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Effect of additives on enzyme-catalyzed polymerization of phenols and aromatic amines

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TABLE OF CONTENTS

1. ABSTRACT

Among biological approaches to the removal of aromatic amines and phenols from wastewater, the so-called enzyme-catalyzed polymerization and precipitation (ECPP) process relies on the use of oxidoreductases acting *via* radical mechanisms and characterized by a rather relaxed substrate specificity, such as laccase, tyrosinase and heme-peroxidases. The main technical constraints of ECPP processes are due to a variety of enzyme deactivation phenomena occurring during catalysis and to the incomplete removal of oxidation products from solution. In order to put ECPP into practice, these drawbacks have to be either counteracted or minimized. Although several approaches, such as enzyme immobilization and reaction engineering, have been proposed to limit these constraints, this review is intended to provide a wide survey on some chemical additives with either protective or coagulating effects that have been so far employed for these purposes.

2. INTRODUCTION

Several manufacturing processes make an extensive use of both aryl amines and phenols as either synthetic intermediates or precursors (1, 2). As a consequence, these compounds are often detected in the process water of many industries including those producing agrochemicals, food, pharmaceuticals polymers, dyestuffs, resins and coatings (3, 4). The majority of these compounds are toxic or suspected carcinogen and some of them, such as phenol, aniline, 2-chlorophenol and 2,4-dichlorophenol, are ranked within the 250 most hazardous pollutants (5). Some of them, such as 4-nonylphenol (NP), *tert*-octylphenol, 17-beta-estradiol and bisphenol A (BPA), are suspected to be endocrine disrupting chemicals (EDC) for hormonal signals in animals and are known to exert irreversible effects on the development of the reproductive organs (6, 7). In addition, chloro-substituted phenols and

anilines are commonly found in chlorinated water, since phenol and aniline can react with chlorine (8, 9).

For these reasons, the discharge of these compounds into surface waters is subjected to strict regulation and their treatment is a matter of substantial activity. To provide an example, in 2006, the United States Environmental Protection Agency's Toxics Release Inventory (US EPA TRI) reported the on- and off-site disposal of 2930 and 612 t of phenol, respectively. In the same year, the National Pollutant Release Inventory (NPRI 2006) of Canada reported 137 t of phenol and its salt being disposed of and, two years earlier, the European Pollutant Emission Register (EPER 2004) certified a direct and indirect release of 2676 t of phenolic compounds (10). While initial effluent concentrations in the industrial wastewater normally range from hundreds to thousands of milligrams per litre (11), maximum discharge limits vary from 0.1 to 5.0 mg l^{-1} in dependence on the national regulation (10).

Conventional treatment methods for the removal of aromatic pollutants from industrial wastewater encompass a variety of adsorption methods, advanced chemical oxidation and microbial processes (12-14). These methods, however, suffer from various drawbacks such as high cost, low selectivity, possible formation of toxic byproducts, high energy requirements and/or operability within a limited pollutant concentration range (15, 16). In several cases, enzyme-based approaches might offer a valuable option to conventional wastewater treatment due to their selectivity, lower susceptibility to shock loading effects than microbial treatments, ability to operate under mild conditions, easy handling and storage of biocatalysts (17-19). In addition, the quantity of solids arising from enzymatic processes are very small in comparison with sludge produced during biological treatment (18, 20). Selectivity for the removal of certain compounds may be important in order to meet increasingly strict regulatory criteria and to either facilitate further treatment by conventional biological systems (11, 21) or for upgrading purposes (22-24).

Among biocatalyst-based approaches to aromatics-containing wastewater, the so-called enzyme-catalyzed polymerization and precipitation (ECPP) process relies on the use of a wide group of oxidoreductases including multi-copper oxidases (MCOs), such as laccase (E.C. 1.10.3.2 *para*-benzenediol: oxygen oxidoreductase) and tyrosinase (E.C. 1.14.18.1 monophenol monoxygenase), and heme-peroxidases (E.C. 1.11.1.7 donor: H_2O_2 peroxidase). These enzymes share the ability to bring about the mono-electronic oxidation of a variety of aromatic pollutants and exhibit a rather relaxed substrate specificity which make them rather versatile catalysts (17, 25, 26). Although the oxidation mechanisms by peroxidases are essentially the same as those by MCOs, the former rely on hydrogen peroxide as the reaction oxidant (26) while both tyrosinase and laccase utilize molecular oxygen (27, 28). The enzyme-generated radicals diffuse from the active site of the enzyme into solution where they undergo non-enzymatic coupling reactions yielding dimers and oligomers (29). These coupling products can spontaneously precipitate out of solution upon attainment of their aqueous solubility limit or can be removed by either filtration or sedimentation (4, 11, 22). In addition, the polymers derived from oxidative coupling seem to exhibit similar mineralization kinetics as naturally occurring lignins and humic acids (30). The basic concept of ECPP was successfully exploited in a pioneering study where horseradish peroxidase (HRP) was found to remove phenols and aromatic amines with 95% or higher efficiency in wastewater (31). Subsequently, process performances of phenol oxidation were found to be independent on HRP purity and an economic analysis proved that the use of crude enzyme preparations resulted in similar costs to those associated with ferrous ions/H_2O_2 catalyst (the so-called Fenton's reagent) (32).

Nonetheless, the main drawback of ECPP is that a large amount of enzyme is required to attain a high removal efficiency; for instance, around 2.4 x 10^5 purpurogallin units of peroxidase per liter wastewater were needed to attain 98% phenol removal from a 53 mM contaminant solution (33). The large biocatalyst requirements are due to the occurrence of deactivation phenomena throughout the enzymatic conversion process (26, 34-37). With regard to peroxidases, three possible deactivation pathways have been described: (i) irreversible inactivation, a form of suicide inhibition, due to the free radicals generated during the catalytic process (36); (ii) inactivation due to end-product polymers formed during the catalytic process which may adsorb the enzyme and co-precipitate it when they exceed their solubility limit in water (33); (iii) excess hydrogen peroxide resulting in the formation of either Compound III or verdohemoprotein (P-670), that are the transient and the permanent catalytically inactive form of the enzyme, respectively (38-40) (Figure 1). It has been suggested that the second mechanism is dominant during peroxidases catalysis (33) and that the third one might be minimized by either determining the optimal oxidant/substrate molar ratio (41) or by gradually adding H_2O_2 with the aid of a peristaltic pump (42). Differently, due to their dependence on molecular oxygen as the reaction oxidant, laccases are apparently only susceptible to the first and the second inactivation mechanism (19, 28). Tyrosinase, instead, is able to catalyze two distinct oxygen-dependent reactions, namely the hydroxylation of monophenols to *ortho*-diphenols (*i.e.,* monophenolase activity) and the oxidation of *ortho*-diphenols to *ortho*-quinones (*i.e.*, diphenolase or catecholase activity) which undergo subsequent non-enzymatic polymerization (27). In this respect, the monophenolase activity of tyrosinase towards certain substrates such *N*,*N*-dimethyltyramine (43) and 4-*tert*-butylphenol (44) was found to be hydrogen peroxide-dependent and a mechanism of enzyme inactivation by H_2O_2 has been proposed (45).

Given that the amount of enzyme required represents the most significant treatment cost, it is of paramount importance to minimize enzyme inactivation phenomena (20). Thus, it has been argued that the accomplishment of this goal might result in both a

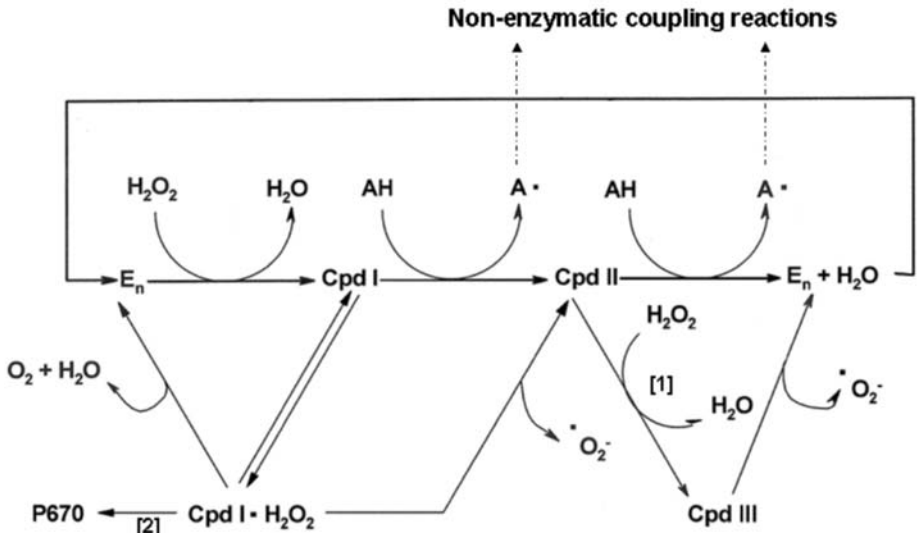

Figure 1. Catalytic cycle and side reactions of peroxidases: E_n is the native enzyme; Cpd I, Cpd II and Cpd III are Compound I, II and III, respectively; Cpd I • H_2O_2 is an intermediate enzyme-hydrogen peroxide complex; P670 is verdohemoprotein; AH and A• are the reducing substrate and its respective radical, respectively. Arrows in bold indicate main catalytic reactions reactions while dotted arrows indicate non-enzymatic reactions (*i.e.*, oxidative coupling) starting from radical species. Reactions [1] and [2] lead to the formation of the transient (*i.e.*, Cpd III) and permanent (*i.e.*, P670) catalytically inactive forms of the enzyme, respectively. Modified from (63).

reduction in enzyme requirements and in the maximization of reaction rates (20, 46).

An additional constraint of these enzymatic applications is the incomplete removal of oxidation products from solution which is due to the accumulation of water-soluble oligomers (33, 41, 46). Depending on the phenol treated, for instance, the soluble oxidation products can be even more toxic than the parent contaminant, as observed for 2-methylphenol, 2-chlorophenol and 2,4-chlorophenol oxidation intermediates (47-49). Besides being toxic, soluble and/or suspended reaction products may increase oxygen demand and alter the permeability of solar radiation in receiving water bodies due to their chromophoric nature (50, 51); thus their removal from the effluent is of paramount importance.

In order to put ECPP into practice, both deactivation phenomena and persistence of soluble oxidation products in solution have to be counteracted. Although several approaches, such as enzyme immobilization (52), reaction engineering (41, 53, 54) and use of redox mediators (55), have been proposed to limit these constraints, the aim of this review is to focus the attention on some organic and inorganic additives so far employed for these specific purposes. Since these substances can be added either before reaction initiation or after its completion, they will be referred within this review to as reaction and post-reaction additives, respectively.

3. REACTION ADDITIVES

A variety of organic substances have been used in ECPP processes with the aim of either reducing the catalyst requirements to accomplish defined pollutant removal yields or to facilitate the removal of persistent soluble oxidation products. In some cases, these two goals are tightly connected since some oxidation intermediates can act as enzyme inhibitors (33, 34). On the basis of their solubility properties, these additives can be further divided into two sub-groups, namely water-soluble and water-insoluble agents, the effects of which are discussed within separated sub-sections.

3.1. Water-soluble reaction additives

3.1.1. Polyethylene glycol

Polyethylene glycol (PEG) was found to limit HRP inactivation during phenol oxidation thus allowing up to 200 times reduction in enzyme requirements (33). In addition, it was found to be a very suitable additive in ECPP processes due to its minimum effective concentration required, easy separation from solution as a co-precipitate with the enzymatic products formed, non-toxicity and cost-efficacy (56). The viability of using PEG as an additive in ECPP applications was confirmed by several subsequent studies and Table 1 provides a synopsis of PEG-assisted conversions of a variety of pollutants and relative incubation conditions where a 90-95% removal was selected as the target clean-up level. To explain the mechanism by which PEG protected the enzyme, Nakamoto and Machida (33) proposed the "sacrificial polymer" theory which postulates the interaction of the additive with the hydrogen binding sites of oxidative coupling products (OCP) thus preventing the enzyme from adhering to the precipitating product. Although their results were largely relevant, the pollutant concentrations used (*i.e.*, 10-30 g l^{-1} phenol corresponding to 106 and 318 mM, respectively) were largely outside the concentration ranges generally encountered in industrial wastewaters (11, 57) and the amounts of additives employed not economically

Table 1. PEG-assisted enzymatic removal of phenolic compounds and aromatic amines.

Compound	Enzyme	pH	Compound concentration (mM)	PEG dose[1] (mg l^{-1})	Optimum enzyme activity[1] (U ml^{-1})		REFR[2]	Reaction time[1] (h)	Oxidant to substrate ratio[1]	Ref.
					With PEG	Without PEG				
Phenol	SBP[5]	6.0[12]	1.0	50	0.60	0.9	1.5	3	1.2	(41)
Phenol	HRP[6]	8.0	1.0	31	0.05	2.0	40	3	1.0	(58)
Phenol	HRP[6]	8.0	10.0	248	0.40	30.0	75	5	1.0	(58)
Phenol	HRP[6]	6.0	100	4000	1.20	240	200	n. r.[3]	0.05	(33)
Phenol	HRP[6]	7.0	1.0	32	0.065	1.44	22.1	12	1.0	(11)
Phenol	HRP[6]	7.0	16.0	194	1.04	22.96	22.1	12	1.0	(11)
Phenol	SBP[5]	7.0	1.0	40	1.35	0.32	4.2	3	2	(64)
Phenol	TP[7]	6.0	0.5	100	1.3	0.23	5.6	3	2	(60)
Phenol	*Tv*L[8]	5.0	1.0	50	0.08	0.08	n.e.[4]	3	0.05	(10)
2-chlorophenol	SBP[5]	7.5[12]	1.0	40	0.19	0.23	1.2	3	0.8	(41)
2-chlorophenol	TP[7]	6.0	0.5	200	1.3	0.23	5.6	3	2.0	(60)
3-chlorophenol	SBP[5]	5.0[12]	1.0	75	0.15	0.65	4.3	3	0.6	(41)
3-chlorophenol	MT[9]	7.0	0.5	400	48.0	48.0	n.e.[4]	6	1.0	(37)
4-chlorophenol	SBP[5]	8.0[12]	1.0	30	0.15	0.2	1.3	3	0.8	(41)
4-chlorophenol	MT[9]	7.0	0.5	400	48.0	48.0	n.e.[4]	3	0.53	(37)
o-cresol	SBP[5]	7.0[12]	1.0	400	0.08	0.6	7.50	3	0.9	(41)
m-cresol	SBP[5]	7.0[12]	1.0	150	0.08	0.75	9.4	3	1	(41)
p-cresol	SBP[5]	7.0[12]	1.0	30	0.4	0.6	1.5	3	0.9	(41)
2,4-dimethylphenol	*Tv*L[8]	5.2	1.0	1	0.004	0.008	2.0	3	0.26	(54)
2,4-dichlorophenol	SBP[5]	7.0[12]	1.0	150	0.04	0.08	2.0	3	0.7	(41)
2,4-dichlorophenol	*Le*L[10]	4.2	8.0	50	0.15	2.00	33.3	15	0.03	(70)
Bisphenol A	SBP[5]	6.5[12]	0.5	60	0.015	0.9	60.0	3	1.2	(41)
Bisphenol A	*Tv*L[8]	5.6	0.5	75	0.004	0.001	4.0	3	0.05	(71)
Bisphenol A	*Tv*L[8]	5.6	1.0	145	0.002	0.01	5.0	3	0.26	(71)
Bisphenol A	*Tve*L[11]	5.0	0.12	5	0.45	0.30	1.5	2	2.16	(46)
Triclosan[13]	*Tve*L[11]	5.0	0.02	50	1.50	3.00	2.0	4	13.3	(55)
Estrone	*Tve*L[11]	6.0	0.004	50	0.8	0.8	1.0	1	n. r.[3]	(81)
Estriol	*Tve*L[11]	6.0	0.004	50	0.8	0.8	1.0	1	n. r.[3]	(81)
Hydroquinone	SBP[5]	6.5	1.5	150	0.005	0.005	1.0	3	1.5	(4)
Resorcinol	SBP[5]	7.5	2.0	150	0.200	0.200	1.0	3	2.0	(4)
Catechol	SBP[5]	6.5	2.5	150	0.025	0.025	1.0	3	1.5	(4)
o-phenylenediamine	SBP[5]	4.5	1.5	150	0.002	0.002	1.0	3	1.5	(4)
m-phenylenediamine	SBP[5]	5.4	2.0	150	0.010	0.010	1.0	3	2.0	(4)
p-phenylenediamine	SBP[5]	5.6	1.5	150	0.005	0.005	1.0	3	1.5	(4)
diphenylamine	*Tv*L[8]	7.0	0.2	200	0.0075	0.002	3.75	3	1.4	(75)

PEG dose, enzyme activity and incubation conditions (*i.e.*, reaction time and oxidant to substrate ratio) enabling 90-95% substrate conversion,[1] calculated by the ratio between the enzyme load in the absence of the additive to that one in its presence,[2] not reported,[3] no effect,[4] soybean peroxidase,[5] horseradish peroxidase,[6] turnip peroxidase,[7] *Trametes villosa* laccase,[8] mushroom tyrosinase,[9] *Lentinula edodes* laccase,[10] *Trametes versicolor* laccase,[11] absence of a buffering system and pH modified by either NaOH or HCl addition,[12] Triclosan, 2,4,4'-trichloro-2'-hydroxydiphenylether.[13]

feasible. This opened the way to an array of investigations aimed to validate the sacrificial polymer theory, to assess the effect of PEG under more realistic pollutant concentrations and to optimize its use. One year later than the study of Nakamoto and Machida (33), the effect of PEG on the HRP-catalyzed phenol removal from a synthetic wastewater was confirmed over a realistic concentration range of the pollutant (1-10 mM) and its dose was largely reduced (58). In particular, the catalyst requirements to attain 95% removal from 1 and 10 mM solutions were reduced by 40 and 75-fold, respectively, at PEG concentrations as low as 31 and 248 mg l^{-1} (58). In a successive study, where 90% removal was selected as the target clean-up level, the use of PEG at 32 mg l^{-1} besides allowing a 22-fold reduction in enzyme requirement, improved the catalytic life-time (CLT), defined as the number of phenol molecules precipitated per molecule of HRP added to solution (11). The same study showed that the removal performances in a continuous stirred tank reactor (CSTR) were higher than in a batch reactor configuration due to the markedly reduced residence time required to accomplish the same degree of phenol removal; comparative experiments conducted on 1, 5 and 10 mM phenol solutions showed that PEG addition reduced enzyme requirements by 92, 91 and 95%, respectively, and enabled 12.5-, 11.3- and 18.4-fold increases in the respective CTLs of HRP (11).

The phenol content of a petroleum refinery wastewater was reduced below the discharge limit by HRP-treatment leading to approx. 58, 78 and 95% reductions of COD, BOD_5 and toxicity, respectively, and the use of PEG (60 mg l^{-1}) resulted in a 4-fold reduction in enzyme requirements (53). Investigations on PEG effects were successively extended to other peroxidases systems from both plant (41, 59, 60) and microbial sources (61, 62). Among them, soybean peroxidases (SBP) was reported to be cheaper than HRP and deemed to be a promising catalyst due to its ability to act on a broad range of compounds and retain its activity over wide ranges of pH and temperature (63). With SBP, it was found that the stimulating effect of PEG in the oxidation of several chloro- and methyl-substituted phenols and BPA was only confined to *m*-cresol and BPA leading to a relative enzyme

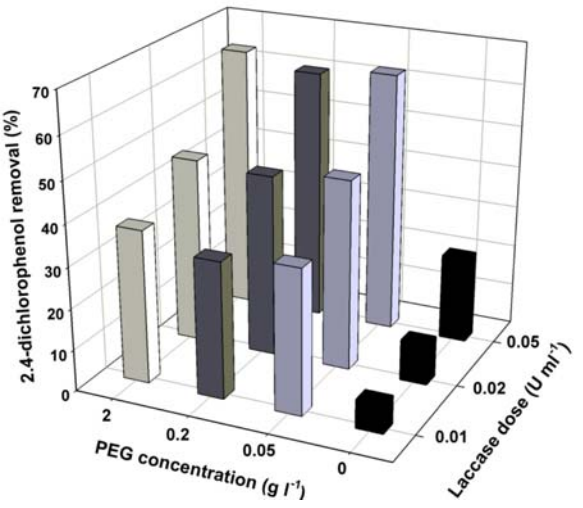

Figure 2. Per cent removal of 2,4-dichlorophenol (8 mM) from solution as a function of laccase dose (0.01, 0.02, 0.05 IU ml^{-1}) and amount of PEG 4000 added to the reaction mixture (0, 0.05, 0.2, 2.0 g l^{-1}). Data are the mean of three parallel experiments and relative coefficients of variation were less than or equal to 7%. Modified from (70).

fold reduction (REFR) of 9.3 and 6.0, respectively, as shown in Table 1 (41). The most efficient PEG in supporting phenol oxidation by SBP had an average molecular weight of 35000 Da and allowed a 4.2-fold reduction in the activity required to attain 95% removal of the pollutant (64). The oxidation performances of a turnip (*Brassica napus*) peroxidase towards a variety of substituted phenols were positively affected by the presence of PEG in the 100-200 mg l^{-1} concentration range enabling an average 5.5-fold reduction in enzyme requirement (60).

To improve process economics, several studies demonstrated that plant materials, such as potato tubers and horseradish roots (65), and soybean hulls (66) were effective carriers of peroxidase activity able to remove phenols, anilines, and other aromatic compounds from water solutions (65). Unfortunately, wastewater treatment applications with plant materials had the same drawbacks (*i.e.*, inhibition of enzyme activity and color formation) as those based on the use of isolated enzymes (65, 67). PEG applied at a relatively moderate concentration of 100 mg l^{-1} enabled high 2,4-dichlorophenol (2,4-DCP) removals (90–95%) by minced horseradish within a broad range of 2,4-DCP concentrations (1.0-9.0 mM) thus extending the applicability of the additive to wastewater treatment application relying on plant parts as enzyme carriers (68).

The effect of PEG, however, appeared to be significantly affected by the structural properties of the target contaminant. This was made evident by the absence of any significant effect of this additive on the conversion of phenylenediamines and benzenediols by soybean peroxidases (4) and diaminotoluenes by both SBP and *Arthromyces ramosus* peroxidases (69). The lack of impact of PEG on the enzymatic transformation of diols and diamines in contrast to that shown on simple phenols and anilines oxidation, is due to the fact that the former group do not form precipitates and thus the additive cannot exert its protection towards the catalyst through the "sacrificial polymer" mechanism (4). The low susceptibility of benzenediols- and aryldiamine-derived oxidation products to precipitate was made evident by the low variations in the total organic content in supernatants of peroxidases-treated solutions despite the degrees of conversion of parent compounds exceeded 80%; thus, this evidence outlined the need of using post-reaction additives as coagulating agents, the effects of which will be discussed later in section 4 of this review (4). PEG addition improved the operational stability of two immobilized turnip peroxidase (TP) preparations employed in the treatment of a synthetic wastewater containing a mixture of chloro-substituted phenols (59); in the same study, the combined use of PEG at 100 mg l^{-1} and a reduced contact time (10 min) allowed an efficiency removal higher than 90% after 10 treatment cycles of a paint wastewater in a batch reactor packed with a covalently-bound TP (59).

The use of PEG as an additive to either suppress or minimize laccase inactivation has been less studied than peroxidases (10, 46, 54, 70-72). In this respect, the first report regarded *Lentinula edodes* laccase (*Le*L), the activity of which required to halve 2,4-DCP concentration from 4, 8 and 16 mM solutions was 35-, 39- and 20-fold lower in the presence of PEG 4000 than in its absence, respectively (70). Figure 2 shows that a 40-fold reduction in PEG concentration did not downgrade the stimulating effect of the polymer on the *Le*L-catalyzed DCP removal (70). In the same study, under the incubation conditions employed for 2,4-DCP conversion, the presence of PEG increased the half-life of *Le*L from 26 to 31 h while the initial reaction velocity was not affected (70). These results were apparently in contrast with those of Modaressi et al. (71) who showed that a pre-incubation of *Trametes villosa* laccase SP-504 (*Tv*L) with PEG in the 25-75 mg l^{-1} concentration range led to a 12-18% increase in the initial velocity of BPA oxidation.

Structure-function analysis revealed that the globular structure of PEG, arising from its tendency to fold upon itself and bind water molecules (73), may be important for prevention of laccase inactivation (72). This hypothesis is consistent with several studies showing that the extent of suppression of minimization of enzyme inactivation tends to increase as the average molecular weight of this polymer increases (33, 46, 68, 74-76). In this respect, it has been shown that PEG is able to bind significantly more water as its average molecular weight is higher than 600 Da (73, 77). In addition, the ability of PEG attached to a surface to reject proteins was reported by some investigators that hypothesized that too much energy was required for a protein to penetrate through the hydrated layer and to reach the surface (78). In the same way, PEG adsorbed onto the polymeric oxidation products could act by repelling peroxidase molecules thus contributing to the reduction in enzyme inactivation (64, 79). Some researchers found the protective effect of PEG on *Tv*L during treatment of a 1 mM BPA solution, the removal of

which required a 5-fold lower enzyme activity than in the absence of the additive (71). The fate of the PEG after the treatment was assessed in the same study since the possible residual presence of the additive might represent an additional burden to subsequent treatment processes. In this respect, several studies showed that the benefits associated with the PEG addition leveled off after a threshold concentration of the additive (64, 71) thus highlighting the need of optimizing its use. The risks associated with the persistence of residual additive in solution after reaction completion had also been reported for gelatin, the use of which led to the formation of colored soluble oxidation products which could not be removed either by adding alum or by centrifugation (80).

In a successive study, *Tv*L-catalyzed oxidative polymerization of 2,4-dimethylphenol (DMP) was studied with the aim of removing at least 95% of its initial concentration after a 3 h incubation time (54); regardless of the initial pollutant concentration, the enzyme activity required to accomplish that task in the presence of PEG was halved as compared to reactions conducted in its absence (Table 1).

The oxidation of triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether), a broad spectrum anti-microbial agent, by *T. versicolor* laccase (*Tve*L) was stimulated in the presence of 50 mg l^{-1} PEG35000, the use of which allowed a two-fold reduction in the activity required to accomplish an approx 90% removal of the contaminant after 90 min (55). It was also observed that the same conversion achieved in 4 h by 1.5 U ml^{-1} laccase in the absence of PEG (*i.e.*,87%) was obtained in approximately 90 min with the same enzyme activity in the presence of the additive (55).

*Tve*L was also investigated for its ability to catalyze the removal of natural *(i.e.,* estrone, 17-beta-estradiol and estriol) and synthetic (*i.e.*, 17-alpha-ethinylestradiol) estrogens from both synthetic and municipal wastewater; high amounts of activity (*i.e.*, 15 U ml^{-1} wastewater) were required to obtain conversions larger than 90% and PEG was unable to protect the enzyme (81).

Regardless of its average molecular weight, PEG was also reported to be unable to both improve the transformation of phenol by mushroom tyrosinase (MT) and to induce precipitation of reaction products (37). The failure of PEG to protect tyrosinase, in contrast to its strong ability to protect peroxidase, was suggested to be due to the differences in nature of the oxidized products of these two enzymes. In this respect, the *o*-quinones formed during the tyrosinase- catalyzed oxidation of phenols, in fact, react to form substantially soluble polymers with low molecular mass (82) and thus PEG may not have the opportunity to protect tyrosinase from precipitating products. In a comparative study examining the phenol removal ability of SBP and *Tv*L from a refinery wastewater, it was found that PEG enabled 10 to 35% reduction in the SBP activity required to achieve 90% conversion while it had no effect on laccase requirement (10).

3.1.2. Miscellaneous water-soluble compounds and polymers

Nakamoto and Machida (33) reported that some additives, such as borate and gelatin, strongly reduced HRP inactivation during phenol oxidation thus leading to significant savings in enzyme requirements (*i.e.,* up to 200-fold reductions). On the one hand, the latter additive, added at a rate of 4 g l^{-1}, led to a 98% phenol removal from a 100 mM synthetic wastewater and similar removal performances were obtained with a wastewater from a semiconductor manufacturing plant (33). On the other hand, the use of borate was considered impractical since its protective effect towards HRP was exerted at concentrations as high as 0.4 M and, due to its insecticidal properties, it was not deemed to be environmentally-sound.

The impact of several water-soluble polymers on the oxidation of naphthol congeners by recombinant laccases from *Polyporus pinsitus* (r*Pp*L), *Myceliophthora thermophila* (r*Mt*L), *Coprinus cinereus* (r*Cc*L) and *Rhizoctonia solani* (r*Rs*L) was investigated by Kulys et al. (72). Kinetic oxygen uptake measurements showed that partial inactivation of laccases occurred during naphthols oxidation and that the presence of the additives led to significant changes in the oxidation profiles. The use of certain polymers, including polyvinyl alcohol (PVA), ficoll, hydroxyethyl-cellulose, dextrans 500, 110 and 20, polyvinylpyrrolidone, albumins and PEG, increased total O_2 consumption without affecting the initial reaction rate (72). With specific regard to PEG, the presence of the additive (50 mg l^{-1}) led to lower inactivation constants of r*Mt*L and r*Cc*L than in its absence (0.014 *vs.* 0.43 and 0.002 *vs.* 0.63 mM^{-1} s^{-1} , respectively) during 1-naphthol oxidation. Interestingly, PVA exerted a higher protective effect on laccase than PEG during 1-naphthol oxidation. In this respect, it was found that PVA had a higher degree of protection than PEG to reactive oxygen species (*i.e.*, superoxide anion, hydroxyl radicals) generated during sonication of *Tv*L (83) and this was ascribed to the different behavior of these two polymers in solution. PVA molecules, unlike PEG, in fact, aggregate instantaneously when dissolved in water, thus creating a hydrophobic layer around the enzyme (83). In this respect, the authors suggested the ability of these polymers to bind hydrophobic naphthyl radicals thus preventing inactivation of laccases (72); it cannot be ruled out, however, an additional protection from reactive oxygen species which might be generated during laccase catalysis (84). Cationic polymers, such as diethylaminoethyl-dextran, poly-*L*-lysine and protasan, did not change total oxygen consumption but reduced the initial naphthol oxidation rate, the latter effect being supposed to be associated with complex formation between positively charged polymers with negatively charged laccases, due to their acidic isoelectric points (72). Finally, negatively charged polymers able to form random coil in solution, such as alginic acid, polyacrylic acid and heparin, were reported to be unable to affect both total oxygen consumption and initial reaction rate (72).

3.1.3. Surfactants

Surfactants were also found to stimulate *in vitro* pollutants degradation by both laccase (85), tyrosinase (74)

and peroxidase (68, 86-89). Having both polar and non-polar domains, surfactants are able to partition at water-oil and water-air interfaces thus reducing the interfacial or surface tension (90, 91). Above a threshold concentration, termed critical micelle concentration (CMC), surfactant molecules tend to cluster together and start forming dynamic aggregates known as micelles (90). With this regard, the effects of surfactants on ECPP processes were found to vary depending on whether they had been added below or above their respective CMC (sub- and supra-CMC conditions, respectively) (85-89).

The use of Dynol 604, an acetylenic-based surfactant able to reduce both equilibrium and dynamic surface tension properties (91), did not affect the initial reaction velocities of oxidation of a variety of phenols by recombinant *C. cinereus* peroxidase (r*Cc*P) under sub-CMC conditions (87). This surfactant, however, enhanced the conversion of naphthol congeners and 1-hydroxypyrene in a dose-response manner and this effect was explained on the basis of its ability to avoid enzyme's active centre clothing by oligomeric oxidation intermediates (87, 88). The increase in light scattering of the reaction mixtures in the presence of Dynol 604 during r*Cc*P-catalyzed naphthol oxidation indicated the formation of oligomers/surfactants aggregates (87).

The adsorption of *C. cinereus* peroxidase (*Cc*P) onto polymeric oxidation products during phenol oxidation was found to be the predominant mechanism of enzyme inactivation and Triton X-100 was able to perform its desorption in a catalytically-active form (89). The *Cc*P requirement for almost quantitative phenol (100 mg l^{-1}) removal was reduced to about one-fourth in the presence of sub-CMC of Triton X-100 (30 mg l^{-1}) with respect to the control reaction where the surfactant had been omitted (86). The same study found that Triton X-100, Triton X-405 and Tween 20, which are non-ionic surfactants containing poly(oxyethene) residues, had a similar ability to enhance phenol oxidation but their stimulating effect was lower than that exerted by PEG 3000 (86).

The removal of 2,4-DCP by HRP markedly increased in the presence of either natural (*i.e.*, rhamnolipids) or synthetic (*i.e.*, Triton X-100, SDS, Nonidet P40 and Tween 20) surfactants (68), that had been added at concentrations lower than their respective CMCs. In another study, under supra-CMC conditions, Tween 80 caused a noticeable reduction in pentachlorophenol (PCP) oxidation by HRP (92). Conversely, an approx. 2.5-fold increase of PCP oxidation was observed under sub-CMC conditions with respect to reaction conducted in the absence of the surfactant (92). Similar results were obtained with *Tve*L, the BPA conversion of which was stimulated by the addition of Triton X-100 only at concentrations lower than or close to its CMC (85). In the same work, endogenous fluorescence spectroscopy studies indicated the occurrence of an interaction between Triton X-100 and laccase, which was beneficial to the folding and the stabilization of laccase (85). The decreased pollutant conversions observed in the presence of surfactant concentrations higher than CMC have been ascribed to the partitioning of the target compound into the micelle pseudo-phase thus precluding its contact with the enzyme (85, 92).

A positive effect of surfactants was also reported for tyrosinase; in particular, the addition of sodium dodecyl sulphate (SDS), Triton X-100 and Tween 20 markedly enhanced 2,4-DCP conversion by *Bacillus thuringiensis* tyrosinase from 36 to 78, 80 and 85%, respectively (74).

In another study, although the addition of the positively charged surfactant alkyl-betaine slightly enhanced BPA conversion by *Tve*L, it was unable to counteract the inhibitory effects of both cyanide (5 mg l^{-1}) and fluoride (25 mM) (46), which are known inhibitors of laccases (93). Although the assessment of a possible protective effect of the additive in the presence of inhibitors is an important task, the tested concentrations were higher than those normally encountered in wastewater. As a matter of fact, the removal of both natural and synthetic estrogens by *Tve*L from municipal wastewater containing a variety of laccase inhibitors (*i.e.*, fluoride, chloride and cyanide) was found to be unaffected with respect to a synthetic waster containing the same concentrations of estrogens (81). Additional degradation studies were performed with HRP and *Tve*L in the presence of ions commonly found in petrochemical effluents (46, 94). Some of these ions (*e.g.*, sulfite and thiosulfate) had a negative effect on pollutant conversion by outcompeting the enzyme for oxidant and this phenomenon was most relevant under oxidant-limiting conditions. Consequently, the extent of inhibition by some of the reducing anions, which is not affected by the presence of additives, could be overcome by higher aeration and hydrogen peroxide/substrate ratios for laccase and peroxidase, respectively (94).

3.2. Water-insoluble reaction additives

3.2.1. Polysaccharides

Among three natural polymers, including cellulose, chitin and chitosan, added to tyrosinase reaction mixtures to remove tyrosinase-catalyzed phenol oxidation products, cellulose was the least effective while chitin, and to a higher extent chitosan, led to extensive removal of soluble colored products (51). Cellulose differs from chitin since the hydroxyl substituent at the C2 position of its monomers is replaced by an *N*-acetylamino group in the latter polymer. It is widely known that carbonyl groups in quinones are easily attacked by the lone pair of the nitrogen in amino group in a nucleophilic reaction leading to covalent C-N bonds; with this regard, chitosan was more effective than chitin in virtue of the lack of a steric hindrance effect exerted by the acetyl group (95). Figure 3 shows that quinone derivatives obtained from the tyrosinase-catalyzed oxidation of *para*-substituted phenols can undergo either Schiff base or Michael's-type addition reaction with the amino substituents of chitosan (96). The removal of enzyme-generated quinones by a two-step approach involving tyrosinase oxidation and subsequent chemisorption of quinone products is of paramount importance since they are often more toxic than their precursors (47, 48, 97).

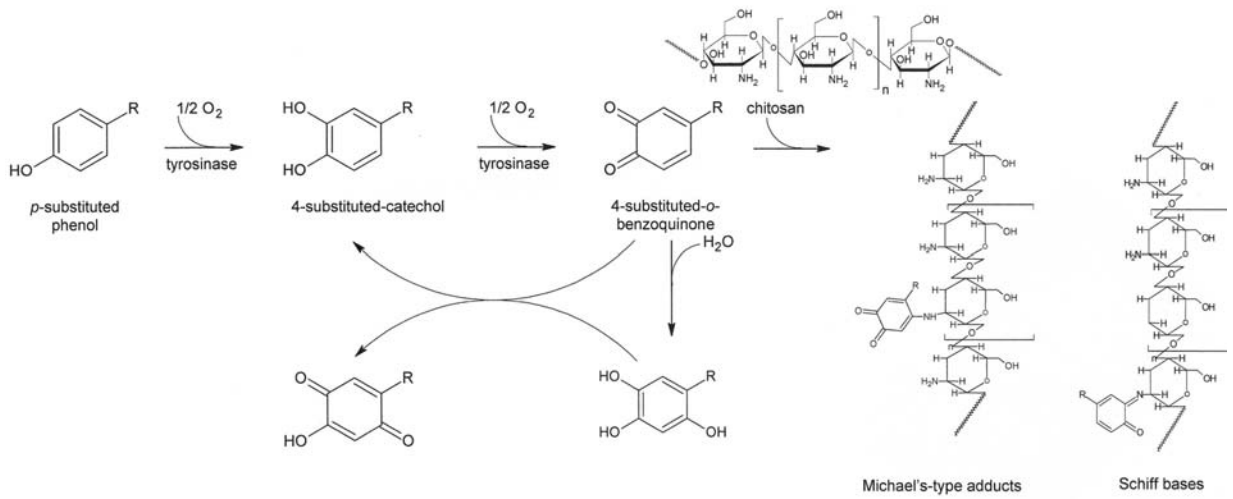

Figure 3. Removal of *para*-substituted phenols by tyrosinase-catalyzed oxidation and subsequent reaction between enzyme-generated *ortho*-benzoquinones and amino groups of chitosan yielding either Schiff bases or Michael's-type adducts. Modified from (96).

This two-step approach was first used to selectively separate phenols from their respective non-phenolic isomers in view of its application for treatment of intermediate process streams (95). Cresol was successfully removed by the MT/chitosan system and no losses of its non-phenolic isomers (*i.e.*, anisole and benzyl alcohol) were observed thus highlighting the selectivity of the approach (95). In the same study, the presence of chitosan markedly enhanced the tyrosinase-catalyzed *para*-cresol conversion and it was suggested that the sequestration of *ortho*-quinones by the polymer avoided their reaction with free amino groups of the enzyme thus minimizing deactivation phenomena (95). Consequently, chitosan exerts both a sorption and a protective effect in tyrosinase conversions (95, 98). In another study, this two-step phenols removal process was found to be advantageous over conventional adsorption onto activated carbon because of the strong binding between quinones and chitosan (50). In fact, quinone-chitosan adsorption enthalpies amounted to -25 kcal mol^{-1}, while those related to phenol/quinone adsorption onto activated carbon were only -7 kcal mol^{-1} (50). In this respect, since adsorption affinities are exponentially dependent on adsorption enthalpies (99), the tyrosinase reaction/chitosan adsorption is a very promising approach for the removal of phenols at trace levels (50). Tyrosinase reaction/chitosan adsorption was also successfully employed for the removal of the polymerization storage inhibitor *tert*-butylcatechol in alternative to multiple-equilibrium-staged operations (100). To this purpose, tyrosinase from either mushroom or bacteria were covalently immobilized to chitosan beads which served as both catalyst and adsorbent for the phenolic inhibitor (100).

In a successive study, although the chitosan addition was very effective in inducing the precipitation of reaction products in the 5-7 pH range, the presence of the polymer did not significantly reduce the amount of tyrosinase required to remove phenol from solution and, thus, was not able to protect tyrosinase from inactivation (101). To explain these findings, that apparently contradicted previous studies (50, 51), Ikehata and Nicell (101) suggested that chitosan might protect the enzyme from inhibition, which limits the initial reaction rate, but not from inactivation, which, instead, negatively affects the extent of transformation. The inefficacy of chitosan above pH 7 was ascribed to an extensive deprotonation of the amino groups of the polymer that might negatively affect its ability to interact with negatively charged suspended reaction products (101). An interesting variant of the tyrosinase oxidation/chemisorption approach, based on moist enzyme-coated chitosan films, was successfully employed for the removal of volatile phenols from the vapor phase (102).

Chitosan was also applied to peroxidase-based systems; for instance, at an optimal concentration of 30 mg l^{-1}, the presence of chitosan led to a 25-fold reduction in the HRP amount in the treatment of a petroleum refinery waste where phenol had been degraded below the discharge limits (53).

3.2.2. Aromatic polymers

The HRP-mediated oxidative coupling reactions of phenol were studied in the presence of lignin and polymethylstyrene (PMS) representing models of natural geosorbents with different and well-defined physicochemical properties (103). Each additive significantly affected peroxidase-catalyzed phenol coupling either by mitigating enzyme inactivation or by participating in cross-coupling reactions or by a combination of these effects. As opposed to PMS, lignin greatly reduced HRP inactivation constants *via* adsorption and the investigators postulated that relatively hydrophilic solids can alleviate peroxidase inactivation by forming enzyme-solid associations (103). An enhanced formation of OCP, however, was observed both in the presence of PMS and lignin and this effect was shown to be due to the occurrence

of cross-coupling reactions. The presence of either aromatic rings or unsaturated C-C bonds were suggested to be the structural requisites of solid-phase materials for their participation in cross-coupling reactions (103). In this respect, cross-coupling reaction of aryloxyradicals with relatively inert chemicals, such as polychlorinated biphenyls and polycyclic aromatic hydrocarbons, has been shown to occur during HRP-catalyzed phenol oxidation (104). The enhancement of formation of OCP in the presence of organic solids such as PMS was discussed on the basis of the mechanisms of HRP catalysis understood to date. With this regard, the HRP-mediated radical production has recently been shown to be a reversible process since the radicals generated during catalysis can abstract electrons from the enzyme intermediates *via* "reverse electron transfer" thus reverting to the original substrate (105). Consequently, in a solids-free system where self-coupling reactions dominate, radical-radical coupling essentially competes with the radical disappearance pathway of reverse electron transfer. The addition of a reactive organic solid matrix, such as PMS, results in an enhanced OCP production in the HRP/phenol system since phenoxyl radicals may react with this matrix, providing an additional pathway by which OCP formation can compete with reverse electron transfer (103).

Similar results were obtained by Wagner and Nicell (106) that observed an enhancement of HRP-catalyzed phenol removal in the presence of both soluble and colloidal constituents of peat moss, the major components of which are humic substances (*i.e.*, humin, humic and fulvic acids). An enhancement of removal of 2,4-DCP by HRP and the occurrence of cross-coupling products had been previously observed when enzymatic reactions were conducted in the presence of both fulvic acids (107) and humic acids (108).

3.2.3. Inorganic additives

Huang and Weber (103) investigated the impact of silica sand on HRP-mediated coupling reactions by selecting solid/water ratios (*i.e.,* 5-50 g l^{-1}) that prevented any physical adsorption of phenol and its dissolved coupling products to the model solid; under those conditions, silica sand was able to mitigate HRP inactivation during phenol oxidation and this protective effect was found to be due to the sorption of the native enzyme and its catalytically active intermediates (*i.e.*, Compound I and Compound II) on the surface of the solid additive (103).

Hydrated aluminosilicate clay minerals have been also shown to positively affect ECPP processes (106, 109). In particular, kaolin and bentonite enhanced the HRP-catalyzed phenol removal (110). Even though HRP was inactivated in fresh bentonite suspensions, the rate of inactivation by the solid was markedly lower than the rate at which enzyme-inactivating reaction products were adsorbed (106). Thus, bentonite exerted its impact through an extension of the enzyme's CLT, resulting in a higher extent of phenol conversion (106). Arseguel and Baboulene (109) also reported that HRP was inactivated when incubated with hydrophobic talc; phenol transformation, however, was markedly enhanced when the mineral was added after reaction initiation (109).

4. POST-REACTION ADDITIVES

This section deals with both organic or inorganic substances which are added after reaction completion with the main aim of removing persistent oxidation products either *via* adsorption or coagulation. Table 2 shows a synoptic frame of the post-reaction additives that have been used after enzyme conversion of both phenolic compounds and aromatic amines with relative doses, contact times and removal yields of oxidation products.

Chitosan was employed as a flocculating agent by adding it at the end of tyrosinase treatment of a synthetic wastewater containing *p*-chlorophenol (98); in this case, chitosan was effective within a limited concentration range (40-90 mg l^{-1}) outside of which flocculation did not occur. To explain this effect, the investigators postulated that in the presence of excess polymer, all adsorptive sites on the suspended particles are occupied by adsorption of individual molecules and thus bridging is minimized in agreement with Michaels's theory (111); in the case of insufficiency of the coagulant, instead, the oxidation products cannot aggregate. Cationic polymer containing amino groups, such as hexamethylene diamine-epichlorohydrin polycondensate (HEP) and polyethyleneimine (PEI), were also effective coagulants of tyrosinase-catalyzed reaction products of chlorinated phenols and anilines and their optimum dosage range was from 10 to 90 mg l^{-1} (98).

In another study, chitosan was found to be better than alum, HEP, polyacrylamide and PEI in the removal of HRP-generated soluble oxidation products of chlorophenols (112); interestingly, a chitosan concentration as low as 2 mg l^{-1} was sufficient to remove more than 95% soluble products (Table 2).

Six different viscosity grades (10-5700 cps) of chitosan and alum were compared for their ability to remove colored oxidation products from phenol oxidation by tyrosinase (101). Alum was scarcely effective in removing soluble oxidation products while, unlike treatments conducted without any additive, dark-brown precipitates and decolorized solutions were obtained in the presence of 100 mg l^{-1} chitosan with the best results being attained with the low viscosity grades (10, 100, and 420 cps). The failure of alum to lead to significant color removal suggested that the phenol oxidation products did not occur in the form of suspended solids and that the small amount of decolorization might be due to either adsorption or entrapment of soluble products into precipitating aluminum hydroxide flocs (101). By contrast, results supported the hypothesis that chitosan exerts its effect *via* chemical interaction of its amino substituents with the carbonyl groups of the colored products; moreover, the highest efficiency obtained with low viscosity grades of the polymer have practical implications, due to the easier preparation of stock solutions thereof derived (101).

Table 2. Removal of enzyme-generated oxidation products by post-reaction additives

Compound	Enzyme source	Precipitating agent	Additive concentration (mg l^{-1})	Adsorption time (min)	Removal soluble oxidation products (%)	Reference
Phenol	MT[1]	Chitin	1400	120	85.4	(51)
Phenol	MT[1]	Chitosan	1400	120	100	(51)
Phenol	MT[1]	Chitosan	40	5	>90	(51)
Phenol	MT[1]	Chitosan	100	180	>90	(101)
Phenol	MT[1]	HEP[8]	30	5	>92	(98)
Phenol	MT[1]	PEI[9]	15	5	>91	(98)
4-methoxyphenol	MT[1]	Chitosan	1400	120	89	(51)
4-chlorophenol	MT[1]	Chitosan	40	5	>90	(98)
2,4-dichlorophenol	BGP[2]	DEAE-cellulose 11	1.0	120	100	(103)
p-cresol	*mel*BT[3]	Chitopearl AL-01	0.025[11]	80	100	(99)
4-n-Heptylphenol	*mel*BT[3]	Chitopearl AL-01	0.025[11]	80	100	(99)
4-n-Nonylphenol	*mel*BT[3]	Chitopearl AL-01	0.025[11]	5	100	(99)
4-tert-Butylphenol	*mel*BT[3]	Chitopearl AL-01	0.025[11]	180	50.2	(99)
2,4-dichlorophenol	*Bt*T[4]	Chitosan	125	n. r.[11]	90	(74)
2,4-dichlorophenol	*Bt*T[4]	Activated Carbon	250	n. r.[11]	80	(74)
2,4-dichlorophenol	HRP[5]	Filtrasorb	10000	180	95	(68)
2,4-dichlorphenol	HRP[5]	Norit	20000	180	95	(68)
2,4-dichlorophenol	HRP[5]	Chitosan	2.0	60	95	(97)
Hydroquinone	SBP[6]	Alum	19	120	60	(4)
Bisphenol A	CIMT[7]	Chitopearl AL-10	0.025[12]	240	100	(110)
Bisphenol T	CIMT[7]	Chitopearl AL-10	0.100[12]	15	60	(110)
Bisphenol C	CIMT[7]	Chitopearl AL-10	0.200[12]	300	98.9	(110)
Bisphenol E	CIMT[7]	Chitopearl AL-10	0.100[12]	300	98.9	(110)
Resorcinol	SBP[6]	Alum	16	120	>80	(4)
Catechol	SBP[6]	Alum	19	120	>80	(4)
Aniline	MT[1]	PEI[9]	40-90	n. r.[12]	>90	(98)
4-cloro-aniline	MT[1]	PEI[9]	40-90	n. r.[12]	>90	(98)
3,4-dichloroaniline	MT[1]	HEP[8]	40-90	5	>90	(98)
o-phenylenediamine	SBP[6]	SDS[10]	144	120	>90	(4)
m-phenylenediamine	SBP[6]	SDS[10]	72	120	>90	(4)
p-phenylenediamine	SBP[6]	SDS[10]	57.7	120	>90	(4)

mushroom tyrosinase,[1] bitter gourd peroxidase,[2] melB tyrosinase,[3] *Bacillus thuringiensis* tyrosinase,[4] horseradish peroxidase,[5] soybean peroxidase,[6] covalently-immobilized mushroom tyrosinase,[7] hexamethylenediamine-epichlorohydrin polycondensate,[8] polyethyleneimine,[9] sodium dodecyl sulphate,[10] not reported,[11] expressed as cm^3 cm^{-3}.[12]

The concept of chemisorption of enzyme-generated phenol oxidation products was successfully exploited in an integrated dephenolization system where a chitosan-packed column located downstream of a polyethersulphone capillary membrane bioreactor containing immobilized tyrosinase facilitated the removal of the colored quinone products from the reactor permeate and enabled the recycling of the phenol-containing wastewater (113). An interesting alternative to this system was reported by Tamura et al. (114) who proposed the use of cross-linked chitosan beads in lieu of commercial ones. With this regard, the removal efficiency of *p*-alkylphenols by covalently immobilized tyrosinase increased with an increase in the amount of cross-linked chitosan beads packed in the column downstream of the enzyme reactor because the rate of quinone adsorption became higher than the rate of enzymatic quinone generation (114).

A further implementation related to the use of this additive was given by use of porous chitosan beads in alternative to acid solutions derived from chitosan flakes. In this respect, it was shown that both linear and branched alkylphenols were completely or effectively removed by *mel*B tyrosinase and subsequent non-enzymatic adsorption on porous chitosan beads (*i.e.*, Chitopearl AL-10) (115). As opposed to another study where little flocculation occurred when chitosan dosage deviated from an optimal concentration range (98), Yamada et al. (115) found that removal efficiency of alkylphenols increased as a function of added chitosan beads; in addition, this additive be readily separated from the reaction solutions after removal of the contaminant (115). For these reasons, the use of chitosan beads is very promising in wastewater applications since they are easier to manage than polymer solution.

Surfactant-mediated separation methods have been also used for the removal of organic species in solution. In particular, adsorptive micellar flocculation with combined SDS and alum (116) was used to remove colored polymeric products after the enzymatic treatment of diphenylamine, while neither SDS nor alum alone were effective in removing these polymeric products (77). The enzymatic conversion of phenylenediamines and benzenediols resulted in color formation without occurrence of any precipitate possibly due to the generation of quinone-like products (4, 69). SDS alone was effective in removing phenylenediamine oxidation products but not the benzenediol ones; conversely, alum alone was effective in removing the latter but not the phenylenediamine products (4). The dominant influence of the cationic nature of the polyaniline products, in attracting the anionic SDS monomer or micelles and in repelling the cationic alum hydrolysis products was surmised to explain these effects (4) thus postulating a charge neutralization rather than a sweep-floc mechanism (117). The removal capacity of benzenediol oxidation products by alum was limited when reactions were performed in phosphate buffer and this was suggested to be due to the formation of aluminum

phosphate gel in lieu of aluminum hydroxide one (4); the hypothesis was confirmed by the higher alum-induced flocculation that was obtained on reaction mixtures that had been run in tap water instead of phosphate buffer (4).

DEAE-cellulose 11 was a very effective adsorbent leading to a quantitative removal of colored oxidation products of phenolic mixture treated by bitter gourd (*Momordica charantia*) peroxidase; this remarkable sorbent capacity was accomplished at 1 mg l^{-1} concentration with a variety of phenolic mixtures (118). In another study, the high phenols binding capacity of Polyclar AT, the fine grade of polyvinylpolypyrrolidone, was used to refine dephenolization obtained by circulating a mixture of phenols through a tubular fixed bed reactor containing co-immobilized *Piricularia oryzae* laccase and MT (119); the use of this approach led to a quantitative removal of naphthol isomers, 2,4-DCP and catechin.

Activated carbon is one of the most common and cheapest adsorbents employed in wastewater treatment and its also exhibits good phenol-binding capacity (120, 121). Activated carbon was also successfully used to remove soluble oxidation products after reaction completion of 2,4-DCP oxidation by both *B. thuringiensis* tyrosinase (74) and HRP (68). Although activated carbon is relatively cheap (121), high amounts of this additive (from 5 to 30 g l^{-1}) were required to obtain percent removals of colored oxidation products higher than 90% (68). In addition, although various techniques *(i.e*., thermal or chemical or wet-air oxidation processes) are currently available for the regeneration of spent activated carbon, their use is not cost-effective.

5. EFFECT OF ADDITIVES ON RESIDUAL TOXICITY

Albeit the use of additives offered important advantages in enhancing enzyme-catalyzed transformation of several pollutants, the possible occurrence of an actual detoxification had to be assessed. Therefore, the detoxification aspect associated with the use of these additives was dealt with in several studies with both laccase and peroxidase (48, 49, 53, 55, 101, 106, 122, 123). In this respect, the use of PEG caused an almost doubling of the residual toxicity in a 0.02 mM triclosan laccase-treated solution as compared with treatment without PEG (55). On the basis of its toxicity towards *Photobacterium phosphoreum (i.e*., EC_{50} = 894 mg l^{-1}), it was estimated that the amount of PEG added to the mixture only accounted for approximately 1% of this increase. The increase in residual toxicity of the solutions treated with PEG was consistent with earlier reports on HRP-catalyzed phenol oxidation in which toxicity increase was ascribed to the enhanced solubility of the oligomeric oxidation products (48, 122). In another study, the use of PEG in *Tve*L-catalyzed BPA oxidation did not significantly affect toxicity with respect of reactions conducted in its absence (46).

However, research has indicated that the particular treatment conditions may significantly affect the quality and quantity of the reaction products that are formed. For example, when PEG was used as a protective additive, there was an increase in the quantity of products that remained in solution following the treatment of phenol (48). In contrast, when the effects PEG and chitosan on residual toxicity of HRP-treated phenol solutions were investigated, it was found that only the latter was able to lead to a time-dependent decline in toxicity with respect to the untreated control (122). Although Sun et al. (50) reported that the chemisorption of reaction products onto chitosan took place within a few minutes, they used a 1000-fold higher chitosan concentration than that employed by Wagner and Nicell (122); thus, this could account for the faster removal rate of reaction products from solution. Synthetic wastewater containing unsubstituted phenol or chloro-substituted phenols were significantly detoxified by adding chitosan either before reaction initiation or after reaction completion with MT (101). Regardless of the presence or the absence of the additives, several strategies were suggested to overcome problems related to the formation of toxic compounds throughout HRP-catalyzed oxidation of phenols (122). Among them, the same investigators suggested that either an increase in the retention time associated with further H_2O_2 supplementation after the completion of the phenol conversion or a controlled H_2O_2 addition in the course of reaction might be advisable for detoxification purposes (122).

Phenol oxidation by HRP was investigated in the presence of bentonite, kaolin and peat moss which were selected as model solid phases which can be encountered in wastewater; HRP-treated solutions containing these additives exhibited markedly lower toxicity than control solutions where these materials had been omitted (106). These results suggested that the nature of reaction products formed in real wastewaters could significantly differ from those obtained in pure phenolic solutions due to the presence of additional components in the former that can interact with reactive phenolic species (106). This was further confirmed by the fact that peroxidase treatments of a petroleum refinery wastewater (53) and a foul condensate from Kraft pulping (123) besides leading to high phenol removal also achieved extensive detoxification.

Samples treated in the presence of 1 g l^{-1} of either bentonite or kaolin were on average 78% and 27% less toxic than the respective reaction mixtures conducted in the absence of these solid phases (106). These differences in the detoxification extents can be ascribed to the different properties of these clay minerals. In this respect, bentonite belongs to the smectites group of clay minerals (124), which are known to expand as they come in contact with water; in particular, the distance between the crystal layers tend to increases as water molecules penetrate into the inter-micellar regions. Consequently, dissolved organic molecules can gain access into the interlayer spaces and become adsorbed. In the case of kaolinites, conversely, adsorption only occurs on the outer surfaces of the mineral (124). Thus, minerals belonging to the smectites group have a much higher capacity to adsorb organic molecules than kaolinites.

6. CONCLUSIVE REMARKS AND PERSPECTIVE

Over the last two decades considerable effort has been devoted to the implementation of ECPP processes by the use of both water-soluble and insoluble additives. Among water-soluble ones, PEG has been shown to be of particular interest as an additive since it significantly reduced the cost of treatment *via* reduction of enzyme requirements (11, 71). The "sacrificial theory", postulated by Nakamoto and Machida (33), has been confirmed by several studies and, in this respect, it has been shown that its protective effect is only confined to those contaminants, the oxidation of which yields substantial amounts of polymers (4). Other limitations associated with the use of PEG are the need to calibrate its amounts in order to avoid its persistence in the treated wastewater and its inability to lead to an extensive detoxification (46, 55, 122). Among water-insoluble additives, instead, chitosan is by far the most interesting one leading to significant protective effects of the catalyst and to a remarkable removal of oxidation products (98, 122). Its use seems to be mainly associated with tyrosinase and the high specificity of the enzyme oxidation/chitosan chemisorption has been even exploited in industrial applications, such as the selective removal of undesired contaminants from intermediate process streams (95, 125) or polymerization storage inhibitors (100).

The application of chitosan has been implemented by the use of either porous and/or cross-linked beads, the use of which has several advantages over acid solutions from chitosan flakes. First of all, the removal of oxidation products is not only efficient within a limited concentration range of the additive (115). Secondly, there is larger surface area available for solid-phase reaction between quinone products and amino groups of the polymer; thirdly, chitosan beads are easily removable from batch reaction and can be confined inside adsorption modules (114, 115). With this regard, the higher affinity of chitosan beads for quinones has been successfully extended from batch reactions to both continuous and recycled systems by inserting a chitosan packed module downstream of an enzyme reactor (113, 114).

Although the use of additives in ECPP applications is promising due to their ability to either reduce enzyme requirements or facilitate removal of persistent oxidation products, it is necessary to gain additional insights into their impact on the residual toxicity of the treated wastewater. First of all, an in-depth structural characterization of reaction products and a mass balance of soluble and insoluble reaction products is needed to understand why the use of certain additives results in a higher toxicity than control reactions (46, 55). In addition, since the large majority of toxicity data have been obtained by the Microtox system, an extended eco-toxicological assessment might be largely advisable.

7. ACKNOWLEDGEMENT

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Abbreviations: BGP: bitter gourd peroxidase; BPA: bisphenol A; *Bt*T: *Bacillus thuringiensis* tyrosinase; *Cc*P: *Coprinus cinereus* peroxidase; CLT: catalytic life-time; CMC: critical micelle concentration; CSTR: continuous stirred tank reactor; 2,4-DCP: 2,4-dichlorophenol; DMP: 2,4-dimethylphenol; EDC: endocrine disrupting chemicals; ECPP: enzyme-catalyzed polymerization and precipitation; HEP: hexamethylene diamine-epichlorohydrin polycondensate; HRP: horseradish peroxidase; MCO: multi-copper oxidases; MT: mushroom tyrosinase; NP, 4-nonylphenol; OCP: oxidative coupling products; PCP, pentachlorophenol; PEG: polyethylene glicol; PEI: polyethyleneimine; PMS: polymethylstyrene; PVA: polyvinyl alcohol; r*Cc*L: recombinant *Coprinus cinereus* laccase; r*Cc*P: recombinant *Coprinus cinereus* peroxidase; REFR: relative enzyme fold reduction; r*Mt*L: recombinant *Myceliophthora thermophila* laccase; r*Pp*L: recombinant *Polyporus pinsitus* laccase; r*Rs*L: recombinant *Rhizoctonia solani* laccase; SBP: soybean peroxidase; SDS: sodium dodecyl sulphate;TP: turnip peroxidase; *Tv*L: *Trametes villosa* laccase; *Tve*L: *T. versicolor* laccase

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