Biodegradation (2011) 22:389–396
DOI 10.1007/s10532-010-9410-8

ORIGINAL PAPER

Biodepollution of wastewater containing phenolic compounds from leather industry by plant peroxidases

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Received: 18 May 2010 / Accepted: 18 August 2010 / Published online: 29 August 2010

Abstract This study deals with the use of peroxidases (POXs) from *Allium sativum, Ipomoea batatas, Raphanus sativus* and *Sorghum bicolor* to catalyze the degradation of free phenolic compounds as well as phenolic compounds contained in wastewater from leather industry. Secretory plant POXs were able to catalyze the oxidation of gallic acid, ferulic acid, 4-hydroxybenzoic acid, pyrogallol and 1,4-tyrosol prepared in ethanol 2% (v:v). Efficiency of peroxidase catalysis depends strongly on the chemical nature of phenolic substrates and on the botanical source of the enzymes. It appeared that POX from *Raphanus sativus* had the highest efficiency. Results show that POXs can also remove phenolic compounds present in industrial wastewater such as leather industry. Removal of phenolic compounds in wastewater from leather industry by POX was significantly enhanced by polyethylene glycol.

Keywords Peroxidase · Leather · Wastewater · Biodepollution · Phenolic compound

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Abbreviations

PEG Polyethylene glycol
POX Peroxidase

Introduction

Phenolic compounds are one of the major classes of organic pollutants generated through various industrial processes, notably in leather industries (Nicell et al. 1993). Other sources of phenolic compounds are pesticides and phenol-containing products such as slimicides, disinfectants, antiseptics and medicinal preparations including mouthwash and sore throat lozenges (de Araujo et al. 2006). The exposure to phenols may result in liver damage and haemolytic anaemia. Respiratory uncoupling is thought to be the main mechanism for the toxic action of some phenols (Ren 2003).

In addition to the acute toxicity, some phenolic pollutants such as bisphenol A and alkylphenols display potential endocrine disrupting activities even at very low concentrations (Singleton and Khan 2003). Although the toxicity and environmental impacts of phenolic compounds are depending on the numbers, types and positions of substituted groups on the aromatic ring(s), some phenolic compounds could be toxic at high concentration to various organisms including humans (NTP 2010). Simple phenols such as pyrogallol is being shown to be toxic because of its mutagenicity (Takemura et al. 2010). Some substituted phenols, such as chlorophenols and alkylphenols, are also highly biorefractory, so that conventional biological processes cannot effectively remove these compounds (Karam and Nicell 1997). Polyphenolic derivatives such as condensed tannins may inhibit microorganisms and enzymes involved in the decomposition of organic pollutants (Scalbert 1991). As a result, the release of these compounds in rivers and sloughs without prior treatment may be responsible for the toxification of aquatic systems.

Conventional processes for removal of phenols from industrial wastewaters include extraction, adsorption on activated carbon, bacterial and chemical oxidation, electrochemical techniques, irradiation, etc. (Liu et al. 2002; Bratkovskaja et al. 2004; Regalado et al. 2004). All of these methods suffer from serious shortcomings such as high costs, incompleteness of purification, formation of hazardous by-products, low efficiency and applicability to a limited concentration range. Nevertheless, these methods are not suitable for treating moderate to high concentrations of phenols (Liu et al. 2002; Regalado et al. 2004). Thus, the development of more effective treatment processes is necessary for the removal of these phenolic pollutants from industrial waste streams and the environment. It has been demonstrated that the oxidation of numerous aromatic compounds, such as phenols and aromatic amines, can be accomplished in an aqueous phase using a variety of enzymes including fungal laccases (Kim and Nicell 2006; Okazaki et al. 2002), tyrosinases (Yoshida et al. 2001), manganese POXs (Hirano et al. 2000), plant secretory POXs (Dec and Bollag 1994; Kobayashi et al. 1998; Caza et al. 1999; Ashraf and Husain 2010) and other enzymes (Sakurai et al. 2001). Among plant oxidases, plant secretory or extracellular POXs, often called class III POXs have been suggested some decades ago for biodepollution of wastewater containing aromatic compounds (Mayer and Harel 1979). Currently, horseradish peroxidase is the most studied enzyme for utilization in decontamination processes. The idea of using POXs for biodepollution is due to the fact they catalyze the polymerization and subsequent precipitation of aqueous phenolic compounds. During POX catalyses, phenols are converted into highly reactive radical species which polymerize by non-enzymatic processes (Hewson and Dunford 1976). The polymeric products are water-insoluble and can be easily removed by solid–liquid operations (Klibanov et al. 1980). The resulting supernatant is free of phenolic compounds, has low smell, less cloudy, and also less toxic. The chemical reactions involved in this catalytic process, the mechanisms of enzyme inactivation and the kinetics of these reactions are discussed elsewhere (Buchanan and Nicell, 1998). The advantage of using crude plant POX in remediation is also the relative cheapness of the crude enzymes and the process is environmental safe compared to other methods (Dec and Bollag 1994). However, to date, it has not been applied on an industrial waste containing phenolic compounds. Crude enzyme extract may be directly incubated with contaminated water instead of using pure enzyme preparation. The

purpose of this work is to assess on laboratory scale the potential use of crude POX extracts from *Allium sativum*, *Ipomoea batatas*, *Raphanus sativus* and *Sorghum bicolor* in order to biodegrade wastewater from local leather industry.

Materials and methods

Chemicals and reagents

Guaiacol, pyrogallol, 1,4-tyrosol, 4-hydroxybenzoic acid, gallic acid, acetic acid and ferulic acid were purchased from Aldrich. H_2O_2 was from Merck. Sodium acetate was purchased from Anachemia Rouses. Bovin serum albumin, catalase (EC 1.11.1.6, 12 400 units per mg protein), polyethylene glycol (average molecular mass of 3350 Da), Folin and Ciocalteu's phenol reagent were acquired from sigma Chemical Co. All other chemicals were of analytical grade.

Wastewater

The wastewater used in these studies was obtained from a leather industry of TAN ALIZ society, in Ouagadougou, Burkina Faso. This stream was selected because it was the main source with the highest concentration of phenols in Ouagadougou (Burkina Faso).

Plant materials

Enzymes were extracted from *Allium sativum, Ipomoea batatas, Raphanus sativus* and *Sorghum bicolor*. To minimize stress related differences in POX biosynthesis, all the plant species were grown in the same farm and in the same natural environment, in Ouagadougou (Burkina Faso), during the rainy-season 2007–2008.

Peroxidase extraction and determination of enzyme activity

Enzyme extracts were prepared by mixing 250 mg of plant material with 1.2 ml of 50 mM Tris–HCl buffer pH 7.3 containing 0.5 M $CaCl_2$ and 5 mM β-mercapto-ethanol, at 4°C for 1 h. After grounding, the homogenate was centrifuged (14000×*g*, 4°C, 45 min) and the resulting supernatant was used as crude extract of POXs. Total protein was quantified by the linearized method of Bradford (Zor and Selinger 1996) using the ratio of A_{620}/A_{450} versus protein concentration. BSA was used as standard. POX activity was measured spectrophotometrically by monitoring the H_2O_2-dependent oxidation of guaiacol, at 25°C. The reaction mixture consisted of 10 µl of 200-fold diluted crude enzyme extract, 20 µl of 100 mM guaiacol, 10 µl of 100 mM H_2O_2 and 160 µl of 50 mM sodium acetate pH 5.0. Control assays in which the enzyme extract or substrates were replaced by buffer were performed. The reaction was initiated by addition of H_2O_2, and monitored at 450 nm, over 30 min. One unit of POX activity (U) is defined as the amount of enzyme releasing 1 µmol of guaiacol radical/min under the assay conditions.

Batch treatment of aqueous phenol

Stock solutions (10 mM) of gallic acid, ferulic acid, 4-hydroxybenzoic acid, pyrogallol and 1,4-tyrosol in 2% ethanol/water (v/v) were prepared. These compounds were selected based on the fact that 4-hydroxybenzoic acid, tyrosol, ferulic acid and gallic acid are among the most important phenolic compounds found in most industrial wastewaters (Borja et al. 1995 Takemura et al. 2010).

Batch reactor for phenolic compound removal contained 0.9 ml of 10 mM stock solution of phenolic compound, 0.1 ml of plant enzymatic extract, 1 ml of 50 mM sodium acetate buffer pH 5 and 0.1 ml of 100 mM H_2O_2. The reaction was initiated by H_2O_2 which was added in assay mixture. After incubation at 25°C aliquots of the reaction mixture were withdrawn at 5 min interval up to 30 min, and the enzymatic reaction was stopped by adding 0.05 ml of stock catalase solution (0.5 mg ml^{-1}). Phenol residual concentration was measured with 5 min interval by the method of Folin and Ciocalteu's (Singleton et al. 1999).

Batch treatment of wastewater from a leather industry

Wastewater samples were centrifuged at 3500×*g* for 30 min to remove suspended materials. The supernatant was analyzed to determine initial concentration of phenolic compounds by the method of Folin and

Ciocalteu's (Singleton et al. 1999). The supernatant was then used to determine the degradation of wastewater phenolic compounds by plant POXs. Batch reactors contained 1 ml of supernatant, 100 µl of 100 mM H_2O_2; 1 ml of 50 mM sodium acetate buffer pH 5.0 and 0–100 µl enzymatic extract. The reaction mixture was stirred continuously during 3 h. Stock catalase solution (0.5 mg ml^{-1}) was added into centrifuge tubes for the purpose of stopping the reaction when required. Sample mixtures were centrifuged at $3500 \times g$ for 30 min. The supernatant was analyzed for it absorbance to determine the concentration of residual phenolic compounds. Reaction controls were used for each incubation to confirm that phenolic compound removal was solely a consequence of POX activity. Control samples were treated concurrently with either in the presence of not of hydrogen peroxide. The effect of polyethylene glycol on the catalytic activity of POX was investigated by adding 100 µl of 100 mM polyethylene glycol in the reaction mixture.

Statistical analysis

All spectrophotometric assays were monitored with a MRX 96-well microplate reader on-line interfaced to a computer (Hewlett Packard). Kinetic data were determined in the linear phase of reaction traces using MRX revelation software version 1CXD-4239 (Dynex Technologies, Inc, USA). The reactions were monitored over 10 min. The initial slopes of the reaction traces caused by enzyme activities were corrected with the slopes of the blanks. All experiments were carried out in triplicate. Analysis of variance (ANOVA) and Student *t*-test ($P = 0.05$, considered as signification) were used to determine statistically significant differences between degradation assays.

Results and discussion

Degradation of pure phenolic substrates

In order to harmonize the used method to quantify levels of phenolic compounds both with pure phenolic compounds and phenolic compounds contained in waste water, the Folin Ciocalteu method has been preferred to other spectrophometric methods. For comparison of the phenol removal efficiencies by peroxidases evaluated in this study, five aqueous phenols was treated with the crude extract from *Allium sativum* bulb, *Ipomoea batatas* tuber, *Raphanus sativus* roots and germinated grain of *Sorghum bicolor*. These four plants display different levels of POX activities. POX specific activities among these plants ranged from 22.1 to 294.6 U/mg. The highest specific activity was found in the bulbs of *Raphanus sativus*, followed by *Sorghum bicolor* and *Ipomoea batatas*. *Allium sativum* showed significantly lower activity than the other three species.

The time course of gallic acid, ferulic acid, 4-hydroxybenzoic acid, pyrogallol and 1,4-tyrosol removal by peroxidase-catalyzed polymerization is shown in Fig. 1. The phenomenon of oxidative coupling of phenols by enzymes was well recognized in the early 1960s (Brown 1967), but its application to wastewater treatment occurred about 15 years later (Klibanov and Morris 1981). Removal experiments were performed both with pure phenolic compounds as well as wastewater containing phenolic compounds.

The incubation of plant extracts with phenolic compounds without H_2O_2 (control sample) did not result in a significant change of the concentration of the initial phenolic compounds. However in the presence of H_2O_2 the concentration of phenolic compounds rapidly decreased (Fig. 1). This suggests that the oxidation of phenolic compounds used in the present study is mainly governed by the activity of POX rather than other endogenous oxidative enzymes such polyphenol oxidases and laccases. Previous works have also shown that POXs are the major oxidative enzymes in sorghum varieties (Dicko et al. 2002, 2006). Five phenolic compounds were incubated with POX extracts and the reaction processes were monitored over a time (Fig. 1). For all the five standard phenolic compounds used as models of aqueous phenol, POXs extract have shown their efficiency to their degradation (Fig. 1).

However, degradation rates were different both between phenolic compounds and the botanical source of POX extracts (Fig. 1). This confirms that the efficiency of POXs on the catalysis is strongly dependant on the chemical nature of substrates (substrate specificity) and on the botanical source of POXs. Sakurai et al. (2001) have found a difference for the degradation of phenol and bisphenol A. The

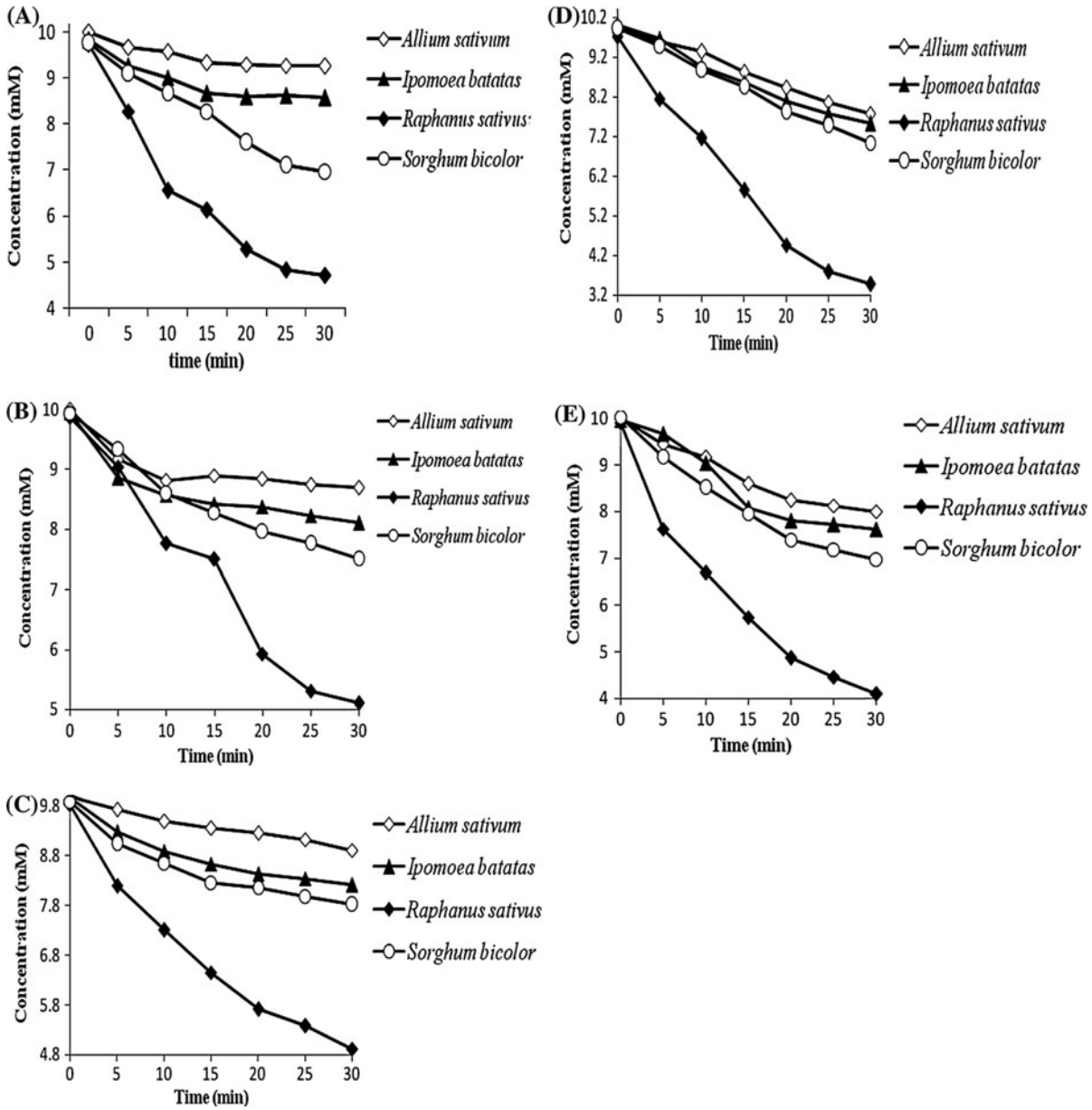

Fig. 1 Degradation of five phenolic compounds as a function of time by plant POXs. Reactions were carried out at pH 5, at 25°C and the reaction mixtures initially contained 10 mM phenolic compound, 5 mM H_2O_2 and 10 µl of enzymatic extract. Enzyme extracts were from *Allium sativum, Ipomoea batatas Raphanus sativus*, or *Sorghum bicolor.* Phenolic compounds were **a** gallic acid, **b** ferulic acid, **c** 4-hydroxybenzoic acid, **d** 1,4-tyrosol, and **e** pyrogallol

most efficient POX extract for the degradation of the five phenolic compounds was the POX from *Raphanus sativus*. Nevertheless, behind *Raphanus sativus*, the extracts of *Ipomoea batatas* and *Sorghum bicolor* also have a significant level of degradation. Several works have demonstrated the ability of POXs to degrade recalcitrant organic compounds like phenols and substituted phenols (Klibanov et al. 1980; Wu et al. 1997; Wright and Nicell 1999; Dalal and Gupta 2006). Some POXs convert virtually all aromatic compounds (phenols and amines) and even some non-aromatic compounds (Dunford 1999).

Degradation of phenolic compounds in wastewater

In order to compare the efficiencies of POXs to degrade phenolic compounds contained in wastewater, the wastewater was incubated with various amounts of the crude POXS from *Allium sativum, Ipomoea batatas, Raphanus sativus* and *Sorghum bicolor.* As found with pure phenolic compounds (Fig. 1), no significant changes in the phenol concentrations were observed in control wastewater samples without the addition of H_2O_2 and POX. Thus, the degradation of wastewater phenolic compounds was essentially due to POXs. In the presence of H_2O_2, the reaction mixtures tend to be uncolored, with the appearance of precipitates, indicating that the degradation of phenolic compounds was effectively caused by POX activities.

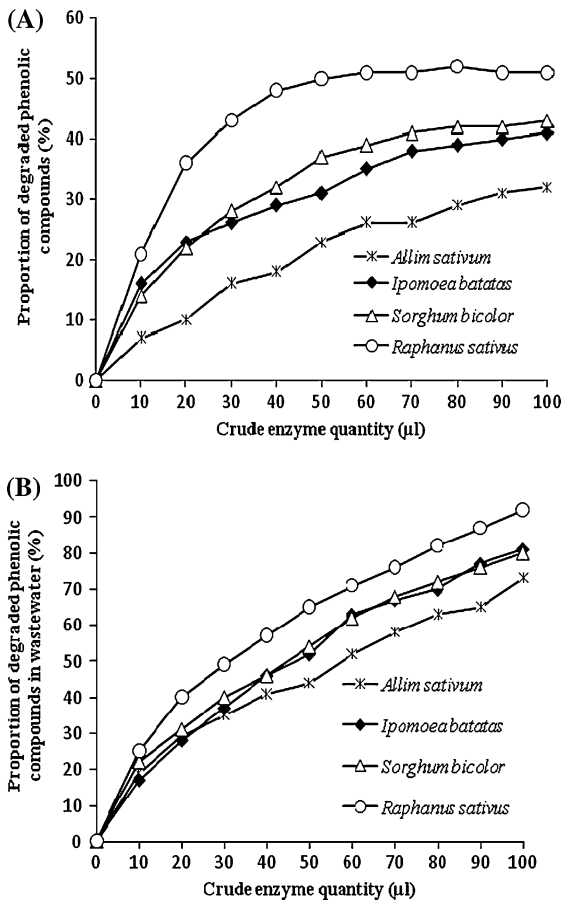

Fig. 2 Degradation of phenolic compounds in wastewater as a function of the amount of enzyme. Enzyme extracts were from *Allium sativum, Ipomoea batatas Raphanus sativus*, or *Sorghum bicolor.* Wastewaters were either incubated with enzyme extracts in the absence of polyethylene glycol (**a**) or in the presence of polyethylene glycol (**b**)

Residual quantities of phenolic compounds were measured following treatment with enzymatic extract and after centrifugation, as a function of enzyme quantities (Fig. 2a). Since polyethylene glycol (PEG) may enhance the action of enzymes in the presence of polymeric compounds (Dalal and Gupta 2006), its effect was also tested in the present study (Fig. 2b).

It clearly appeared that the addition of PEG in the reaction medium considerably enhanced the degradation of phenolic compounds (Fig. 2a and b). Without PEG, the maximal transformation of phenol by the POXs of the four plants ranged from 32 to 55%. The other advantage of using PEG is that, it is relatively a nontoxic organic compound (Kinsley and Nicell, 2000; Cheng et al. 2006; Gonzalez et al. 2008). In addition, it is a biodegradable compound and has a little impact on environment.

The best degradation was obtained with the extract from *Raphanus sativus* and the lowest, with the extract from *Allium sativum*. With the addition of PEG in the reaction medium, the yield of degradation increased rapidly as function of the amount of enzyme (Fig. 2b). The highest degradation yield was obtained with POXs from *Raphanus sativus* and the lowest with POXs from *Allium sativum*. The maxima yields of degradation obtained in the experimental conditions were 93, 76, 77, and 72% for POXs from *Raphanus sativus*, *Sorghum bicolor*, *Ipomoea batatas*, and *Allium sativum,* respectively. The comparison between the phenol degradation by POX without and with PEG showed a significant difference ($P < 0.05$) between the two methods. With PEG, the quantity of POX from *Raphanus sativus* necessary for degradation of phenolic compounds was lower than the amount necessary for the same level of degradation in the absence of PEG (Fig. 2a and b). Furthermore, the degradation was more efficient in the presence of PEG. The comparison of the profiles of the degradation kinetics (Fig. 2a and b) indicated that POX may be fully inactivated before a complete degradation of all phenolic compounds. Apparent inactivation of peroxidase during phenol polymerizing reaction was found to be mainly caused by

adsorption of peroxidase by reaction products. Nevertheless, PEG enhanced the yield of degradation probably by minimizing the interaction between free POX molecules and degradation products during the catalysis. Indeed, monitoring the degradation of pentachlorophenol by POX from *Cochlearia armoracia,* Zhang and Nicell (2000) also found similar results. Other studies on phenol removal by horse radish peroxidase have shown that PEG improved the phenol removal efficiency by forming a protective layer around the active centre of enzyme which prevents the attack of free phenoxy radicals formed in the catalytic cycle (Cheng et al. 2006). Most of the phenoxy radicals interact with PEG due to their greater affinity with PEG than the enzyme, thus preventing the adsorption of the reaction product on the active sites of enzyme molecules (Tonegawa et al. 2003; Gonzalez et al. 2008). Thus as previously found (Ikehata et al. 2003, 2005), the addition of PEG in the reaction mixture increased both the rate and the yield of degradation. The enzyme reaction in this method can be considered as a modified type of ping-pong kinetics, referred as peroxidase ping-pong by Dunford (1991).

Conclusion

Crude plant extracts containing peroxidase activities can be used for biodepollution of wastewater containing phenolic compounds. Peroxidases from *Allium sativum, Ipomoea batatas, Raphanus sativus* and *Sorghum bicolor* are able to catalyze the degradation of phenolic compounds such as gallic acid, ferulic acid, 1,4-tyrosol, pyrogallol, 4-hydroxybenzoic acid from wastewater. Among these plants *Raphanus sativus* is the most interesting source of POX for the biodepollution of phenolic compounds. This first study on a pilot assay on the elimination of phenolic compounds by peroxidases may serve as a basis for an experimental application in industrial effluents.

Acknowledgments This research project was supported trough financial assistance from the Fondazione Lelio and Lisli Basso-Issoco, Italy, and Agence Universitaire de la Francophonie (AUF-GP3A), France.

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