sens, il a été démontré que les oligomères générés ne présentent plus de similarité structurelle avec l'estrogène, résultant en une élimination de l'activité xénoestrogénique de ces composés qui s'avère directement associée à la transformation des composés parents.

De façon à augmenter les possibilités d'utilisation de la laccase dans des bioprocédés, différentes techniques d'immobilisation ont été étudiées, à savoir l'immobilisation covalente sur un support solide et l'immobilisation par réticulation d'agrégats d'enzymes (CLEAs). De façon générale, les méthodes utilisées ont permis d'augmenter la stabilité de la laccase vis-à-vis de différents dénaturants chimiques, physiques et biologiques. De plus,

cette isolubilisation de la laccase a permis une réutilisation de l'enzyme et une utilisation de façon continue du biocatalyseur dans différents types de bioréacteurs. Toutefois, les procédures d'immobilisation choisies ont un impact significatif sur l'activité enzymatique, la récupération de cette activité, la stabilité et les caractéristiques cinétiques du biocatalyseur formé.

En premier lieu, la laccase de *C. polyzona* a été immobilisée sur de la terre de diatomées (Celite ®) silanisée en utilisant les dialdéhydes glyoxal et glutaraldéhyde comme agents réticulant. L'impact de la formation de réticulations intra-enzymatiques lors de l'immobilisation de l'enzyme sur le support a aussi été étudié. Il apparaît que le mode d'immobilisation choisi a un impact significatif sur l'activité du biocatalyseur, sur la récupération de cette activité ainsi que sur sa stabilité. En ce sens, les procédures impliquant la réticulation intra-moléculaire de l'enzyme produisent un biocatalyseur ayant une faible activité enzymatique et une grande stabilité contre la dénaturation thermique et chimique. La co-immobilisation de la laccase avec la BSA n'a pas pu générer de microenvironnements favorables résultant en une amélioration de sa stabilité comme dans le cas des CLEAs formés par co-réticulation. L'analyse des paramètres cinétiques des biocatalyseurs formés indique que l'immobilisation covalente de la laccase résulte en une diminution de l'affinité apparente et du taux maximum de réaction du biocatalyseur pour le substrat ABTS comparativement à la laccase libre.

Le biocatalyseur solide formé par immobilisation de la laccase sur la Celite à l'aide de glutaraldéhyde a été utilisé dans un réacteur garni (PBR) pour l'élimination en continu de ces xénobiotiques. Les performances de ce PBR ont été maintenues sur 5 cycles d'élimination consécutifs de près de 200 minutes. Finalement, ces catalyseurs ont démontré une grande flexibilité d'opération. Les paramètres cinétiques du biocatalyseur utilisé vis-à-vis de ces perturbateurs endocriniens démontrent que ce procédé d'oxydation biocatalytique présente une affinité décroissante pour le NP, le TCS et le BPA.

Comme l'immobilisation d'enzymes sur un support solide génère des biocatalyseurs présentant de faibles activités massiques, la laccase a été insolubilisée par la formation de CLEAs. Cette méthode a permis de générer un biocatalyseur présentant une activité massique laccase de 148 Ug^{-1} suite à l'optimisation des conditions de précipitation et de réticulation de l'enzyme. Les CLEAs ainsi formés présentent une plus grande stabilité face

à différents dénaturants chimiques, biologique et physiques que l'enzyme libre. De plus, en référant aux constantes de Michaelis-Menten des CLEAs et de la laccase libre relatives à l'oxydation de l'ABTS, il apparaît que les capacités catalytiques des CLEAs (sans ajout de BSA) sont supérieures à celles de la laccase libre. L'addition de BSA lors de la formation des CLEAs de laccase permet une stabilisation du catalyseur face à la dénaturation thermique tout en diminuant l'activité massique de ce dernier. Cet ajout de BSA provoque une diminution des performances biocatalytiques du CLEAs formé.

Ces CLEAs ont été utilisés dans un réacteur à lit fluidisé pour l'élimination en continu de ces substances chimiques. Ce système a démontré qu'il est possible d'obtenir une transformation efficace de ces composés en utilisant une faible quantité de biocatalyseur. Toutefois, il s'est avéré difficile de maintenir la fluidisation de ces catalyseurs solides.

De façon à augmenter l'utilisation des CLEAs pour le traitement en continu des perturbateurs endocriniens ciblés, un réacteur à perfusion a été développé. Celui-ci est constitué d'un panier réalisé à l'aide d'un média métallique filtrant. Ce panier contient les CLEAs et est agité à l'aide d'une hélice marine à trois pales. Le réacteur est alimenté en continu par l'intérieur de ce panier. L'effluent traité quitte le panier par gravité. Ce type de réacteur permet de conserver les CLEAs confinés dans le panier et par conséquent permet de maintenir un environnement favorable à la conversion du NP, BPA et TCS. En ce sens, l'activité biocatalytique à l'intérieur de ce panier s'est avérée stable sur une période de sept jours durant lesquels ces perturbateurs endocriniens ont été éliminés en continu.

Les conditions d'agitation de ce réacteur ont un impact significatif sur la stabilité et l'activité apparente du biocatalyseur. Ainsi, une augmentation de la vitesse de rotation de l'hélice marine résulte en une augmentation de l'activité enzymatique apparente et en une diminution de la stabilité des CLEAs. En particulier, l'apparition de vortex dans le système favorise le transfert de masse entre le substrat et le catalyseur, ce qui augmente l'activité apparente du catalyseur et augmente l'interface liquide/gazeux ce qui résulte en une diminution de sa stabilité. Il est donc impératif de sélectionner des conditions d'opération permettant un équilibre entre la stabilité et l'activité enzymatique du catalyseur.

En utilisant de telles conditions, ce système a pu être utilisé pour efficacement éliminer le NP, BPA et TCS et ce, de façon continue. Pour ce faire, le comportement cinétique du

biocatalyseur de type Michaelis-Menten a été combiné au comportement d'un réacteur à fonctionnement continu (CSTR). Le modèle développé s'est avéré adéquat pour prédire l'efficacité d'élimination du système.

Le développement de bioprocédés utilisant des laccases immobilisées sour forme de CLEAs offre différents avantages pour le développement de nouvelles biotechnologies tant environnementales qu'industrielles. En ce sens, la formation de CLEAs permet une augmentation de la stabilité (contre la dénaturation physique, chimique et biologique) de l'enzyme, la production d'un catalyseur présentant une forte activité massique (et par extension une faible activité volumique), la possibilité de développer des bioréacteurs compacts, l'absence de support solide impliquée dans l'immobilisation de cette enzyme, la diminution du volume de déchets à disposer une fois le catalyseur inactivé et la possibilité d'utiliser ce catalyseur dans différentes configurations de réacteurs. Particulièrement, le réacteur à perfusion développé s'avère une configuration de réacteur permettant une utiliastion aisée de ce type de biocatalyseur.

À la lumière de ces résultats, il paraît évident que l'immobilisation de la laccase de *C. polyzona*, tant par liaison covalente à un support solide que par la formation de CLEAs, représente une avenue prometteuse pour l'utilisation de cette enzyme dans des procédés de bioremédiation d'effluents aqueux contaminés par différents perturbateurs endocriniens phénoliques. En effet, cette approche permet une stabilisation, une utilisation en continu du biocatalyseur et une flexibilité dans le design du bioréacteur ce qui, globalement, a un impact direct sur les coûts liés à l'utilisation de catalyse enzymatique dans des applications environnementales. De plus, ces biocatalyseurs solides représentent des outils prometteurs pour la synthèse organique de produits d'intérêts (polyphénols, modification de polymères phénoliques, fonctionnalisation de la lignine, etc.) qui, jusqu'à ce jour, ont été réalisés en utilisant de la laccase soluble ou des moyens chimiques. Cette approche permettra de produire en continu ces produits tout en diminuant la contamination par le biocatalyseur présent et ce, dans des conditions qui, jusqu'à ce jour pouvait s'avérer non favorables à la laccase. Qui plus est, ces approches, de par leur capacité de stabilisation de la laccase vis-à-vis différents solvants organiques, augmentent les possibilités d'utilisation de la laccase pour la synthèse dans des milieux organiques.

Il faut toutefois noter que l'ensemble des résultats présentés précédemment traitent uniquement de l'élimination de ces composés dans une matrice aqueuse idéale, c'est à dire, constituée d'eau distillée uniquement. Cependant, les effluents aqueux réels contiennent des particules en suspensions, des sels, des polluants organiques, des microorganismes, etc. qui peuvent influencer le comportement des biocatalyseurs développés dans ce projet et la biodisponibilité de ces substrats. Il apparaît donc impératif, comme suite à ce projet, de tester ces systèmes dans des situations non-idéales afin de démontrer leur réelle efficacité. Aussi, il serait fort intéressant de développer des biotraitements utilisant ces enzymes pour l'élimination de ces perturbateurs endocriniens adsorbés sur des matrices solides telles des boues ou des sols. Il est également à noter que le devenir à long terme des polyphénols formés suite à l'action oxydative de la laccase sur ces perturbateurs endocriniens n'est pas documenté. Il s'avère donc impératif de déterminer la stabilité de ces composés dans différentes matrices environnementales de façon à s'assurer que l'activité estrogénique des composés parents ne peut pas être retrouvée suite à l'action des différents acteurs physico-chimique présents dans le milieu récepteur.

Finalement, ce travail a démontré que la laccase de *C. polyzona*, sous sa forme libre et surtout immobilisée, est un catalyseur offrant une grande capacité d'élimination de ces polluants en solutions aqueuses et que cette enzyme peut être utilisée dans le développement de biotechnologies axées sur l'élimination de ces composés. Toutefois, dans une perspective globale où les répercussions à long terme sont considérées, une approche de traitement du problème par un moyen bio-physico-chimique, quelle qu'elle soit, s'avère limitée. En effet, ces approches déresponsabilisent les consommateurs et les producteurs vis-à-vis de l'impact de leur choix et gestes sur l'environnement (au sens large, incluant l'économie, la société et l'écosphère). De ce constat découle la nécessité d'adopter une démarche holistique qui tient compte du contexte et des répercussions de tous problèmes ou phénomènes sur cet environnement. Une approche responsable et durable pourrait reposer sur le développement et l'utilisation de produits de substitution à ces polluants qui ne présenteraient pas ces effets sur les organismes présents dans les milieux récepteurs, fabriqués selon des méthodes responsables et ce, à des coûts compétitifs.

ANNEXE 1

CRIBLAGE DE SOLUTIONS ENZYMATIQUES PROVENANT DE DIFFÉRENTES SOUCHES DE WRF

Cette annexe présente les résultats obtenus lors d'un criblage préliminaires effectué pour déterminer la solution enzymatique à utiliser au cours de ce travail. Pour ce faire, nous avons utilisé différentes solutions enzymatiques provenant de différentes souches de WRF s'avérant de bonnes productrices de laccases. Les resultants obtenus lors de ces essais préliminaires ont justifiés l'utilisation de de la laccase de *C. polyzona*.

A1.1 Méthodologie

A1.1.1 Organismes et conditions de culture

Les souches de WRF testées sont les suivantes: *C. polyzona* (MUCL 38443), *Pycnoporus sanguineus* (MUCL 41582), *P. coccineus* (MUCL 38527) provenant de la mycothèque de l'Université Catholique de Louvain, *Pleurotus dryinus* et *P. ostreatus* de la Collection de basidiomycètes de l'Institut de biochimie et de biotechnologie (Tbilisi, Georgia) et *Ganoderma stipitatum, Lentinus swartzii* et *Lentinus crinitus* provenant de la Collection de la Faculté des sciences exactes et naturelles de l'Université de Antioquia (Medellin, Colombie).

Les souches *C. polyzona*, *P. dryinus* et *P. Ostreatus*, ont été cultivées dans 100 ml du milieu suivant : glucose, 10.0 g l^{-1}; NH_4NO_3, 1.0 g l^{-1}; KH_2PO_4, 0.8 g l^{-1}; Na_2HPO_4, 0.4 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1}; yeast extract, 2.0 g l^{-1}. Le pH du milieu, avant autoclavage, a été ajusté à 6.0 en utilisant du NaOH (1 M).

Les souches *P. coccineus* et *P. sanguineus* ont été cultivées dans 100 ml d'un milieu contenant 20 g l^{-1} d'extrait de malt.

Finalement, les souches *Ganoderma stipitatum, Lentinus swartzii* et *Lentinus crinitus* ont été cultivées dans 100 ml du milieu suivant: glucose, 10 g l^{-1}; tartrate d'ammonium, 0.2 g l^{-1}; Tween 80, 0.5 g l^{-1}; acide tartrique, 3 g l^{-1}; extrait de levures, 0.2 g l^{-1}; $MnSO_4$, 40 mg l^{-1}

et KH_2PO_4, 0.8 g l^{-1}. Le pH initial du milieu a été ajusté à 5.0 en utilisant du NaOH (2M) avant autoclavage.

Les différentes fermentations ont eu lieu dans des Erlenmeyer de 250 ml à 27°C. Les milieux étaient agités orbitalement à 150 rpm. La fermentation a eu lieu pendant 10 jours. Par la suite, les milieux ont été filters et les filtrats recueillis ont été utilises comme source de laccases.

A1.1.2 Elimination du NP, du BPA et du TCS à l'aide des solutions enzymatiques

Les essais préliminaires d'élimination du NP, du BPA et du TCS ont été réalisés sur des solutions aqueuses contenant initialement 5 mg l^{-1} de chacun de ces perturbateurs endocriniens et 100 U l^{-1} de laccase et ce, à une température de 30°C et un pH de 5. Le milieu réactif était le suivant : NP, BPA ou TCS, 5 mg l^{-1}; tampon phosphate (0.5M) / citrate (0.5M) et 1% v/v de méthanol. Le volume du milieu réactif était de 5 ml. Les milieux ont été agités orbitalement à 150 rpm dans des erlenmeyers de 50 ml. Ces traitements préliminaires ont été réalisés pendant 1 heure.

A1.1.3 Extraction

Le NP a été extrait en utilisant une cartouche C18 d'extraction solide. Préalablement, la cartouche a été conditionnée avec 4 ml de méthanol et 4ml d'eau distillée. Le milieu réactif a été élué à un débit d'environ 2 ml min^{-1} à l'aide d'un dispositif sous vide. Le NP résiduel a été élué en utilisant 3 ml d'acétate d'éthyle et 6 ml de dichlorométhane. La phase organique a été évaporée sous un jet d'azote. Le NP a été dissout dans 100 µl de méthanol.

Le BPA et le TCS ont été extraits de la solution aqueuse en utilisant de l'acétate d'éthyle. Cette extraction liquide/liquide a été effectuée en utilisant un ratio volumique de 1/1 (solution traitée/acétate d'éthyle). La solution a été acidifiée à un pH de 2 en utilisant du HCl (37% w/w), agitée pendant 20 minutes à l'aide d'un barreau magnétique (300 rpm) puis congelée pendant 16 heures. La phase organique a, par la suite, été récupérée et évaporée sous un jet d'azote. Le BPA et le TCS ont été dissouts dans 100 µl de méthanol.

A1.1.4 Analyse par HPLC

L'analyse quantitative de ces perturbateurs endocriniens a été realisée par HPLC (contrôleur 600, injecteur automatique 717 plus, détecteur PDA 996, Waters corporation). La colonne utilisée était une colonne Adsorbosphere XL (Alltech). La debit de la phase mobile était de 1 ml m^{-1}. Cette phase était constituée des solutions suivantes: (A) acetate d'éthyle + 1% (v/v) d'eau bi-distillée et (B) tetrahydrofurane + 5 % (v/v) d'eau bitistillée. Le gradient suivant a été impose au système: Initialement l'éluent était constitué de 100% A, puis cette concentration en A a été réduite linéairement à 87.5% sur une période de 5 minutes. Finalement, cette concentration est gardée constante pendant 15 minutes. La détection du NP, BPA et TCS s'est effectuée à une longueur d'onde de 277 nm.

A1.2 Résultats

Le Tableau A1.1 présente les différents résultats préliminaires obtenus à l'aide des différentes solutions enzymatiques préliminairement testées.

TABLEAU A1.1. ÉLIMINATION DES PERTURBATEURS ENDOCRINIENS NP, BPA ET TCS, PRÉSENTS EN SOLUTION À UNE CONCENTRATION DE 5 mg l^{-1}, APRÈS UN TRAITEMENT DE 1H EN UTILISANT 100 UI^{-1} DE LACCASE À UNE TEMPÉRATURE DE 30°C ET UN pH DE 5[a]

Souches de WRF	Élimination (%)		
	NP	BPA	TCS
P. dryinus	N.D.	5 ± 0.1	N.D.
P. ostreatus	N.D.	10 ± 1	N.D.
C. polyzona	100 ± 7	94 ± 3	40 ± 7
P. coccineus	N.D.	50 ± 2	25 ± 5
P. sanguineus	N.D.	75 ± 6	N.D.
G. stipitatum	N.D.	22 ± 3	40 ± 6
L. swartzii	N.D.	37 ± 2	15 ± 3
L. crinitus	N.D.	25 ± 2	17±1

[a] Moyenne de triplicats ± écart-type
N.D. non déterminée

Ces résultats démontrent que la laccase de *C. polyzona* démontre une meilleure capacité d'oxidation de ces composés que les laccases provenant des autres souches de WRF testées. En référant à cette capacité oxidative, la laccase sécrétée par *C. polyzona* a été utilisée pour ce travail.

ANNEXE 2

CINÉTIQUE DE TYPE MICHAELIS-MENTEN

Le mécanisme de la réaction entre la laccase et ces substrats peut être représenté par le mécanisme suivant :

I. La laccase (E) réagit avec le substrat phénolique (S) de façon à former un complexe enzyme-substrat (E·S) :

$$RH + laccase \xrightarrow{k_1} [RH \bullet laccase] \qquad \text{(A2.1)}$$

II. Ce complexe peut se décomposer et reformer de la laccase et du substrat phénolique libres :

$$[RH \bullet laccase] \xrightarrow{k_2} RH + laccase \qquad \text{(A2.2)}$$

III. Ce complexe peut également réagir avec l'oxygène dissout dans le milieu pour former le radical phénoxy, de l'eau et récupérer la laccase :

$$[RH \bullet laccase] + O_2 \xrightarrow{k_3} R^{\bullet} + H_2O + laccase \qquad \text{(A2.3)}$$

Ces étapes, schématisées à l'aide des équations A2.1 à A2.3 simplifient le processus enzymatique, mais peuvent être utilisées pour décrire les phénomènes observés dans cette étude.

Soit E, S, O, E·S et Z représentant respectivement l'enzyme, le substrat phénolique, l'oxygène, le complexe enzyme substrat et les produits de la réaction. Les étapes A2.1 à A2.3 peuvent être schématisées de la façon suivante :

$$S + E \xrightarrow{k_1} E \bullet S \qquad \text{(A2.4)}$$

$$E \bullet S \xrightarrow{k_2} E + S \qquad \text{(A2.5)}$$

$$E \bullet S + O \xrightarrow{k_3} Z + E \qquad \text{(A2.6)}$$

Le taux d'élimination du substrat ($-r_s$) est donnée par l'expression A2.7.

$$-r_s = k_1[E][S] - k_2[E \bullet S] \qquad \text{(A2.7)}$$

Le taux de formation du complexe enzyme-substrat (r_{ES}) est, quant à lui, donné par l'expression A2.8.

$$r_{ES} = k_1[E][S] - k_2[E \bullet S] - k_3[O][E \bullet S] \qquad \text{(A2.8)}$$

Comme la laccase catalyse la réaction d'oxydation, la concentration totale en laccase ($[E]_t$) est constante et est exprimée par l'équation A2.9.

$$[E]_t = [E] + [E \bullet S] \qquad \text{A2.9}$$

En réarrangeant l'expression A2.9 de façon à exprimer la concentration de laccase libre dans le milieu ([E]), nous obtenons l'expression A2.10.

$$[E] = [E]_t - [E \bullet S] \qquad \text{A2.10}$$

En combinant les équations A2.10 et A2.9 et en faisant l'hypothèse que le système est en état pseudo stationnaire nous obtenons l'expression A2.11. L'hypothèse d'état pseudo-stationnaire suppose que le taux de formation du complexe enzyme-substrat est égal à zéro (Fogler, 1999). Cette hypothèse est soutenue par la courte durée de vie de ce complexe due à la grande réactivité de ce dernier.

$$r_{ES} = 0 = k_1([E]_t - [E \bullet S])[S] - k_2[E \bullet S] - k_3[O][E \bullet S] \qquad \text{A2.11}$$

En résolvant pour obtenir une expression permettant de déterminer la concentration en complexe, nous obtenons l'expression A2.12.

$$[E \bullet S] = \frac{k_1[E]_t[S]}{k_1[S] + k_2 + k_3[O]} \qquad \text{A2.12}$$

Puis, en combinant les expressions A2.7 et A2.10 l'équation A2.13 est obtenue.

$$-r_s = k_1([E]_t - [E \bullet S])[S] - k_2[E \bullet S] \qquad \text{A2.13}$$

Par la suite, en soustrayant l'expression A2.11 de A2.13 l'équation A2.14 est obtenue.

$$-r_s = k_3[O][E \bullet S] \qquad \text{A2.14}$$

Finalement, en substituant l'équation [E·S] par l'expression A2.12 dans l'équation A2.14, nous obtenons la forme générale du taux de conversion du substrat (A2.15).

$$-r_s = \frac{k_1 k_3[E]_t[S][O]}{k_1[S] + k_2 + k_3[O]} \qquad \text{A2.15}$$

Au cours des différents essais effectués, la concentration en oxygène dans le milieu réactif a toujours été en excès. De ce fait, cette concenration est considérée constante. Par conséquence, posons :

$$k_3' = k_3[O] \qquad \text{A2.16}$$

Regroupons également les constantes k1, k2 et k_3' dans la constante K_m selon l'expression A2.17.

$$K_m = \frac{k_2 + k_3'}{k_1} \qquad \text{A2.17}$$

En divisant le numérateur et le dénominateur de l'expression A2.15 par la constante k_1, l'expression A2.18 est obtenue.

$$-r_s = \frac{k_3^{'}[E]_t[S]}{[S]+K_m} \qquad \text{A2.18}$$

L'expression A2.18 représente la forme générale de l'équation cinétique dite de Michaelis-Menten (Michaelis et Menten, 1913). La constante K_m est appelée constante de Michaelis-Menten. Elle représente la concentration de substrat à laquelle le taux de réaction représente la moitié du taux maximal de réaction (voir Figure A2.1). Ce taux maximal de réaction (V_{max}) est fonction de la concentration en enzyme présente en solution et est donnée par l'expression A2.19.

$$V_{\max} = k_3^{'}[E]_t = k_{cat}[E]_t \qquad \text{A2.19}$$

Dans l'équation A2.19, la constante k_{cat} représente le taux maximal de réaction par quantité d'enzyme (dans ce travail, par unité massique d'enzyme).

Globalement, nous obtenons l'équation A2.20.

$$-r_s = \frac{k_{cat}[E]_t[S]}{[S]+K_m} = \frac{V_{\max}[S]}{[S]+K_m} \qquad \text{A2.20}$$

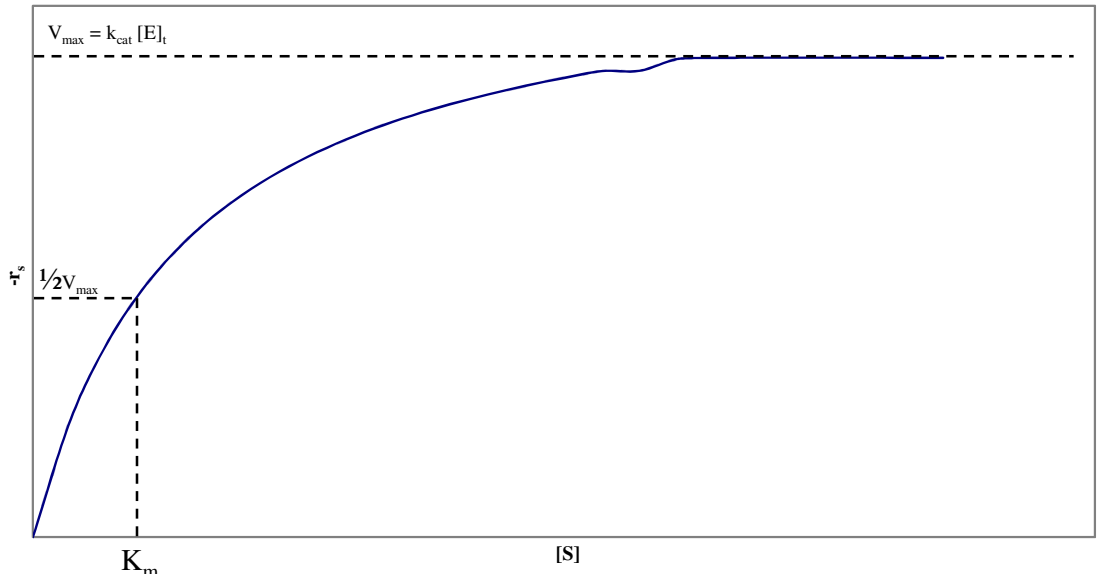

Figure A2.1. Représentation de la cinétique de Michaelis-Menten

A2.2 References

Fogler, H. S., 1999. Elements of chemical eraction engineering. Prentice Hall PTR, Upper Saddle River.

Michaelis, L. and Menten, M.L., 1913. Die Kinetik der Invertinwirkung. Biochem. Z. 49, 333-369.

ANNEXE 3

TAUX DE REACTION EN FUNCTION DE LA CONCENTRATION INITIALE EN SUBSTRAT

Cette annexe présente les taux de réaction en fonction de la concentration initiale en substrat (ABTS et/ou EDCs) de façon à démontrer que i) la laccase de *C. polyzona* a un comportement cinétique de type Michaelis-Menten et ii) que la gamme de concentrations en substrat utilisée était suffisamment étendue.

A3.1 Laccase libre et immobilisée sur Celite® R-633 (résultats présentés au Chapitre 5)

La Figure A3.1 présente le taux de réaction de la laccase libre et des biocatalyseurs solides CR633-GLU et CR633-SIM-GLU en fonction de la concentration initiale en ABTS et ce, à pH 3 et à température ambiante. La Figure A3.2, quant à elle, présente le taux de réaction du biocatalyseur CR633-GLU en fonction de la concentration initiale en perturbateurs endocriniens et ce, à pH 5 et à 20°C.

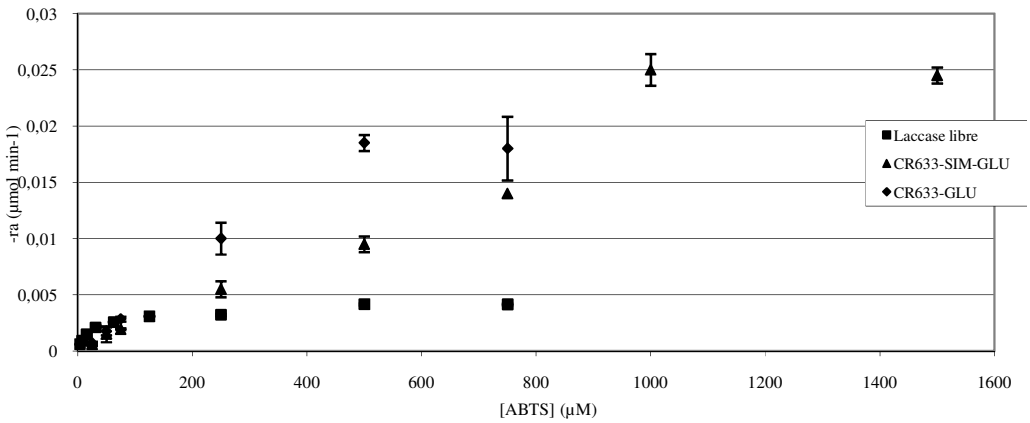

Figure A3.1. Taux de réaction de la laccase libre et des catalyseurs solides CR633-GLU (30 mg) et CR633-SIM-GLU (30 mg) en fonction de la concentration initiale en ABTS à pH 3 et 20°C. Moyenne de 3 valeurs ± écart-type

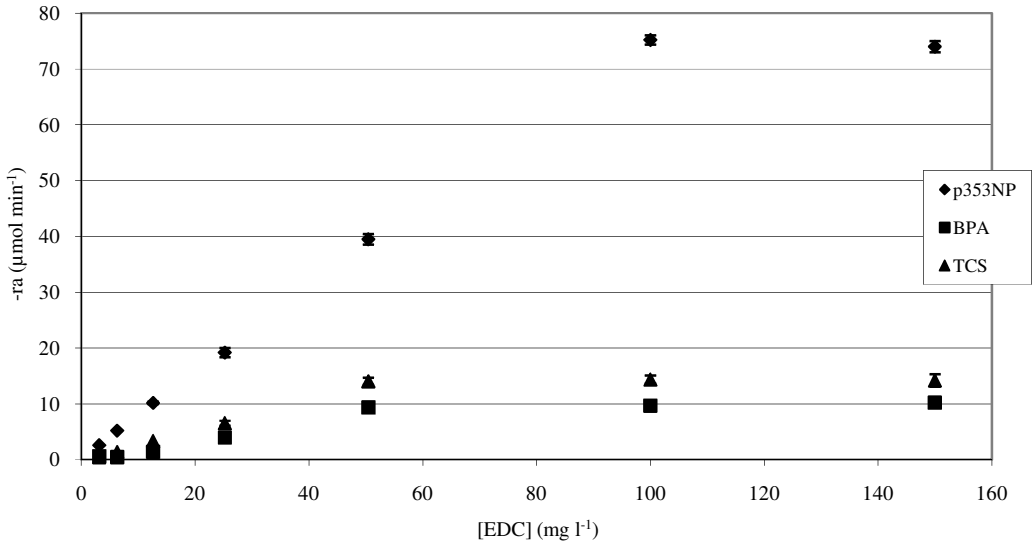

Figure A3.2. Taux de réaction du catalyseur solide CR633-GLU en fonction de la concentration initiale en perturbateurs endocriniens p353NP, BPA et TCS à pH 5 et 20°C. Moyenne de 3 valeurs ± écart-type

A2.2 Laccase immobilisée sous forme de CLEAs (résultats présentés au Chapitre 6)

La Figure A3.3 présente le taux de réaction des biocatalyseurs formés par réticulation d'aggrégats d'enzymes (CLEA) en fonction de la concentration initiale en ABTS et ce, à pH 3 et à température ambiante. La Figure A3.4, quant à elle, présente le taux de réaction du biocatalyseur CLEA en fonction de la concentration initiale en perturbateurs endocriniens et ce, à pH 5 et à 20°C.

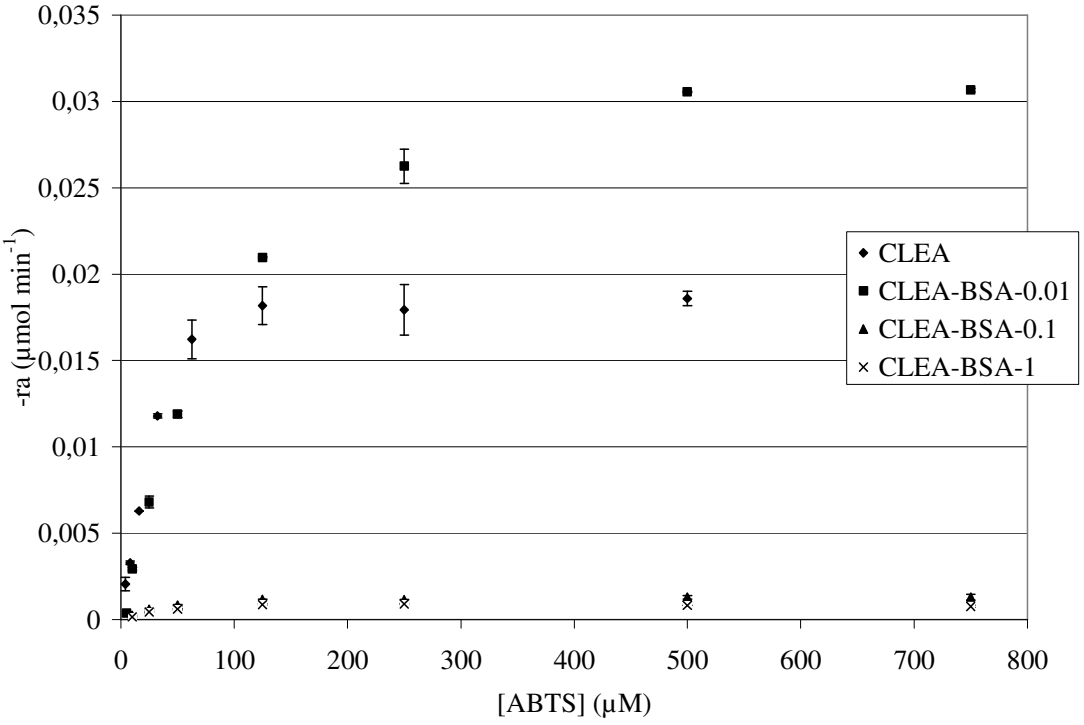

Figure A3.3. Taux de réaction des catalyseurs solides CLEA (20 µg), CLEA-BSA-0.01 (300 µg), CLEA-BSA-0.1 (285 µg) et CLEA-BSA-1 (1000 µg) en fonction de la concentration initiale en ABTS à pH 3 et 20°C. Moyenne de 3 valeurs ± écart-type

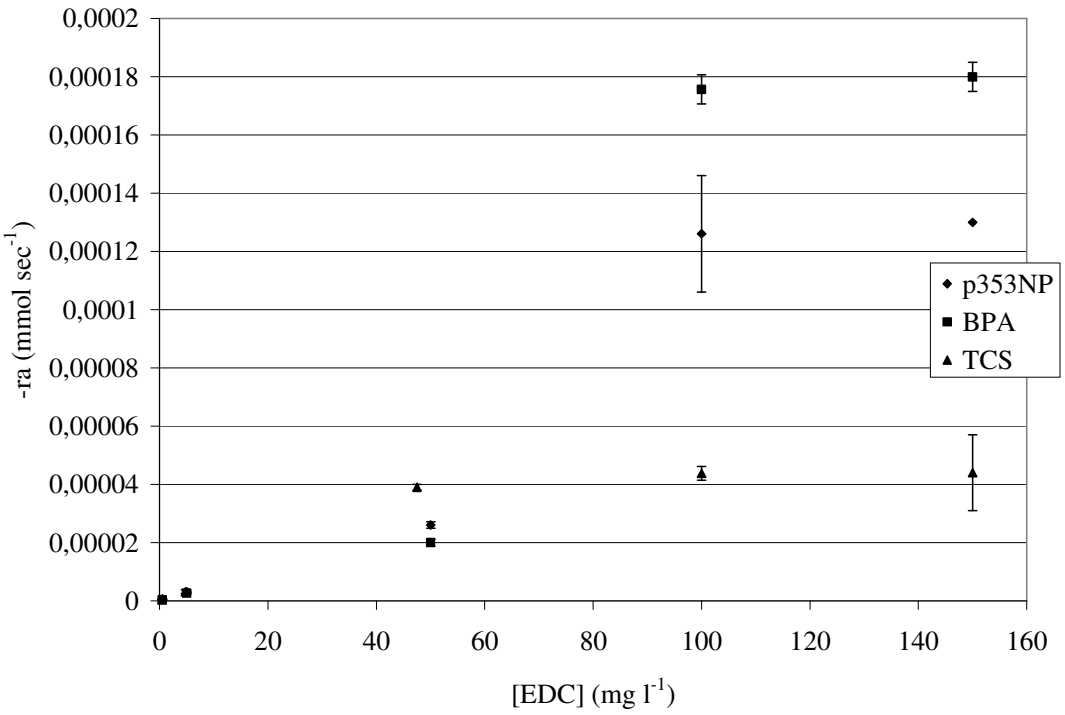

Figure A3.4. Taux de réaction du catalyseur solide CLEA en fonction de la concentration initiale en perturbateurs endocriniens p353NP, BPA et TCS à pH 5 et 20°C. Moyenne de 3 valeurs ± écart-type